MOLECULAR CHARACTERISATION OF SOME MULTI-DRUG RESISTANT *SALMONELLA ENTERICA* OF HUMAN ORIGIN IN SOUTHEAST NIGERIA

BY

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CERTIFICATION

This is to certify that this research study was carried out by Onyenwe Nathaniel Ejikeme under my supervision, in the Department of Pharmaceutical Microbiology, Faculty of Pharmacy, University of Ibadan, Nigeria.

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DEDICATION

This work is dedicated to the almighty God, my beloved Children, Miracle (Ada), Chizitere (Precious), Godswill (Osinachi), Ifeanyi (Francis), and wife Oluchi (Norah).

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ABSTRACT

There has been an increase in the occurrence of antibiotic resistance among *Salmonella enterica*, one of the commonest causative agents of *Salmonella* infections. Fluoroquinolones and third generation cephalosporins are usually the drugs of choice in the management of *Salmonella* infections. Previous reports have indicated common occurrence of multi-drug resistance (MDR) including resistance to β-lactams and fluoroquinolones among clinical Gram-negative organisms. However, there is paucity of information on the genetic determinants of resistance to β-lactam and fluoroquinolones from *S. enterica* in Southeast Nigeria. This study screened for the presence of Extended-Spectrum Beta-Lactamases (ESBL) and mutations in *gyrA* and *parC* genes of *S. enterica* from human origin in the Southeast Nigeria.

Twenty-five *S. enterica* isolates from stool of patients suspected to have *Salmonella* infection were collected from each of four hospitals (one teaching hospital and three Federal Medical Centres) in Southeast Nigeria between July and September, 2010. The isolates were confirmed using Microbact identification kit®. Antibiogram for the isolates was determined by disc-diffusion based on Clinical and Laboratory Standards Institute breakpoints. Five commonly used antibiotics (amoxicillin-clavulanic acid, cefotaxime, ceftriaxone, ciprofloxacin and levofloxacin) in the treatment of *Salmonella* infections were selected for determination of Minimum Inhibitory Concentrations (MIC) against the isolates using broth-dilution method. Isolates resistant to two or more different classes of antibiotics were classified as MDR. Isolates resistant to fluoroquinolones and cephalosporins were exposed to mutagens for R-plasmid curing, and ESBL were detected phenotypically using Double-Disk Synergy Test. Genomic and plasmid DNA of mutagen treated and untreated isolates were extracted by boiling and alkaline lysis, respectively. Polymerase chain reaction was used to amplify bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ among the ESBL positive isolates, and Quinolone Resistance Determining Regions (QRDR) among fluoroquinolone resistant isolates, followed by sequencing of the QRDRs. Antibiogram data were analysed using ANOVA at $p = 0.05$.

The 100 clinical isolates collected were confirmed to be *S. enterica*. Percentage resistance obtained was: amoxicillin-clavulanic acid (87%), chloramphenicol (80%), amoxicillin (80%), co-trimoxazole (78%), sparfloxacin (78%), streptomycin (77%), gentamicin (51%), ceftazidime (44%), perfloxacin (29%), ciprofloxacin (29%), ofloxacin (28%), cefotaxime (27%), ceftriaxone

(22%) and levofloxacin (22%). Eighty of the 100 isolates were MDR and the ranges of MICs of the selected antibiotics were: amoxicillin-clavulanic acid (≥ 50 µg/mL), cefotaxime (6.25 - 25 µg/mL), ceftriaxone (6.25 – 12.5 µg/mL), ciprofloxacin (6.25 – 12.5 µg/mL) and levofloxacin (12.5 - 25 µg/mL). Of the 100 isolates, nine MDR isolates carrying R-plasmid were cured. Thirty six of the MDR isolates produced ESBL phenotypically, of which 13 were $bla_{\text{CTX-M}}$ positive. DNA sequencing revealed single point mutations in *gyrA* at amino acid positions Asp-87-Gly, Asp-87-Asn and Ser-83-Tyr in 55 (68.8%), and double mutation in *parC* at positions Asp-87-Gly in 14 (17.5%). There was significant difference in the activity of the individual antibiotics against the isolates.

The occurrence of mutations in *gyrA* and *parC* genes, and chromosomal $bla_{\text{CTX-M}}$ were responsible for fluoroquinolones and cephalosporins resistance, respectively in some of the *Salmonella enterica* from Southeast Nigeria. Hence, alleviating the fear of easy spreading of quinolone and cephalosporin resistant isolates.

Keywords: Multi-drug resistant *Salmonella enterica*, R-plasmids, quinolone-resistance determining region, $bla_{\text{CTX-M}}$, fluoroquinolones

Word count: 499.

LIST OF MAIN ABBREVIATIONS

Abbreviation	Full Meaning
MDR	Multidrug Resistance
BLAST	Basic Local Alignment Search Tool
DNA	Deoxyribonucleic acid
bla	Beta-Lactamase
bla CTX-M	Cefotaximase
bla SHV	Beta-Sulfhydryl variable
bla TEM	Beta-Temoniera
ESBLs	Extended-Specrum Beta-Lactamases
MIC	Minimum Inhibitory Concentration
CLSI	Clinical Laboratory Standards Institute
PCR	Polymerase Chain Reaction
µg/mL	Microgram per millilitres
*Hind*III	*Haemophilus influenzae* III
Qnr	Quinolone Resistant Protein
g*yrA*	DNA gyrase enzyme
parC	Topoisomerase IV enzyme
Qnr-B	Quinolone Resistant Protein Class B

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CHAPTER ONE
INTRODUCTION

1.1. Background

1.1.1. Description of *Salmonella enterica*

Typhoid fever is a systemic infection caused by *Salmonella enterica* serotype *typhi*. This is a highly adapted human specific pathogen and possesses remarkable mechanism for persistence in host (Mushtaq, 2006). Most of the disease burden occurs in developing countries due to poor sanitary conditions (Parry *et al*., 2002; Parry and Beeching, 2009).

In the late 1980s, some *S. typhi* and *S. paratyphi* strains (multidrug resistant) developed plasmid-mediated resistance simultaneously to all of the first line antibacterial agents, which are ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole (TMP-SMZ) (Parry and Beeching, 2009; Brusch *et al*., 2010). The use of antimicrobial agents in any environment creates selection pressure that favours the survival of antibiotic-resistance pathogens (White *et al.,* 2007). According to the infectious disease report that was released by the World Health Organisations in 2000, such organisms have become increasingly prevalent world wide (White *et al*., 2003).

Multi-drug resistant *Salmonella typhi* (MDRST) is defined as *Salmonella typhi* resistant to all first line antibiotics i.e. chloramphenicol, ampicillin, and trimethoprim-sulphamethoxazole (Mushtaq, 2006). Multiple outbreaks of infections with these resistant strains occurred in India, Pakistan, Bangladesh, Vietnam, Middle East and Africa (Thong, *et al*., 2000; Connerton *et al*., 2000; Mirza *et al*., 2000). These strains were also found to be resistant to sulphonamide, tetracycline and streptomycin. Amoxicillin and trimethoprim-sulphamethoxazole were effective alternatives till the end of 1990s when strains resistant to all the first line anti-salmonella drugs used at that time, were reported (Mushtaq, 2006). This resistance rarely develops during course of treatment but instead results from clonal dissemination of individual multi-drug resistant *S. typhi* or from transfer of R-plasmid. However, there are reports from some areas, of strains, fully susceptible to all first line drugs (Mushtaq, 2006).

Fluoroquinolones are now recommended by most authorities for the treatment of typhoid fever. They are highly effective against susceptible organisms, yielding a better cure rate than cephalosporins. Unfortunately, resistance to first-generation fluoroquinolones is widespread in many parts of Asia (Brusch *et al.,* 2010). In recent years, third-generation cephalosporins have been used in regions with high fluoroquinolone resistance rates, particularly in South Asia and Vietnam. Unfortunately, sporadic resistance has been reported, so it is expected that these will become less useful over time (Brusch *et al*., 2010). Threlfall and Ward (2001) have reported that *S. typhi* with decreased sensitivity to ciprofloxacin is endemic in several Asian countries, and incidence of such strains has increased in travelers from the Indian subcontinent. They suggested 3rd generation cephalosporins such as ceftriaxone or cefotaxime as possible alternatives, and in their study, it was assured that all strains were sensitive to these drugs.

Reduced susceptibility to fluoroquinolones has become a major problem mostly in Asia (Hawkey, 2003; Mushtaq, 2006). Outbreak with such strains affected eight thousand people and killed 150 people in Tajikistan in 1997. Isolates responsible for this outbreak of fluoroquinolone resistance had their MIC to be ten times of those fully susceptible to the drug. This decreased susceptibility is resulting in treatment failure (Mushtaq, 2006). At the moment the emergence of resistant strains to two major second line drugs like ciprofloxacin and ceftriaxone is posing a major problem (Ackers *et al*., 2000). Antibiotic resistance is a moving target and reports are quickly outdated, thus recommendation regarding antibiotic treatment must not be taken with a grain of salt (Brusch *et al.,* 2010), if the origin of the infection is unknown, the combination of a first-generation fluoroquinolone and a third-generation cephalosporin should be used (Brusch *et al.,* 2010). Based on these facts, this research was aimed to carry out molecular characterisation of some multidrug resistant clinical isolates of *Salmonella enterica* from human origin from Southeast part of Nigeria and to detect the resistance determinants, mutations in the *gyrA* and *parC* genes and possible variations of resistant gene involved.

1.2 Justification for this study:

There has been an increase in the multidrug-resistance of *Salmonella enterica*, the causative agent of typhoid fever in Nigeria (Yah, 2007). This organism also causes other diseases such as enteric fever, salmonella septicaemia and gastroenteritis, thereby indicating the organism as an important human pathogen of these nosocomial infections (Cheesbrough, 2006). The increasing clinical importance of these infections has led to the search for information on the prevalence of multidrug resistance pathogens such as *Salmonella enterica* in Africa including Nigeria (Yah, 2010).

Notably, high level of antimicrobial resistance and multidrug resistant *Salmonella enterica* isolates have not been reported in the Southeast region of Nigeria, thereby leading to scarcity of published or documented data in this region. Amongst the drugs implicated in Nigeria and other parts of the world were chloramphenicol and co-trimoxazole which have been the fronline drugs for the treatment of the infections caused by *S. enterica,* and resistance to these drugs has become a real challenge especially in the developing world (Brusch *et al*., 2010). Currently, the generations of antibiotics implicated were the quinolones (fluoroquinolone) and cephalosporins (Mustaq, 2006; Yah, 2007). Hence, the need to determine and characterize the genetic determinants associated with the resistance to the currently implicated drugs using phenotypic and molecular techniques in the Southeast Nigeria.

1.3 Overall aim for this study

The overall aim of this study is to characterize the resistance determinants of the multidrug resistant clinical isolates of *Salmonella enterica* of human origin, in other to identify the types of genes involved in their acquisition and their possible locations in the organism from the Southeast part of Nigeria.

1.4 Specific objectives for the study

1. To determine the antibiogram and Minimum Inhibitory Concentrations (MIC) of antibiotics mostly used against *Salmonella enterica* infections in Southeast Nigerian hospitals.
2. To determine and characterize the *Salmonella* isolates producing Extended Spectrum Beta-lactamase (ESBL) enzymes such as *bla*-CTX-M, *bla*-SHV, *bla*-TEM-1 and plasmid-mediated quinolone resistant gene such as *Qnr*B gene type.
3. To determine the Quinolone Resistance Determining Regions (QRDRs) in *gyrA* and *ParC* of *Salmonella enterica.*
4. To determine the resistant plasmid and genetic location of the different resistance determinants harbored by the resistant *Salmonella enterica.*

CHAPTER TWO
LITERATURE REVIEW

2.1 Characteristics of *Salmonella enterica*

Salmonellae are Gram-negative, motile aerobic rods that characteristically ferment glucose and manose but fail to ferment lactose or sucrose, they are pathogenic for humans or animals by the oral route (Cheesbrough, 2006). Salmonellae grow readily on simple media. The selective media for the organisms from faces contain brilliant green, cholate, selenite, tetra chlorate, or citrate to suppress the growth of coliforms. Formerly classified as separate species, DNA hybridization studies have now shown that all pathogenic Salmonellae belong to a single species, *Salmonella enterica* which is subdivided into 7 subspecies (subsp). *S. enterica* subsp. enterica has over 2000 serovars which can cause disease in humans (Cheesbrough, 2006). For convenience the Serovars (first letter in capital) are written in an abbreviated form, e.g, the accepted abbreviation for *S.enterica* subsp. Enteric Serovar Typhi is *S. typhi* (italics is not used for the serovar) (Cheesbrough, 2006). They usually use citrate as their sole carbon source for growth and produce hydrogen sulphide.

Multidrug-resistant *Salmonella typhi* has become a problem in a developing country like Nigeria and in developed countries like the United States and United Kingdom. This chronic infection appears to be peculiar to isolates from temperate climate zones. Strains native to Africa do not give rise to chronic infection. The commonest group of carriers are women in their 50's with gallstones (Brusch *et al.,* 2010). Typhoid fever appears to be a disease that has been associated with man since close to the first appearance of hominids and it may have first infected human ancestors anywhere from 200,000 to two million years ago. The bacterium can survive in contaminated water, but does not have any host other than man. There are over 1,000 different strains of the bacterium of which only a few cause typhoid (Mushtaq, 2006). *Salmonella typhi*, a potentially lethal organism was successfully managed with the introduction of chloramphenicol. Since then emergence of resistant strains began and now Multidrug resistant *Salmonella typhi* (MDRST) has become a real challenge especially in the developing world. There have been reports from different parts of the world about resistance pattern. Most reports from

developing countries are showing MDRST strain (Ackers *et al*., 2000). The original clinical indication of chloramphenicol was in the treatment of typhoid, but with the universal presence of multi-drug resistant *Salmonella typhi* it is seldom used for this indication except where the organism is known to be sensitive (Mandal *et al*., 2010). Chloramphenicol susceptibility test following disk diffusion is not enough for its re-selection in the treatment of typhoid fever, (Gautam *et al*., 2002) and therefore, it is imperative to compare the Minimum Inhibitory Concentration values (MICs) of chloramphenicol for the sensitive isolates with chloramphenicol MICs for the resistant isolates. The genes for antibiotic resistance in *S. typhi* and *S. paratyphi* are acquired from *Escherichia coli* and other Gram-negative bacteria via plasmids (Brusch *et al.,* 2010). The plasmids contain cassettes of resistance genes that are incorporated into a region of the *Salmonella* genome called an integron. Some plasmids carry multiple cassettes and immediately confer resistance to multiple classes of antibiotics. This explains the sudden appearance of MDR strains of *S. typhi* and *S. paratyphi*, often without intermediate strains that have less-extensive resistance (Brusch *et al.,* 2010).

The primary target of the organism is the intestine. It attacks through tissues that are a part of the immune system called Peyer's patches. These tissues on the inner surface of the intestine are normally the first line of defense against food and water-borne infection, but the typhoid bacterium subverts them (Cheesbrough, 2006). It does not have the cell surface features that normally trigger a defensive response but it can fool the cells of the patches to take it in without attacking it. The infected tissues become inflamed and the intestine begins to lose function, leading to diarrhoea or constipation. There may be bleeding of the intestine leading to bloody stools. In severe cases, the disease may punch holes in the intestine leading to peritonitis (infection of the abdominal cavity) and death (Cheesbrough, 2006). Even if the patient recovers from the infection, it may leave residual damage, such as the formation of attachments of the damaged areas of the intestine to the abdominal wall, or the development of a chronic infection that leads to the patient becoming a carrier (Cheesbrough, 2006). A closely related bacterium *Salmonella typhimurium* causes a similar disease in the mouse, but does not affect man and the combination of the pathogen and the mouse is used as a model to study the human disease. Resistance to fluoroquinolones is evolving in an ominous direction.

Fluoroquinolones target DNA gyrase and topoisomerase IV, bacterial enzymes that are part of a complex that uncoils and recoils bacterial DNA for transcription (Brusch *et al*., 2010). Patients in the United Kingdom and United States detected strains resistant to all three first-line drugs (ampicillin, trimethoprim-sulphamethoxazole and chloramphenicol) were reported to be infected with Multi-drug Resistant *Salmonella typhi* (MDRST) (Brusch *et al*., 2010), while ciprofloxacin and ceftriaxone resistant isolates were reported as a result of traveling to the developing world especially Southeast Asia (Ackers *et al*., 2000). In high-prevalence areas outside the areas mentioned above, the rate of intermediate sensitivity or resistance to fluoroquinolones is 3.7% in the Americas (*P* =.132), 4.7% (*P* =.144) in sub-Saharan Africa, and 10.8% (*P* =.706) in the Middle East.

Therefore, for strains that originate outside of south or Southeast Asia, the WHO recommendations may still be valid—that uncomplicated disease should be treated empirically with oral ciprofloxacin and complicated typhoid fever from these regions should be treated with intravenous ciprofloxacin (Brusch *et al*., 2010). A single-point mutation *gyrA* confers partial resistance. If a second *gyrA* point mutation is added, the resistance increases somewhat. However, a mutation in *parC* added to a single *gyrA* mutation confers full in vitro resistance to first-generation fluoroquinolones. Clinically, these resistant strains show a 36% failure rate when treated with a first-generation fluoroquinolone such as ciprofloxacin. The risk of relapse after bacterial clearance is higher in both partially and fully resistant strains than in fully susceptible strains (Brusch *et al*., 2010). According to Mushtaq (2006), susceptibility testing on 350 isolates reported that 16% of the isolates were MDRST. No resistance was reported on ciprofloxacin, ceftriaxone, gentamicin and kanamicin (Ackers *et al*., 2000). Nadeem and colleagues reported from Quetta, Pakistan that 69% of isolates were found to be MDRST (Nadeem *et al*., 2002). In yet another report from Bahawalpur, Pakistan: 53.8% of isolates were found MDRST and all strains were sensitive to fluoroquinolones and third-generation cephalosporins (Munir *et al*., 2001). In their study, 28 isolates were checked against all three first-line anti-salmonella drugs of which 18 (65%) isolates turned out to be MDRST (Munir *et al*., 2001). The third-generation fluoroquinolone, gatifloxacin, appears to be highly effective against all known clinical strains of *S. typhi* both *in vitro* and *in vivo*.

However, any two of a number of *gyrA* mutations, when added to the *parC* mutation, confer full *in-vitro* resistance. Although such a combination is yet to be discovered *in-vivo*, all of these mutations exist in clinical (Brusch *et al*., 2010). Studies by Ackers *et al*. (2000) and Nadeem *et al*. (2002) demonstrated all the isolates including MDRST were fully susceptible to ciprofloxacin and ceftriaxone, while data in Mushtaq (2006) reported that out of 18 MDRST four of the isolates were found resistant to ciprofloxacin, four resistant to ceftriaxone and two of them were found resistant to both of these drugs. The overall resistance to ciprofloxacin and ceftriaxone was found to be 19.2% and 17.9% respectively (Mushtaq, 2006). Threlfall and Ward (2001) reported decreased sensitivity to ciprofloxacin and suggested possible alternatives as ceftriaxone and cefotaxime, and reassured that organisms were fully susceptible to these drugs. According to Mushtaq (2006) resistance to ceftriaxone was 21.1% out of 76 isolates that were found resistant to cefotaxime as well. Cases of *Salmonella typhi* resistant to ciprofloxacin and ceftriaxone have been reported from Bangladesh (Mushtaq, 2006).

Pattern of *S. typhi* resistance is changing rapidly. MDRST and strains resistant to ciprofloxacin and ceftriaxone are a major threat in developing world (Mushtaq, 2006). In some areas strains fully susceptible to all first-line anti-salmonella drugs (ampicillin, trimethoprim-sulphamethoxazole and chloremphenicol) have re-emerged (Mushtaq, 2006). Mushtaq (2006) reported that 65 isolates were tested against two or more first-line anti-salmonella drugs and found that they were sensitive to at least two first-line drugs, and only one isolate was found sensitive to all three first-line drugs. The author also reported that resistance to trimethoprim/sulphamethoxazole and chloramphenicol was found to be 94.2% and 65.3% respectively. Similarly, resistance to cefuroxime was 53.1%, cefaclor 49.2% and amoxiclavulanic acid 42.5% of the isolates (Mushtaq, 2006).

Cui *et al.* (2008) in their study reported a high incidence of quinonlone-resistant *S. typhimurium* isolates might have been affected by several factors. First, patients infected by antimicrobial drug-resistant *S. typhimurium* strains had higher rates of hospitalization than the patients infected by susceptible strains (Martin *et al*., 2004 ; Varma *et al*., 2005), and the isolates in their study were from a university-affiliated medical centre that usually treats patients with severe illness (Cui *et al*., 2008). In China, inappropriate prescriptions

might be even more common because antimicrobial drug prescriptions in hospitals were a source of profit (Cui *et al*., 2008). Cui *et al*., (2008), stated that though they do not have the patient antimicrobial drug–use information, but the easy access to antimicrobial drugs raises the possibility that before the collection of stool specimens, the outpatients might have taken fluoroquinolones after the onset of the illness. Secondly, because livestock products are a common source of salmonellosis (Cui *et al.,* 2008), the dissemination of ciprofloxacin-resistant *S. typhimurium* might have been facilitated by the use of fluoroquinolones in livestock production. Lastly, use of other antimicrobial drugs, such as ampicillin, gentamicin, or streptomycin, may also contribute to the spreading of fluoroquinolone-resistant *S. typhimurium* because all the ciprofloxacin-resistant isolates were also resistant to 8-11 additional antimicrobial drugs, they used in their study (Cui *et al.,* 2008).

Hakanen *et al*. (2001) stated that when looking for reasons for the rapidly increased quinolone resistance in travelers' *Salmonella* isolates, three issues must be considered, such as; transferable resistance, mutational resistance, and clonal spread. However, transferable fluoroquinolone resistance appears to be rare in bacteria *in vivo* (Hakanen *et al*., 2001). Thus, either clonal spread or resistance due to mutations in chromosomal genes remains the potential mechanism accounting for the high level of reduced fluoroquinolone susceptibility in Southeast Asia (Hakanen *et al*., 2001). The emergence of mutation-based resistance may be fostered by selection pressure caused by the use of antimicrobial agents in either human medicine or agriculture (Hakanen *et al*., 2001). In Europe, none of the fluoroquinolones licensed for humans is approved for animal use, although many other quinolone preparations are allowed for the treatment of livestock, poultry, and fish. The policy is stricter in the United States, where the only quinolone licensed for food animals is enrofloxacin (Hakanen *et al*., 2001). According to Hakanen *et al*. (2001), no conclusions can be drawn on a potential link between the reduced fluoroquinolone susceptibility of salmonellae and the use of quinolones in animal husbandry in the area studied. In direct contrast, treatment with first-generation quinolones (e.g., nalidixic acid) is known to cause rapid emergence of resistance in the family of Enterobacteriaceae (Hakanen *et al*., 2001). Another topic of major interest involves the potential influence of antimicrobial use in travelers on infections with

quinolone-resistant *Salmonella* strains (Hakanen *et al*., 2001). However, strains with reduced fluoroquinolone susceptibility are currently not identified in any microbiological laboratory worldwide according to current CLSI recommendations, with MIC 4 µg/mL of ciprofloxacin as a breakpoint for resistance (CLSI, 2007). These breakpoint values are considered adequate, as the clinical importance of the reduced fluoroquinolone susceptibility of salmonellae remains unproven. Nevertheless, it is suggested that laboratories worldwide aim at recognizing these less susceptible strains, to reveal their eventual clinical impact and that laboratories use the nalidixic acid screening test (CLSI, 2007) or the E-test to aid identification (Hakanen *et al*., 2001). The CLSI (2014), reported in 2013, that the ciprofloxacin, levofloxacin, and ofloxacin MIC interpretive criteria (breakpoints) was revised for *Salmonella* and the disk diffusion breakpoints for ciprofloxacin, in part to better detect these fluoroquinolone resistance mechanisms in *Salmonella* spp. Though, at present, no levofloxacin or ofloxacin disk diffusion breakpoints for *Salmonella* have been established by the CLSI (CLSI, 2014; Deak *et al.,* 2015).

The recent professional guideline for the treatment of typhoid fever in South Asia was issued by the Indian Association of Pediatrics (IAP) in October, 2006 (Brusch *et al*., 2010). Although these guidelines were published for pediatric typhoid fever, the authors felt that they are also applicable to adult cases. For empiric treatment of uncomplicated typhoid fever, the IAP recommends cefixime and, as a second-line agent, azithromycin. For complicated typhoid fever, cefriaxone was recommended. Aztreonam and imipenem are second-line agents for complicated cases (Brusch *et al*., 2010). The authors believe that the IAP recommendations have more validity than the WHO recommendations for empiric treatments of typhoid fever in both adults and children (Brusch *et al*., 2010).

2.2 *Salmonella enterica* infections in man

Salmonella infections represent a major health problem worldwide, particularly in the developing countries where they are recognized as the most frequent cause of morbidity and mortality (Yah, 2010). Mortality is highly associated with infants under one year of age (South Australia Department of Health, 2008). The impact of lives lost, together with

the high costs to local public health care systems, makes prevention and control a priority (Yah, 2010). Antibiotic resistant *Salmonella* are of global concern because they affect both developed and developing countries due to increased international travel (Yah, 2010). Antimicrobial drug resistance has become increasingly common in *S. enterica*, which can complicate therapy (Ajibade *et al*., 2010).

These concerns have been further reinforced in recent years by the emergence of antimicrobial resistance among the major groups of the enteric pathogens. The presence of antibiotic- resistant bacteria from hospitalized patients has been documented (Yah, 2010). Reports have shown that the resistance of gastroenteric *Salmonella* strains to these antimicrobial agents is in large part due to the production of extended-spectrum β-lactamases (ESBLs) encoded on plasmids, as well as on the chromosome (David and Frank, 2000); Yujuan *et al*. (2006) and Yah (2010). According to Momtaz *et al*. (2002), the development of *S. typhi* strains that are resistant to antibiotics historically used to treat *S. typhi* infection has forced physicians to prescribe fluoroquinolones or third-generation cephalosporins. So far, data from Momtaz *et al.* (2002) study showed that neither quinolone resistance nor third-generation cephalosporin resistance has emerged in Egypt (Momtaz *et al*., 2002). However, extrapolation from data in the literature suggests that quinolone resistance is likely to develop unless use of this drug class is restricted (Momtaz *et al*., 2002). Also Momtaz *et al*. (2002) stated that resurgence in chloramphenicol-susceptible *S. typhi* strains has been reported in recent years. They further stated that eighty percent of Indian strains isolated in 1991-1993 were susceptible to chloramphenicol, compared with only 33.3% of such strains isolated in 1990-1991 ($P < .001$). This observation was attributed to the restricted use of chloramphenicol for 2.5 years (Momtaz *et al*., 2002).

2.2.1 Antigenic structure of *S. enterica*

While Salmonellae are initially detected by their biochemical characteristics, groups and species must be identified by antigenic analysis. Like other enterobacteriace, Salmonellae possess several O antigens and different H antigens (Adeleke *et al*., 2006). Some Salmonellae have capsular antigens, referred to as Vi, which may be associated with

virulence (Cheesbrough, 2006). Organisms may possess H antigens and become immotile and loss of O antigen is associated with change from smooth to rough. Colony which forms Vi antigen may be lost partially or completely (Cheesbrough, 2006; Adeleke *et al*., 2006).

2.2.2 Pathogenesis of *S. enterica*

Salmonella typhi and perhaps *S. paratyphi* A and *S. schottmulleri* (formerly *S. paratyphi* B) are primarily infective for humans, and infection with these organisms implies acquisition from a human source (Cheesbrough, 2006). The rest majority of salmonellae, however, are chiefly pathogenic in animals (e.g poultry, pigs, rodents, cattle, pets and in many others) that constitute the reservoirs for human infections (Cheesbrough, 2006). The organisms are acquired through the oral route, usually with contaminated food and drinks. The mean infective dose for humans is 10^5 - 10^8 Salmonellae to produce clinical and subclinical infection. Among the host factors that contribute to resistance to Salmonellae infection are gastric acidity, normal intestinal microbial flora and local intestinal immunity (IgA). In humans, salmonellae produce 3 main types of disease (enteric fever, Salmonellae septicaemia and gastroenteritis) but mixed forms are also frequent (Cheesbrough, 2006).

2.2.3 The enteric fever

Enteric fever including typhoid and paratyphoid fever is caused by *S. enterica serovar. typhi*. The disease usually begins insidiously after an incubation period of 7 - 14 days, with malaise, anorexia and headache, followed by the onset of fever. The ingested organisms multiply in the gastrointestinal tract, and some enter the intestinal lymphatics, from which they are disseminated throughout the body by the blood stream and are excreted in urine (Cheesbrough, 2006). The bile is a good culture medium for *S. typhi*, and so luxuriant growth occurs in the biliary tract and provides a continued flow of organisms into the small bowel (flat patches of lymphoid tissue situated in the small intestine but mainly in the ileum, seat of infection in typhoid fever) where they tend to localize in the payer's patches.

Their ability to persist in the biliary tract may result in a chronic carrier state, with continued excretion in the faeces (Cheesbrough, 2006). Fever often increases in a step-like manner and is accompanied by relative bradycardia (slow rate of heart contraction resulting in slow pulse rate). Diarrhoea is usually absent. Cough and signs of bronchitis may be present, "rose spots", which last for only a few days, may appear in the truck, and sphenomegaly and leucopenia are common (Cheesbrough, 2006). After the third week the fever usually subsides. In fatal case the most prominent (excessive formation of cells.) lesion found at autopsy is lymphoid hyperplasia, ulcerations in the payer's patches may lead to intestinal haemorhages or perforation of the bowel (Cheesbrough, 2006). In active lesions, bacilli are often detectable in the phagocytic monocular cells, where they can multiply intracellular bacilli seen to be protected from the bacteriolytic action of specific Antibody, which appears in the blood long before the disease subsides. The paratyphoid fever caused by *S. parartyphi* A and B is usually milder and have shorter incubating period (1 - 10 days). Bacterial infection occurs early; fever usually lasts for 1-3 weeks and rose spots are rare (Cheesbrough, 2006).

2.2.4 *Salmonella* septicaemia

It is characterized by high intermitent fever, and focal suppurative lesions may develop almost anywhere in the body including the brainy tract, kidneys, heart, spleen, meninges, joints and lungs. Prolonged septicaemia of this type is most commonly caused by *S. choleraesuis* (Cheesbrough, 2006).

2.2.5 Gastroenteritis

This is most commonly the kind of Salmonellae infection, primary confined to the gastrointestinal tract. The most frequent cause in the U.S is *S. typhimurium* (Cheesbrough, 2006). Symptoms begin 8 - 48hour after the consumption of contaminated food, with diarrhea, coughing from mild to fulminate (developing quickly with rapid termination) from the sudden and or let or set (food poisoning). Headache, chills and abdominal pain are followed by nausea, vommitting, and diarrhoea, accompanied by fever lasting from 1 - 4 days. Blood cultures are rarely positive, but the organisms can usually be cultured from faeces (Cheesbrough, 2006).

2.2.6 Carriers

Following active salmonellosis, the organisms occasionally become established in the host, who then continues indefinitely to excrete as many as 10^6 - 10^9 *S. typhi* per gram of faeces. The source is usually a chronic superlative focus in the bilary tract. When treated with broad – spectrum antibiotics carriers may come down with active Salmonellae disease (Cheesbrough, 2006).

2.2.7 Laboratory diagnosis of *Salmonella* infection

A diagnosis of salmonella's infection is made by isolation of this organism. Isolation from blood or urine establishes the diagnosis, but a salmonellae organism isolated from the faeces is not necessarily the cause of the individual's illnesses. Blood culture is often positive in the first week of the disease. Bone marrow cultures may be useful, urine cultures may be positive after the second week. Stool specimens must be taken repeatedly, in enteric fevers, the stools are positive from the second and third weeks or, in gastroenteritis, during the first week (Cheesbrough, 2006).

2.2.8 Immunity to *Salmonella* infection

Infection with *S. typhi, S. Paratypli and S. chottmulleri* usually confers certain dose of immunity. However, relapses may occur in 2 - 3 weeks after recovery in spite of antibodies (IgA) which may prevent attachment of salmonellae to intestinal epithelium (Cheesbrough, 2006).

2.2.9 Treatment of *Salmonella* infection

Definitive treatment of typhoid fever (enteric fever) is based on susceptibility. As a general principle of antimicrobial treatment, intermediate susceptibility should be regarded as equivalent to resistance. Between 1999 and 2006, 13% of *S. typhi* isolates collected in the United States were multidrug resistant (Multidrug-resistant *S. typhi* is, by definition, resistance to the original first-line agents, ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole.) as stated by Brusch *et al*. (2010).

Until susceptibilities are determined, antibiotic prescription should be empiric, for which there are various recommendations (Brusch *et al*., 2010). For typhoid fever, and salmonellae septicemia, chloramphenicol has long been the drug of choice, but chloramphenicol resistant strains have appeared (Galanis *et al*., 2006). Based on antibiotic sensitivity testing parenteral ampicillin and trimethoprime-sulfamethaxazole are usually effective alternatives. Plasmid mediated resistance to many antimicrobials has been observed (Helms *et al.,* 2002). Response to therapy is usually rapid, but because the organisms tend to survive within phagocytic cell, relapses frequently occur unless the patient is treated for at least 2 weeks (Cheesbrough, 2006). Antibiotics are not indicated in salmonellae gastroenteritis (except in the very young and those over 60) since the disease is brief but limited to the gastroenteritinal tract. In addition, the unnecessary use of antibiotics prolongs salmonellae excretion, promotes the incidence of the carrier state, and favours the acquisition of resistance by the infection strain (Cheesbrough, 2006). In schistomiasis endemic areas there is a high indence of chronic *S. typhi* and *S. paratypi A*. infections and carriers (Cheesbrough, 2006). The salmomellae colonize adult schistosome flukes (protected from antibiotics). An immume complex disorder of the kidneys can occur in those with urinary schistomiasis (nephrotyphoid), characterized by fever, oedema, marked albuminuria and haematuria. Infection with *S. typhi* can also cause osteomyelitis and typhoid arthritis particularly in those with sickle cell disease and thalassaemia (Cheesbrough, 2006). This can be biochemically differentiated from other Salmonellae by being citrate negative, not producing gas and forming only small amount of H_2S. Isolates of *S. typhi* can be identified serologically (Cheesbrough, 2006). In eliminating the carrier state the bactericidal ampicillin is much more effective than the bacteriostatic chloramphenicol. Prolonged treatment with large doses of ampicillin is effective in 60% - 80% of cases. In carriers who relapse after one or more course of therapy cholecytectomy truncates the carrier state in 98 out of 100 cases (Galanis *et al*., 2006).

2.2.10 Prevention of *Salmonella* infection

Most important in preventing the man to man transmissions of typhoid fever have been;

i) Proper sewage disposal and Pasteurization of milk

ii) Maintenance of unpolluted water supplies

Exclusion of chronic carriers as food handlers *S. typhi* infects only humans and its control is relatively feasible. The incidence of typhoid fever in them has been declining steadily (eg from 5593 cases in 1942 to 398 in 1977).

Eradication of human disease due to salmonellae that infect animals as well as man is difficult and also elimination of the animal reservoir is impossible (Zhao *et al.,* 2006). Domestic fouls probably constitutes the largest reservoir of almonellae having been isolated from 4% of apparently healthy chickens or turkeys and from many other domestic and wild animals including; turtles meat and pooled preparations of dived eggs. Practical ways for controlling salmonellae in animals only recently started and improved techniques of food processing should lower the incidence of human infections (Gautam *et al*., 2002).

2.3 *Salmonella* infections associated with animal

Salmonella is recognized as one of the major food-borne pathogens in the United States, causing an estimated 1.4 million cases of illness, approximately 20,000 hospitalizations, and more than 500 deaths annually (Paveen *et al.,* 2007). *Salmonellae* are a common cause of foodborne disease worldwide (Galanis *et al*., 2006). Although a growing number of human salmonellosis cases are associated with contaminated fruits and vegetables, traditionally, illness has been linked with consumption of contaminated food of animal origin, especially poultry and poultry products (Roy *et al.*, 2002). More problematic is the fact that antimicrobial resistance, in particular multidrug resistance (MDR), is being increasingly identified among numerous *Salmonella* serotypes recovered from animals and humans worldwide (Zhao *et al*., 2006). The levels and degree of resistance vary globally and are influenced by antimicrobial use practices in humans and animals and geographical variations in the epidemiology of *Salmonella* infections (Zhao *et al.,* 2006). *Salmonella* isolates showing resistance to clinically important antibiotics have been reported since the early 1960s, when most of the reported resistance was limited to a single antibiotic (Paveen *et al*., 2007*).* However, since the mid-1970s, there has been an increasing trend of *Salmonella* isolates exhibiting MDR phenotypes worldwide. The recovery of antimicrobial-resistant *Salmonella* in foods of animal origin has raised

concerns that the treatment of human salmonellosis may be compromised because antimicrobial-resistant strains appear to be more often associated with severe disease than are susceptible isolates (Helms *et al*., 2002); of significant concern is the isolation of *Salmonella* exhibiting decreased susceptibility to fluoroquinolones (e.g., ciprofloxacin) and extended-spectrum cephalosporins (e.g., ceftiofur and ceftriaxone) because these two antimicrobial agents are important in treating *Salmonella* infections in adults and children, respectively (Gupta *et al*., 2003). The majority of these antimicrobial-resistant phenotypes in *Salmonella* and other pathogens are gained from extrachromosomal genes that may impart resistance to an entire antimicrobial class. In recent years, a number of these resistance genes have been associated with large transferable plasmids on which may be other DNA mobile elements, such as transposons and integrons. Recent data indicate that different resistance determinants can amass in linked clusters, such that antimicrobials of a different class or substances such as disinfectants or heavy metals may select for MDR in bacteria (Harbottle *et al*., 2006). Although resistance, in particular MDR, appears to be most serious in certain serotypes, this situation may be shifting. Thus, there is a continuing need for increased surveillance of antimicrobial- resistant phenotypes in *Salmonella* isolates of animal and human origin on a global basis. The role of meat and poultry products in the dissemination of antimicrobial-resistant zoonotic bacterial pathogens is well documented (Larkin *et al.,* 2004), though, the hygienic standards for meat production was quites high (Paveen *et al*., 2007).

In most developed countries faecal contamination of meat products cannot be completely prevented. Procedures such as handling during processing also may contribute to cross-contamination among carcasses (Paveen *et al.,* 2007). Recently, several investigators suggested that processing conditions may play a significant role in promoting and influencing the selection of pathogens, including antimicrobial-resistant variants (Logue *et al.,* 2003). However, the factors that contribute to this selection have yet to be fully evaluated. Several studies have been conducted on the prevalence and antimicrobial resistance of *Salmonella* in processed poultry, poultry products, and poultry processing plants (Jam and Chen, 2006; Paveen *et al*., 2007).

2.4 *Salmonella* and the quinolones

The quinolone class of antibiotics comprises a relatively large and expanding group of synthetic compounds, and has since their discovery in the early 1960s evolved to become important and effective agents in the treatment of a wide range of bacterial infections (Haugum *et al*., 2006). The quinolones inhibit the bacterial enzymes, DNA gyrase and DNA topoisomerase, both of which are essential for bacterial DNA replication (Corbett *et al*., 2004). Bacterial type II DNA topoisomerases are A2B2 hetero tetramers (Corbett *et al*., 2004). DNA gyrase is composed of two *GyrA* and two *GyrB* subunits, encoded by *gyrA* and *gyrB* (Wang, 2002; Haugum *et al*., 2006) and topoisomerase by two *ParC* and two *ParE* subunits, encoded by *parC* and *parE* Type. DNA topoisomerases catalyse the ATP-dependent transport of one intact DNA double helix which give several topological transformations, including relaxation of positively or negatively supercoiled DNA and decatenation unlinking of double strand DNA rings (Wang, 2002). Quinolones act by binding to complexes that form between DNA and gyrase or topoisomerase and formation of this quinolone-enzyme-DNA complex that contains broken DNA inhibits DNA synthesis. Important mechanisms for quinolone resistance are mutations accumulating in the genes encoding DNA gyrase and topoisomerase *gyrA*, *gyrB, parC* and *parE* (Haugum *et al*., 2006; Hawkey, 2003). In *Salmonella and Escherichia coli*, the majority of mutations in DNA gyrase are found between residues *67* and *106* in *gyrA*, in a region called the quinolone resistance-determining region (QRDR). While some mutations in *parC* in salmonellae have been found (Eaves *et al*., 2004), between residues *57* and *84*, they may only be required to achieve high-level resistance (Hopkins *et al.,* 2005). Mutations in *gyrB* and *parE* are considered rare in salmonellae (Hopkins *et al*., 2005; Haugum *et al.,* 2006). Mutations in *gyrB* in salmonellae are found between residues *420* and *464*, and mutations in *parE* in salmonellae are found between residues 453 and 512 (Hopkins *et al*., 2005; Haugum *et al*., 2006). Other reported mechanisms for quinolone resistance are active efflux mechanisms, and decreased outer membrane permeability. In *E. coli*, the AcrAB-TolC system is an efflux system involved in multi-drug resistance (Cloeckaert and Chaslus-Dancla, 2001; Hopkins *et al*., 2005; Haugum *et al*., 2006). Over-expression of the AcrAB efflux pump has also been shown in strains of *Salmonella enterica* serovar *Typhimurium S. typhimurium* with reduced susceptibility to

fluoroquinolones (Cloeckaert and Chaslus-Dancla, 2001). There have been some reports on plasmid-mediated quinolone resistance, but only in *Klebsiella* and *E. coli* (Hopkins *et al.,* 2005; Haugum *et al*., 2006). Among salmonellae, and especially *Salmonella enterica* serovars *Enteritidis*, Hadar, *typhimurium* and Virchow, there have been reports of increase in quinolone resistance (Threlfall, 2002). In a previous study it was discovered that a geographically dependent distribution of *gyrA* mutation at codon *83* and *87* in *S. hadar* (Lindstedt *et al*., 2004). Earlier studies have observed that in salmonellae, the relative frequency of different mutations in *gyrA* was dependent on the quinolone antibiotic used for selection (Levy and Marshall, 2004; Haugum *et al*., 2006), and that the position and type of amino acid substitution in *gyrA* varied with the serovar (Haugum *et al*., 2006).

Among *S. typhi* isolates obtained in the United States between 1999 and 2006, 43% were resistant to at least one antibiotic. Nearly half of *S. typhi* isolates found in the United States now come from travelers to the Indian subcontinent, where fluoroquinolone resistance is endemic (Brusch *et al.,* 2010). The rate of fluoroquinolone resistance in the South and Southeast Asia and, to some extent, in East Asia is generally high and rising (Brusch *et al*., 2010). Susceptibility to chloramphenicol, TMP-SMZ, and ampicillin in these areas is rebounding. In Southeast Asia, MDR strains remain predominant, and some acquired resistance to fluoroquinolones by the early 2000s. Fluoroquinolones have become the first-line drugs for the treatment of typhoid fever (Hirose *et al*., 2002). Fluoroquinolones are active drugs against isolates of the *Salmonella* species (Hakanen *et al.,* 2001). There are several reports, however, of treatment failures when these antimicrobials have been used to treat *Salmonella* infections caused by strains with reduced fluoroquinolone susceptibility (Hakanen *et al*., 2001). However, some *Salmonella enterica* serovar *typhi* strains with decreased susceptibilities to fluoroquinolones have been already reported (Hirose *et al.,* 2001; Deak *et al.,* 2015). The emergence and spread of these organisms have been reported in developing countries (Hirose *et al.,* 2002). There is evidence that the incidence of strains that are resistant to nalidixic acid with decreased susceptibilities to the most recent fluoroquinolones used for the treatment of typhoid fever is on the increase. In most strains, the acquired fluoroquinolone resistance was attributed to mutations in the genes encoding DNA gyrase

(*gyrA, gyrB*) (Hirose *et al.,* 2002) or DNA topoisomerase IV (*parC, parE*) (Hirose *et al.,* 2002). According to Hirose *et al*. (2002), the mutations responsible for fluoroquinolone resistance in the *gyrA, gyrB, parC*, and *parE* genes of *Salmonella enterica* serovar *typhi* and serovar *Paratyphi A* were investigated and the sequences of the quinolone resistance-determining region (QRDRs) of the *gyrA* gene in clinical isolates which showed decreased susceptibilities to fluoroquinolones had a single mutation at either the *Ser-83* or the *Asp-87* codon, and no mutations were found in the *gyrB, parC*, and *parE* genes. According to Hirose *et al*. (2002), these findings indicate that *gyrA* mutations are of principal importance for the fluoroquinolone resistance of serovars *typhi and Paratyphi A*. Double mutations at positions *83* and *87* of the *gyrA* amino acid sequence were also reported in clinical isolates of serovar *Schwarzengrund*, which caused nosocomial infections in the United States and which exhibited ciprofloxacin resistance (Hirose *et al*., 2002). Although strains with high-level fluoroquinolone resistance due to double mutations at codons *83* and *87* in the *gyrA* amino acid sequence have not been found in clinical isolates of serovars *typhi and Paratyphi A*, several cases of the failure of treatment for typhoid fever due to strains with decreased susceptibilities to fluoroquinolones have been reported (Hirose *et al.*, 2002). The difference in fluoroquinolone resistance between two closely related species may be explained by differences in outer membrane permeabilities for fluoroquinolones and differences in active efflux activities (Hirose *et al*., 2002).

Fluoroquinolone resistance in *Salmonella enterica* is of clinical importance because ciprofloxacin is the drug of choice for treating invasive human salmonellosis (Eaves *et al*., 2004). Fluoroquinolone resistance in *S. enterica* is usually mediated by at least one mutation in a DNA topoisomerase gene *gyrA* resulting in elevated ciprofloxacin and levofloxacin MICs (0.12 to 0.5 µg/ml) according to Deak *et al.* (2015). However, in clinical human and veterinary isolates of *Salmonella* spp., mutations are usually confined to *gyrA*. Whilst a single mutation in *gyrA* on its own is not sufficient for clinical resistance to fluoroquinolones, a *gyrA* mutation is a good marker indicating that fluoroquinolones should not be chosen for treating the respective infection (Randall *et al*., 2005). According to Gaind *et al*. (2006) analysis revealed that in *S. typhi and paratyphi A*, a single *gyrA* mutation (*Ser-83-->Phe* or *Ser-83-->Tyr*) was associated with reduced

susceptibility to ciprofloxacin (MICs 0.125-1 mg/L); an additional mutation in *parC* (*Ser-80-->Ile*, *Ser-80-->Arg*, *Asp-69-->Glu* or *Gly-78-->Asp*) was accompanied by an increase in ciprofloxacin MIC (> or = 0.5 mg/L) (Gaind *et al*., 2006). They further stated that three mutations conferred ciprofloxacin resistance: two in *gyrA* (Ser-83-->Phe and *Asp-87-->Asn* or *Asp-87-->Gly*) and one in *parC*. This is the first report of *parC* mutations in *S. typhi*. Ciprofloxacin-resistant *S. typhi and S. paratyphi A,* differed in their MICs and mutations in *gyrA* and *parC*. Moreover *S. typhi* harboured a 50 kb transferable plasmid carrying a class 1 integron (*dfrA15/aadA1*) that confers resistance to co-trimoxazole and tetracycline but not to ciprofloxacin (Gaind *et al*., 2006). Pulse field gel electrophoresis (PFGE) revealed undistinguishable *XbaI* fragment patterns in ciprofloxacin-resistant *S. typhi* as well as in *S. paratyphi A* isolates and showed that ciprofloxacin-resistant *S. typhi* have emerged from a clonally related isolate with reduced susceptibility to ciprofloxacin after sequential acquisition of a second mutation in *gyrA* (Gaind *et al*., 2006). The presence of a plasmid-borne integron in ciprofloxacin-resistant *S. typhi* may lead to a situation of untreatable enteric fever (Gaind *et.al.,* 2006). According to Haugum *et al.* (2006), reporting their study on the effect of quinolone antibiotics and chemicals on mutation types in *Salmonella enterica* serovars Enteritidis, confirmed *gyrA* codon *83* and codon *87* as the main targets for mutations in *S. enteritidis.*

According to Hakanen *et al*. (2001), based on their preliminary report on fluoroquinolone, stated an increasing trend in quinolone resistance among Salmonella isolates classified as being of foreign origin in Finland. Among all 1,210 *Salmonella* isolates, 78 (6.4%) exhibited reduced susceptibility to ciprofloxacin (MIC greater than 0.125µg/ml) (Hakanen *et al*., 2001). Hakanen *et al*. (2001) conclusively reported that their analysis clearly showed that in the era of frequent international connections, microbes may be easily transmitted from one place to another. Correspondingly, factors furthering the emergence and spread of antimicrobial resistance in any country may soon have an impact on resistance of bacterial pathogens, or even of normal human flora, in faraway regions, even different continents. On this basis, the emergence of antimicrobial resistance in any part of the world may have a global bearing and thus deserves universal attention (Hakanen *et al*., 2001).

2.5 Effects of quinolone administration

All quinolones cause erosion of cartilage in weight-bearing joints. They may cause convulsions, increased intracranial pressure, toxic psychosis, CNS stimulation (i.e.nervousness, lightheadedness, confusion, hallucinations) and should not be used by anyone with seizure disorders, or cerebral arteriosclerosis (Haugum *et al*., 2006). There have been deaths due to anaphylactic shock, and cardiovascular collapse. Also occurring are tingling, itching, facial swelling, and difficult breathing. At the first sign of a rash anyone taking ciprofloxacine and having diarrhoea should immediately check with his prescribing physician (Haugum *et al*., 2006). Antibacterial drugs may kill off normal intestinal flora, resulting in an overgrowth of clostridia. It produces a toxin that is a primary cause of "antibiotic-associated- colitis". Achilles and other tendon ruptures requiring surgical repair, resulting in prolonged disability can occur from quinolone use. Hence, it is adviced to discontinue ciprofloxacin and consult your physician, if you experience pain, inflammation, or tendon rupture (Hirose *et al*., 2002; Haugum *et al.,* 2006).

Crystaluria (particles out of solution in urine) may occur, particularly if the urine is alkaline. While taking ciprofloxacin, hydration should be monitored (8 - 8oz glasses of water daily minimum) and drink orange or cranberry juice, or apple cider vinegar (2 teaspoon with 1 teaspoon honey in 8 oz water) to maintain acidity of the urine. Photosensitivity (sunburn) is reported to occur easily (Haugum *et al*., 2006). Although more than 2,500 serotypes have been reported, *Salmonella enterica serotype Typhimurium* is 1 of the leading serotypes causing salmonellosis worldwide (Galanis *et al*., 2006). Fluoroquinolones such as ciprofloxacin are strongly recommended for treatment of severe *S. typhimurium* infections in adults (Guerrant *et al*., 2001; Cui *et al*., 2008).

2.6 Antibiotic resistance associated with extended-spectrum beta-lactamase (ESBL) production

Among Gram-negative bacteria, the emergence of resistance to expanded-spectrum cephalosporins has been a major concern. It appeared initially in a limited number of bacterial species that could mutate to hyperproduce their chromosomal class C β-lactamase. A few years later, resistance appeared in bacterial species not naturally-producing AmpC enzymes *(K. pneumoniae, Salmonella spp., P. mirabilis)* due to the production of TEM- or SHV-type ESBLs. Characteristically, such resistance has included oxyimino- (for example ceftizoxime, cefotaxime , ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam aztreonam), but not 7-alpha-methoxy-cephalosporins (cephamycins) (Paterson *et al*., 2003 ;Woodford *et al*., 2006). Plasmid-mediated AmpC β-lactamases represent a new threat, since they confer resistance to 7-alpha-methoxy-cephalosporins (cephamycins) such as cefoxitin or cefotetan are not affected by commercially-available β-lactamase inhibitors, and can, in strains with loss of outer membrane porins, provide resistance to carbapenems (Woodford *et al*., 2006). Studies have also shown that resistance to broad-spectrum β-lactams is highly mediated by extended-spectrum β-lactamase (ESBL) enzymes, increasing the world health problem in clinical settings (Yujuan & Ling, 2006; Valverde *et al*., 2008; Yah, 2010). According to Yah (2010), the plasmid-borne β-lactamases are also competent enough to hydrolyze β-lactam antibiotics, as well as the mechanism of resistance to β-lactam agents among gram-negative bacteria (Yah, 2010).

Members of the family Enterobacteriaceae commonly express plasmid-encoded β-lactamases (e.g., TEM-1, TEM-2, and SHV-1) which confer resistance to penicillins but not to expanded-spectrum cephalosporins. In the mid-1980s, a new group of enzymes, the extended-spectrum b-lactamases (ESBLs), was detected (First detected in Germany in 1983) (Philippon *et al*., 2002; George *et al*., 2005). ESBLs are beta-lactamases that hydrolyze extended-spectrum cephalosporins with an oxyimino side chain. These cephalosporins include cefotaxime, ceftriaxone, and ceftazidime, as well as the oxyimino-monobactam aztreonam. Thus ESBLs confer resistance to these antibiotics and related oxyimino-beta lactams (Philippon *et al*., 2002). In general, an isolate is suspected

to be an ESBL producer when it shows in vitro susceptibility to the second-generation cephalosporins (cefoxitin, cefotetan) but resistance to the third-generation cephalosporins and to aztreonam (Woodford *et al*., 2006). Moreover, one should suspect these strains when treatment with these agents for Gram-negative infections fails despite reported in vitro susceptibility. Once an ESBL-producing strain is detected, the laboratory should report it as "resistant" to all penicillins, cephalosporins, and aztreonam, even if it is tested (in vitro) as susceptible (Woodford *et al*., 2006). Associated resistance to aminoglycosides and trimethoprim-sulfamethoxazole, as well as high frequency of co-existence of fluoroquinolone resistance, creates problems. Beta-lactamase inhibitors such as clavulanate, sulbactam, and tazobactam in vitro inhibit most ESBLs, but the clinical effectiveness of beta-lactam/beta-lactamase inhibitor combinations cannot be relied on consistently for therapy (Woodford *et al*., 2006). Cephamycins (cefoxitin and cefotetan) are not hydrolyzed by majority of ESBLs, but are hydrolyzed by associated AmpC-type β-lactamase (AmpC type β-lactamases are commonly isolated from extended-spectrum cephalosporin-resistant Gram-negative bacteria.

The characteristics of *β*- lactamases is based on the nucleotide and amino acid sequences in these enzymes (Paterson *et al*., 2003). AmpC β-lactamases (also termed class C or group 1 are typically encoded on the chromosome of many Gram-negative bacteria including *Citrobacter*, *Serratia* and *Enterobacter* species where its expression is usually inducible (Philippon *et al*., 2002). Also, β-lactam/β-lactamase inhibitor combinations may not be effective against organisms that produce AmpC-type β-lactamase (Lee *et al*., 2004). Sometimes these strains decrease the expression of outer membrane proteins, rendering them resistant to cephamycins. In vivo studies have yielded mixed results against ESBL-producing *K. pneumoniae*. (Cefepime, a fourth-generation cephalosporin, which has demonstrated an in-vitro stability in the presence of many ESBL/AmpC strains). In typical circumstances, genes from TEM-1, TEM-2, or SHV-1 are altered in their amino acid configuration around the active site by mutations (Kim *et al*., 2006). This extends the spectrum of β-lactam antibiotics susceptible to hydrolysis by these enzymes. The ESBLs are frequently plasmid encoded. Plasmids responsible for ESBL production frequently carry genes encoding resistance to other drug classes (for example, aminoglycosides). Therefore, antibiotic options in the treatment of ESBL-producing

organisms are extremely limited (Kim *et al*., 2006). Carbapenems are the treatment of choice for serious infections due to ESBL-producing organisms, yet carbapenem-resistant isolates have recently been reported (Kim *et al*., 2006). ESBL-producing organisms may appear susceptible to some extended-spectrum cephalosporins. However, treatment with such antibiotics has been associated with high failure rates (Philippon *et al*., 2002; George *et al*., 2005). According to Woodford *et al*. (2006) CTX-M beta-lactamases (class A) enzymes were named for their greater activity against cefotaxime than other oxyimino-beta-lactam substrates (e.g., ceftazidime, ceftriaxone, or cefepime). Rather than arising by mutation, they represent examples of plasmid acquisition of beta-lactamase genes normally found on the chromosome of *Kluyvera* species, a group of rarely pathogenic commensal organisms. These enzymes are not very closely related to TEM or SHV beta-lactamases in that they show only approximately 40% identity with these two commonly isolated beta-lactamases (Woodford *et al*., 2006). More than 80 CTX-M enzymes are currently known. Despite their name, a few are more active on ceftazidime than cefotaxime (Woodford *et al*., 2006). They have mainly been found in strains of *Salmonella enterica* serovar *Typhimurium* and *E. coli*, but have also been described in other species of Enterobacteriaceae and are the predominant ESBL type in parts of South America. (They are also seen in Eastern Europe) CTX-M-14, CTX-M-3, and CTX-M-2 are the most widespread. CTX-M-15 is currently (Woodford *et al.,* 2006) the most widespread type in *E. coli* the UK and is widely prevalent in the community (Woodford *et al*., 2006).

Although the inhibitor-resistant β-lactamases are not ESBLs, they are often discussed with ESBLs because they are also derivatives of the classical TEM- or SHV-type enzymes (Bradford, 2001). These enzymes were at first given the designation IRT for inhibitor-resistant TEM β-lactamase; however, all have subsequently been renamed with numerical TEM designations. There are at least 19 distinct inhibitor-resistant TEM β-lactamases. Inhibitor-resistant TEM β-lactamases have been found mainly in clinical isolates of *E. coli*, but also some strains of *K. pneumoniae*, *Klebsiella oxytoca*, *P. mirabilis*, and *Citrobacter freundii* (Bradford, 2001). Although the inhibitor-resistant TEM variants are resistant to inhibition by clavulanic acid and sulbactam, thereby showing clinical resistance to the beta-lactam/ lactamase inhibitor combinations of

amoxicillin-clavulanate (co-amoxaclav), ticarcillin-clavulanate, and ampicillin /sulbactam, they normally remain susceptible to inhibition by tazobactam and subsequently the combination of piperacillin / tazobactam, although resistance has been described. To date, these beta-lactamases have primarily been detected in France and a few other locations within Europe (Bradford, 2001). Also, according to Bradford (2001), Paterson *et al*. (2003), George *et al*. (2005), TEM-1 is the most commonly-encountered Beta-lactamase in Gram-negative bacteria which belong to the TEM beta-lactamases (class A). Up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1. Also responsible for the ampicillin and penicillin resistance seen in *H. influenzae* and *N. gonorrhoeae* in increasing numbers. Although TEM-type beta-lactamases are most often found in *E. coli* and *K. pneumoniae*, they are also found in other species of Gram-negative bacteria with increasing frequency.

The amino acid substitutions responsible for the ESBL phenotype cluster around the active site of the enzyme changed its configuration, allowing access to oxyimino-beta-lactam substrates (Bradford, 2001; Paterson *et al*., 2003; George *et al*., 2005). Opening the active site to beta-lactam substrates also typically enhances the susceptibility of the enzyme to b-lactamase inhibitors, such as clavulanic acid. Single amino acid substitutions at positions *104, 164*, *238*, and *240* produce the ESBL phenotype, but ESBLs with the broadest spectrum usually have more than a single amino acid substitution. Based upon different combinations of changes, currently *140* TEM-type enzymes have been described. TEM-10, TEM-12, and TEM-26 are among the most common in the United States (Bradford, 2001; Paterson *et al*., 2003; George *et al*., 2005), while SHV-1 which is in the SHV beta-lactamases (class A) shares 68 percent of its amino acids with TEM-1 and has a similar overall structure, the SHV-1 beta-lactamase is responsible for up to 20% of the plasmid-mediated ampicillin resistance in this species of *E. coli* and *K. pneumoniae*. SHV-1, the original member of the SHV β-lactamase family, is present in most strains of *Klebsiella pneumoniae* and may be either chromosomally or plasmid mediated (Bradford, 1999). ESBLs in this family also have amino acid changes around the active site, most commonly at positions *238* or *238* and *240*. More than 60 SHV varieties are known. They are the predominant ESBL type in Europe and the United States and are found worldwide. SHV-5 and SHV-12 are among the most common

(Paterson *et al*., 2003). While ESBL-producing organisms were previously associated with hospitals and institutional care, these organisms are now increasingly found in the community. CTX-M-15-positive *E.coli* are a cause of community-acquired urinary infections in the UK, (Woodford *et al*., 2006) and tend to be resistant to all oral β-lactam antibiotics, as well as quinolones and sulfonamides (Woodford *et al*., 2006).

2.7 Overview of the quinolones

The quinolones are a family of synthetic broad-spectrum antibiotics. The term quinolones (s) refers to potent synthetic chemotherapeutic antibacterials (Nelson *et al*., 2007; Ivanov, and Budanov, 2006). The first generation of the quinolones begins with the introduction of nalidixic acid in 1962 for treatment of urinary tract infection in humans (Sanofi-Aventis, 2008). Quinolones and fluoroquinolone are chemotherapeutic bactericidal drugs, eradicating bacteria by interfering with DNA replication. The other antibiotics used today, (e.g., tetracyclines, lincomycin, erythromycin, and chloramphenicol) do not interact with components of eukaryotic ribosomal particles and, thus, have not been shown to be toxic to eukaryotes, (Murray *et al*., 2006) as opposed to the fluoroquinolone class of drugs. Other drugs used to treat bacterial infections, such as penicillins and cephalosporins, inhibit cell wall biosynthesis, thereby causing bacterial cell death, as opposed to the interference with DNA replication as seen within the fluoroquinolone class of drugs (Ambrose and Owens, 2000). Quinolones inhibit the bacterial DNA gyrase or the topoisomerase II enzyme, thereby inhibiting DNA replication and transcription (Ambrose and Owens, 2000). Quinolone-induced DNA damage was first reported in 1986 (Lamb, 2008; Lardizabal, 2009). There continues to be debate as to whether or not this DNA damage is to be considered one of the mechanisms of action concerning the severe and non-abating adverse reactions experienced by some patients following fluoroquinolone therapy (Sissi and Palumbo, 2003; Lardizabal, 2009).

Recent evidence has shown that topoisomerase II is also a target for a variety of quinolone-based drugs. Quinolones can enter cells easily via porins and, therefore, are often used to treat intracellular pathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae*. For many Gram-negative bacteria, DNA gyrase is the target,

whereas topoisomerase IV is the target for many Gram-positive bacteria. It is believed that eukaryotic cells do not contain DNA gyrase or topoisomerase IV. However, there is debate concerning whether the quinolones still have such an adverse effect on the DNA of healthy cells, in the manner described above, hence contributing to their adverse safety profile. This class has been shown to damage mitochondrial DNA (Kaplowitz, 2005). Resistance to quinolones can evolve rapidly, even during a course of treatment. Numerous pathogens, including *Staphylococcus aureus*, enterococci, and *Streptococcus pyogenes* now exhibit resistance worldwide (Jacobs, 2005). Widespread veterinary usage of quinolones, in particular in Europe, has been implicated (Nelson *et al*., 2007). There are three known mechanisms of resistance (Robicsek *et al*., 2006). Some types of efflux pumps can act to decrease intracellular quinolone concentration (Ambrose and Owens, 2000). In Gram-negative bacteria, plasmid-mediated resistance genes produce proteins that can bind to DNA gyrase, protecting it from the action of quinolones. Finally, mutations at key sites in DNA gyrase or topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drugs' effectiveness. They prevent bacterial DNA from unwinding and duplicating (Hooper, 2001). Quinolones in comparison to other antibiotic classes have the highest risk of causing colonization with MRSA and *Clostridium difficile*. Finally, mutations at key sites in DNA gyrase or Topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drug's effectiveness (Liu and Mulholland, 2005). A general avoidance of fluoroquinolone is recommended based on the available evidence and clinical guidelines (Muto *et al*., 2003; Tacconelli *et al*., 2008; Vonberg, 2009). Though it is generally accepted that nalidixic acid is to be considered the first quinolone drug, this has been disputed over the years by a few researchers that believed that chloroquine, from which nalidixic acid is derived, should be considered the first quinolone drug, rather than nalidixic acid. Since the introduction of nalidixic acid in 1962, more than 10,000 analogs have been synthesized but only a handful have found their way into clinical practice (Stacy and Childs, 2000). Floroquinolones are not recommended as first-line antibiotics for acute sinusitis, as this condition is usually self-limiting, and the risks outweigh the benefits in comparison to other antibiotic classes (Karageorgopoulos *et al*., 2008; Le Saux, 2008). The basic pharmacophore, or active structure, of the fluoroquinolone class is based upon the

quinoline ring system (Schaumann, and Rodloff, 2007). The addition of the fluorine atom at C6 is what distinguishes the successive-generation floroquinolones from the first-generation quinolones. It has since been demonstrated that the addition of the C6 fluorine atom is not a necessary requirement for the antibacterial activity (Ambrose and Owens, 2000). The first generation is rarely used today. A number of the second-, third-, and fourth-generation drugs have been removed from clinical practice due to severe toxicity issues or discontinued by their manufacturers. The drugs most frequently prescribed today consist of Avelox (moxifloxacin), Cipro (ciprofloxacin), Levaquin (levofloxacin), and, to some extent, their generic equivalents.

First-generation quinolone;

- cinoxacin (Cinobac) (Removed from clinical use, according to Oliphant and Green (2002),
- flumequin (Flubactin) (Genotoxic carcinogen)(Veterinary use)
- nalidixic (NegGam, Wintomylon)[1] (Genotoxic carcinogen)
- oxolic acid (Uroxin) (Currently unavailable in the United States)
- piromidic acid (Panacid) (Currently unavailable in the United States)
- pipemidic acid (Dolcol) (Currently unavailable in the United States)
- rosoxacin (Eradacil) (Restricted use, currently unavailable in the United States) (Oliphant and Green, 2002).

Second-generation quinolone;

The second-generation class is sometimes subdivided into "Class 1" and "Class 2" (Oliphant, and Green, 2002)

-ciprofloxacin (Zoxan, Ciprobay, Cipro, Ciproxin)
- enoxacin (Enroxil, Penetrex) (Removed from clinical use)
-fleroxacin (Megalone, Roquinol) (Removed from clinical use)
-lomefloxacin (Maxaquin) (Discontinued in the United States)
- nadifloxacin (Acuatim, Nadoxin, Nadixa) (Currently unavailable in the United States)
- norfloxacin (Lexinor, Noroxin, Quinabic, Janacin) (restricted use)
- ofloxacin (Floxin, Oxaldin, Tarivid) (Only as ophthalmic in the United States)
- pefloxacin (Peflacine) (Currently unavailable in the United States)

-rufloxacin (Uroflox) (Currently unavailable in the United States) (Oliphant and Green, 2002).

Third-generation quinolone;

Unlike the first- and second-generations, the third-generation is active against *Streptococcus* (Ambrose and Owens, 2000)

-balofloxacin (Baloxin) (Currently unavailable in the United States),

- grepafloxacin (Raxar) (Removed from clinical use),

- levofloxacin (Cravit, Levaquin) ,

-pazufloxacin (Pasil, Pazucross) (Currently unavailable in the United States)

-sparfloxacin (Zagam) (Currently unavailable in the United States),

- temafloxacin (Omniflox) (Removed from clinical use),

-tosufloxacin (Ozex, Tosacin) (Currently unavailable in the United States) (Oliphant and Green, 2002; Ambrose and Owens, 2000).

Fourth-generation quinolone;

Fourth generation fluoroquinolones act at DNA gyrase and topoisomerase IV (Gupta, 2009). This dual action slows development of resistance.

-clinafloxacin (Currently unavailable in the United States (Ambrose and Owens, 2000)

-gatifloxacin (Zigat, Tequin) (Zymar -opth.) (Tequin removed from clinical use)

-gemifloxacin (Factive)(Currently unavailable in the United States)

- moxifloxacin (Avelox,Vigamox) (restricted use).

-sitafloxacin (Gracevit) (Currently unavailable in the United States)

-trovafloxacin (Trovan) (Removed from clinical use) Oliphant and Green, 2002)

- prulifloxacin (Quisnon) (Currently unavailable in the United States) in development (Ambrose and Owens, 2000)

- garenoxacin (Geninax)(Application withdrawn due to toxicity issues)

- delafloxacin

The quinolones have been widely used in agriculture, and several agents that have veterinary but not human use exist such as; danofloxacin (Advocin, Advocid) difloxacin (Dicural, Vetequinon), enrofloxacin (Baytril) Ibaflin), marbofloxacin (Marbocyl, Zenequin), orbifloxacin (Orbax, Victas), sarafloxacin (Floxasol, Saraflox, Sarafin) (Ambrose and Owens, 2000).

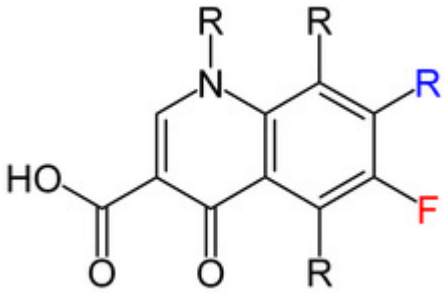

Figure 2.1: Essential structure of all quinolone antibiotics (Sanofi-Aventis, 2008): The blue drawn remainder of R is usually piperazine; if the connection contains fluorine (red), it is a fluoroquinolone.

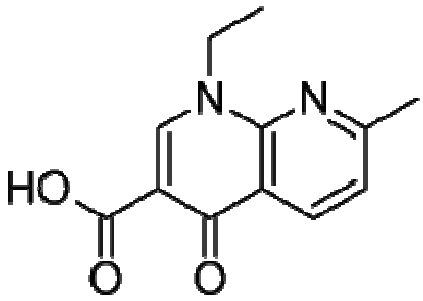

Figure 2.2: Structure of Nalidixic acid (Sanofi – Aventis, 2008).

2.8 Levofloxacin

Levofloxacin, a fluoroquinolone is a synthetic chemotherapeutic antibiotic of the quinolone class (Nelson *et al*., 2007) and is used to treat severe or life-threatening bacterial infections or bacterial infections that have failed to respond to other antibiotic classes (Liu and Mulholland, 2005; MacDougall *et al*., 2005). It is sold under various brand names, such as Levaquin and Tavanic, the most common. In form of ophthalmic solutions, it is known as Oftaquix, Quixin, Edolev, L-flox and Iquix etc. The Systematic

(IUPAC) name is (S)-7-fluoro-6-(4-methylpiperazin-1-yl) -10-oxo-4-thia-1-azatricyclo[7.3.1.$0^{5,13}$] trideca-5(13),6,8,11-tetraene-11-carboxylic acid with Molecular formulae $C_{18}H_{20}FN_3O_4$ and molar mass of 361.368g/mol. (Nelson *et al*., 2009; Corbett and Berger, 2004).

Levofloxacin is a chiral fluorinated carboxyquinolone. Investigation of ofloxacin, an older drug that is the racemic mixture, found that the l form [the (-)-(*S*) enantiomer] is more active. This specific component is levofloxacin (Janssen Pharmaceuticals, 2008). Levofloxacin interacts with a number of other drugs, as well as a number of herbal and natural supplements. Such interactions increase the risk of cardiotoxicity and arrhythmias, anticoagulation, the formation of non-absorbable complexes, as well as increasing the risk of toxicity (DrugBank, 2009). Levofloxacin is associated with a number of serious and life-threatening adverse reactions as well as spontaneous tendon ruptures and irreversible peripheral neuropathy. Such reactions may manifest long after therapy had been completed and in severe cases may result in life-long disabilities. Hepatoxicity has also been reported with the use of levofloxacin. (Albrecht, 2007; Albrecht, 2008). Levofloxacin is a broad-spectrum, third-generation fluoroquinolone antibiotic and optically active L-isomer of ofloxacin with antibacterial activity. Levofloxacin diffuses through the bacterial cell wall and acts by inhibiting DNA gyrase (bacterial topoisomerase II), an enzyme required for DNA replication, RNA transcription, and repair of bacterial DNA. Inhibition of DNA gyrase activity leads to blockage of bacterial cell growth (Liu, and Mulholland, 2005)

Levofloxacin was first patented in 1987 (Levofloxacin European patent – Daiichi Pharmaceutical Co., Ltd.) and was approved by the United States Food and Drug Administration on December 20, 1996 (Nelson *et al*., 2007) for use in the United States to treat severe and life-threatening bacterial infections. Within a significant number of medical publications and books, levofloxacin was described as a second generation fluoroquinolone (Lamb, 2008) whereas within a number of medical web sites it has been described as a third-generation fluoroquinolone (Lamb, 2008). Levofloxacin is considered to be same as ofloxacin by the U.S. Food and Drug Administration (FDA), with the exception of the potency shown *in vitro* against mycobacteria. In vitro, it is, in

general, twice as potent as ofloxacin, whereas d-ofloxacin is less active against mycobacteria (Nelson *et al*., 2007). Levofloxacin is limited to the treatment of proven serious and life-threatening bacterial infections such as urinary tract infections, community-acquired pneumonia, skin and skin structure infections, nosocomial pneumonia, chronic bacterial prostatitis (Albrecht, 2007; Albrecht, 2008).

Levofloxacin has shown moderate activity against anaerobes, and about twice as potent as ofloxacin against *Mycobacterium tuberculosis* and other mycobacteria, including *Mycobacterium avium* complex (Nelson *et al*., 2007; Albrecht, 2007; Albrecht, 2008). Although claimed to be effective, levofloxacin is not to be considered a first line agent for inhalational anthrax in the pediatric population due to severe adverse reactions involving the musculoskeletal system and other serious adverse reactions, including fatalities (Nelson *et al*., 2007; Albrecht, 2007; Albrecht, 2008).

The Center For Disease Control (CDC) revoked its recommendation regarding the use of fluoroquinolones (ciprofloxacin) as a first line agent in treating anthrax (in part) due to the risk of adverse reactions documented within the Antimicrobial Postexposure Prophylaxis for Anthrax study (aka Cipro 60-day study), (Dolui *et al*., 2007). However, the fluoroquinolones are licensed to treat lower respiratory infections in children with cystic fibrosis in the UK (Dolui *et al*., 2007; Johnson & Johnson, 2009). Caution should be exercised in prescribing to patients with liver disease. Levofloxacin is also considered to be contraindicated in patients with epilepsy or other seizure disorders (Albrecht, 2008; Janssen Pharmaceuticals, 2008). Research indicates that the fluoroquinolones can rapidly cross the blood-placenta and blood-milk barriers, and are extensively distributed into the fetal tissues (Cahill *et al*., 2005). Peak concentration in human breast milk is similar to levels attained in plasma. Breast-feeding mothers that take levofloxacin may expose their infants to severe adverse reactions, and pregnant women are at risk of spontaneous abortions and birth defects (Nardiello *et al*., 2002; Cahill *et al*., 2005). For this reason, the prescription of levofloxacin is contraindicated during pregnancy. Other fluoroquinolones have also been reported to be present in the mother's milk and are passed on to the nursing child (Cahill *et al.,* 2005; Shin *et al.,* 2003). Oral and I.V. Levofloxacin is not licensed for use in the pediatric population, except as noted above,

due to the risk of serious, life-threatening and permanent injury to the pediatric patient (Nardiello *et al*., 2002). Within one study, a pediatric patient has a 3.8% chance of experiencing a serious musculoskeletal adverse event (Noel *et al*., 2007).

However, the two most recent pediatric studies involving the use of levofloxacin indicated paediatric patient has a greater than 50% chance of experiencing one or more adverse reactions (Noel *et al*., 2007). Within the first paediatric study, it is stated that 275 (52%) levofloxacin-treated subjects experienced one or more adverse event, out of the total 712 subjects tested. Serious adverse events were reported in 33 (6%) levofloxacin-treated subjects. Two serious adverse events in levofloxacin-treated subjects resulted in fatal outcomes. Within the second pediatric study, 122 experienced one or more adverse events, out of the 204 subjectes tested. Twelve subjects (6%) discontinued the drug study due to an adverse event. Seven subjects (3%) experienced 8 serious adverse events (Albrecht, 2008).

Levofloxacin pharmacokinetics is linear and predictable after single and multiple oral or I.V dosing regimens. Levofloxacin is rapidly and, in essence, completely absorbed after oral administration. Peak plasma concentrations are usually attained one to two hours after oral dosing. The plasma concentration profile of Levofloxacin after I.V administration was similar and comparable in extent of exposure (AUC) to that observed for levaquin tablets when equal doses (mg/mg) are administered (Janssen Pharmaceuticals, 2008; Noel *et al*., 2007). Levofloxacin is excreted largely unchanged in the urine. Its mean terminal plasma elimination half-life ranges from approximately 6 to 8 hours following single or multiple doses of Levofloxacin given orally or intravenously (Janssen Pharmaceuticals, 2008; Noel *et al*., 2007). Levofloxacin is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria.

Levofloxacin should be used only to treat or prevent infections that are proven or strongly suspected to be caused by susceptible bacteria (Albrecht, 2008). Normally, levofloxacin should only be used in patients who have failed at least one prior therapy. It is reserved for use in patients who are seriously ill that require immediate hospitalization (Johnson and Johnson, 2004; Janssen Pharmaceuticals, 2008; Shin *et al*., 2003). Though, it was

considered to be a very important and necessary drug required to treat severe life threatening bacterial infections. The associated prescription abuse of levofloxacin remains unchecked, which has contributed to the problem of bacterial resistance. The overuse of antibiotics, such as what happens with children suffering from otitis media, has given rise to super-bacteria that are resistant to antibiotics entirely (Fraunfelder and Fraunfelder, 2009; Shin *et al*., 2003). Fluoroquinolones, including levofloxacin, had become the most commonly prescribed class of antibiotics to adults in 2002. Nearly half (42%) of these prescriptions were for conditions not approved by the FDA, such as acute bronchitis, otitis media, and acute upper respiratory tract infection, according to a study that was supported in part by the Agency for Healthcare Research and Quality (Linder *et al*., 2005; Grépinet *et al*., 2008; Shin *et al*., 2003) In addition, they are commonly prescribed for medical conditions that are not even bacterial to beging with such as viral infections, or those to which no proven benefit exists.

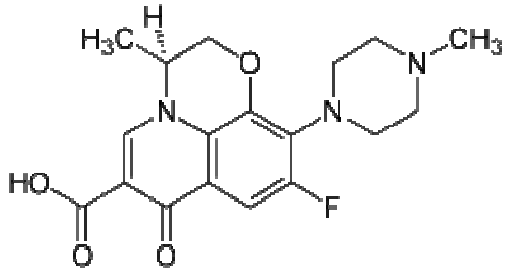

Figure 2.3: Structure of Levofloxacin (Sanofi-Aventis, 2008)

2.9 Ciprofloxacin

The Systematic (IUPAC) name of ciprofloxacin is 1-cyclopropyl- 6-fluoro- 4-oxo- 7-piperazin- 1-yl- quinoline- 3-carboxylic acid. The molecular formula is $C_{17}H_{18}FN_3O3$ with the molar mass 331.346 (Nelson *et al.,* 2007). Ciprofloxacin hydrochloride (USP) is

the monohydrochloride monohydrate salt of ciprofloxacin. Ciprofloxacin is a synthetic chemotherapeutic antibiotic of the fluoroquinolone class of drug (Nelson *et al*., 2007) and was was first patented in 1983 by Bayer A.G. and subsequently approved by the United States Food and Drug Administration (FDA) in 1987. Ciprofloxacin has 12 FDA-approved human uses and other veterinary uses, but it is often used for non-approved uses (off-label) (Bayer Corporation, 2001; Nelson *et al*., 2007). Ciprofloxacin interacts with other drugs, herbal and natural supplements, and thyroid medications (Cooper *et al*., 2005). It is a broad-spectrum antibiotic that is active against both Gram-positive and Gram-negative bacteria and functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV (Nelson *et al*., 2007) enzymes necessary to separate bacterial DNA, thereby inhibiting cell division (Jam and Chen, 2006)

This mechanism can also affect mammalian cell replication. In particular, some congeners of this drug family (for example those that contain the C-8 fluorine) display high activity not only against bacterial topoisomerases but also against eukaryotic topoisomerases and are toxic to cultured mammalian cells and *in vivo* tumor models. Although quinolones are highly toxic to mammalian cells in culture, its mechanism of cytotoxic action is not known. Recent studies have demonstrated a correlation between mammalian cell cytotoxicity of the quinolones and the induction of micronuclei (Jam and Chen, 2006) As such some fluoroquinolones may cause injury to the chromosome of eukaryotic cells (Jam and Chen, 2006).

Oral and intravenous fluoroquinolones are not licensed by the U.S. FDA for use in children due to the risk of permanent injury to the musculoskeletal system, with two exceptions as outlined below (Connerton *et al*., 2000; Galanis *et al*., 2006). Within the studies submitted in response to a Pediatric Written Request, the rate of arthropathy was reported to be 9.3% and 13.6% in one month and year respectively (Connerton *et al*., 2000), as such the pediatric use of ciprofloxacin is restricted to proven complicated urinary tract infections and pyelonephritis due to *E. coli* and inhalation anthax (Bayer Corporation, 2001). Although claimed to be effective, ciprofloxacin is not to be considered a first line agent for inhalation anthrax in the pediatric population (Threlfall *et al*., 2001; Galanis *et al*., 2006). The CDC revoked its recommendation regarding the use

of ciprofloxacin as a first line agent in treating anthrax due to the unacceptable risk documented within the Antimicrobial Postexposure Prophylaxis for Anthrax study (aka Cipro 60 day study) (Dolui *et al*., 2007). However, the fluoroquinolones are licensed to treat lower respiratory infections in children with cystic fibrosis in the U.K (Bayer Corporation, 2001). Current recommendations by the American Academy of Pediatrics noted that the systemic use of ciprofloxacin in children should be restricted to infections caused by multidrug resistant pathogens or when no safe or effective alternatives are available (Robicsek *et al*., 2006).

Ciprofloxacin is not recommended to treat CAP (community acquired pneumonia) as a stand-alone first-line agent. The current guidelines (Infectious Diseases Society of America) (Dolui *et al.,* 2007) stated that, in very limited circumstances, ciprofloxacin or levofloxacin should be combined with other drugs such as a ßeta-lactam drug to treat specific CAP infections, but neither drug was recommended to be used separately as a stand-alone first-line agent (Nelson *et al*., 2007). In addition, the current guidelines stated that: "Data exist, suggesting that resistance to macrolides and older fluoroquinolones (ciprofloxacin and levofloxacin) resulted in clinical failure (Nelson *et al.,* 2007). Other studies have shown that repeated use of fluoroquinolones predicted an increased risk of infection with fluoroquinolone-resistant pneumococci (Hooper, 2001). As noted above, under licensed use, ciprofloxacin was also considered to be contraindicated for the treatment of certain sexually transmitted diseases by some experts due to bacterial resistance (Hooper, 2001). Due to growing prevalence of antibiotic resistance to the fluoroquinolones in the Southeast Asia, the use of ciprofloxacin in patients have been discouraged (Nelson *et al*., 2007; Brusch *et al*., 2010). Ciprofloxacin is also considered contraindicated within the pediatric population (except for the indications outlined under licensed use above), pregnancy, nursing mothers, and in patients with epilepsy or other seizure disorders (Brusch *et al*., 2010).

The fluoroquinolones have also been reported as being present in the mother's milk and are passed on to the child, which may increase the risk of the child suffering from this syndrome as well, even though the child had never been prescribed with or taken any of the drugs found within this class (Brusch *et al*., 2010). Overdose of ciprofloxacin may

result in reversible renal toxicity. Treatment of overdose included emptying of the stomach via induced vomiting or by gastric lavage. Careful monitoring and supportive treatment, monitoring of renal function and maintaining adequate hydration are recommended by the manufacturer (Brusch *et al*., 2010). Ciprofloxacin is commonly used for urinary tract and intestinal infections (traveler's diarrhoea) and was once considered a powerful antibiotic of last resort (Jacobs, 2005), used to treat especially persistent infections. Not all physicians agreed with this assessment, as evidenced by its wide-spread use to treat minor infections as well as non-approved uses. As a result, in recent years, many bacteria have developed resistance to this drug, leaving it significantly less effective than it would have been otherwise (Jacobs, 2005) Resistance to ciprofloxacin and other fluoroquinolones may evolve rapidly, even during a course of treatment. Numerous pathogens, including *Staphylococcus aureus*, enterococci, and *Streptococcus pyogenes* now exhibit resistance worldwide (Tacconelli *et al*., 2008). Widespread veterinary usage of the fluoroquinolones, particularly in Europe, has been implicated (Nelson *et al*., 2007).

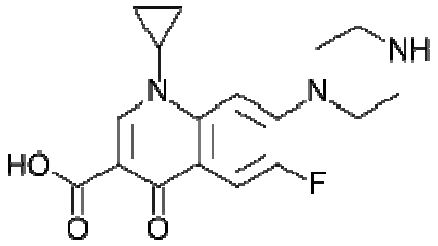

Figure 2.4: Structure of ciprofloxacin (Sanofi-Aventis, 2008).

2.10 Cephalosporins

The Cephalosporins are a class of β-lactam antibiotics originally derived from the fungus *Acremonium*, which was previously known as *Cephalosporium* (Stork, 2006; Pegler and Healy, 2007). Cephalosporins are indicated for the prophylaxis and treatment of

infections caused by susceptible bacteria. First-generation cephalosporins are active predominantly against Gram-positive bacteria, and successive generations have increased activity against Gram-negative bacteria (albeit often with reduced activity against Gram-positive organisms) (Richard and Ronald, 2009; Kollef, 2009).

The cephalosporin nucleus can be modified to gain different properties. Cephalosporins are sometimes grouped into "generations" by their antimicrobial properties (Stork, 2006). The first generation cephalosporins were designated first-generation cephalosporins, whereas, later, more extended-spectrum cephalosporins were classified as second-generation cephalosporins. Each newer generation of cephalosporins has significantly greater Gram-negative antimicrobial properties than the preceding generation, in most cases with decreased activity against Gram-positive organisms. Fourth-generation cephalosporins, however, have true broad-spectrum activity (Pichichero, 2006). Cephalosporins are bactericidal and have the same mode of action as other ß-lacatam antibiotics (such as penicillins) but are less susceptible to penicillinases. Cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell walls. The peptidoglycan layer is important for cell wall structural integrity. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as penicillin-binding proteins (PBPs). PBPs bind to the D-Ala-D-Ala at the end of peptidoglycan precursors to crosslink the peptidoglycan. Beta-lactam antibiotics mimic the D-Ala-D-Ala site, thereby competitively inhibiting PBP crosslinking of peptidoglycan (Kollef, 2009).

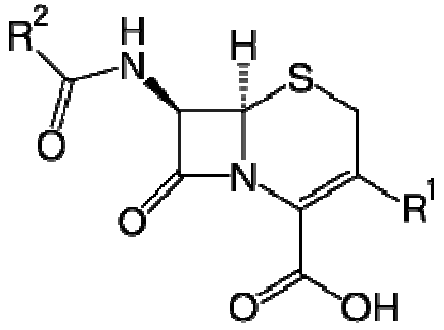

Figure 2.5: Core structure of the cephalosporins (Sanofi-Aventis, 2008).

2.11 Cefotaxime

The systematic IUPAC name of cefotaxime is (6*R*,7*R*,*Z*)-3-(acetoxymethyl)-7-(2-(2-aminothiazol-4-yl)-2-(methoxyimino)acetamido)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid. Its chemical formular is $C_{16}H_{17}N_5O_7S_2$, while the molecular weight is 455.47g/mol. Cefotaxime is a third-generation cephalosporin antibiotic (Bonnet, 2004). Like other third-generation cephalosporins, it has broad spectrum activity against Gram positive and Gram negative bacteria. In most cases, it is considered to be equivalent to ceftriaxone in terms of safety and efficacy (Bonnet, 2004).

The *syn*-configuration of the methoxyimino moiety confers stability to β-lactamase enzymes produced by many Gram-negative bacteria. Such stability to β-lactamases increases the activity of cefotaxime against otherwise resistant Gram-negative organisms. Cefotaxime inhibits bacterial cell wall synthesis by binding to one or more of the penicillin-binding proteins (PBPs) which in turn inhibits the final transpeptidation step of peptidoglycan synthesis in bacterial cell walls, thus inhibiting cell wall biosynthesis(Onyenwe *et al*., 2012). Bacteria eventually lyse due cell wall autolytic enzymes (autolysins and murein hydrolases) while cell wall assembly is arrested. Cefotaxime is used for infections of the respiratory tract, skin, bones, joints, urogenital system, meningitis, and septicemia. It generally has good coverage against most Gram-negative bacteria, with the notable exception of *Pseudomonas* (Bonnet, 2004). It is also effective against most Gram-positive cocci except *Enterococcus*. It is active against penicillin-resistant strains of *Streptococcus pneumoniae*. It has modest activity against the anaerobic *Bacteroides fragilis*. Cefotaxime crosses blood-brain barrier better than Cefuroxime in meningitis.

Cefotaximases (CTX-M) are class - A β-lactamases that in general present higher levels of hydrolytic activity against cefotaxime than against ceftazidime. Ceftazidime MICs for organisms producing these enzymes are sometimes in the susceptible range (Bonnet, 2004). Many laboratories use ceftazidime resistance alone as an indicator of extended-spectrum β-lactamase production. For this reason, CTX-M-producing isolates may be missed by routine susceptibility testing performed by clinical microbiology laboratories (Bonnet, 2004). CTX-M enzymes comprise a rapidly growing family distributed both

over wide geographic areas and among a wide range of bacteria of clinical significance (Bonnet, 2004; Batchelor *et al*., 2005).

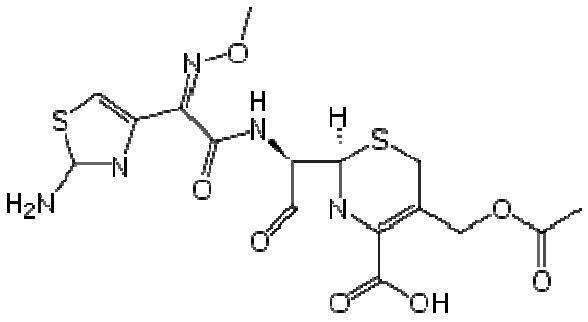

Figure 2.6: Structure of Cefotaxime (Sanofi-Aventis, 2008).

2.12 Ceftriaxone

The systemic IUPAC name of ceftriaxone is (6*R*,7*R*)-7-{[(2*Z*)-2-(2-amino-1,3-thiazol-4-yl)->2- (methoxyimino)acetyl]amino}-3-{[(2-methyl-5,6-dioxo-1,2,5,6-tetrahydro-1,2,4-triazin-3-yl) thio]methyl}-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid. The formular is $C_{18}H_{18}N_8O_7S_3$, while the molar mass is 554.58 g/mol. Ceftriaxone is a third-generation cephalosporin antibiotic. Like other third-generation cephalosporins, it has broad spectrum activity against Gram-positive and Gram-negative bacteria (Bradley *et al.,* 2009). In most cases, it is considered to be equivalent to cefotaxime in terms of safety and efficacy. Resistance to ceftriaxone, the drug of choice for invasive salmonella disease, is a public health concern, especially with respect to children, since fluoroquinolones, which can also be used to treat this disease, are not approved for use in children (Fey *et al.,* 2000).

Ceftriaxone is often used (in combination, but not direct, with macrolide and/or aminoglycoside antibiotics) for the treatment of community-acquired or mild to moderate health care-associated pneumonia. It is also a choice drug for treatment of bacterial

meningitis. In pediatrics, it is commonly used in febrile infants between 4 and 8 weeks of age who are admitted to the hospital to exclude sepsis. The dosage for acute ear infection in the very young is 50mg/kg I.M, one dose only) (Bradley *et al.,* 2009). It has also been used in the treatment of Lyme disease, typhoid fever and gonorrhea. Intravenous dosages may be adjusted for body mass in younger patients and is administered every 12–24 hours, at a dose that depended on the type and severity of the infection (Bradley *et al*., 2009). Treatment for chlamydia infection was also recommended (usually with azithromycin) unless it is specifically ruled out (Barclay, 2007). It must not be mixed or administered simultaneously (within 48 hours) with calcium-containing solutions or products for patients younger than 28 days old (Bradley *et al*., 2009), even via different infusion lines (rare fatal cases of calcium- ceftriaxone precipitates in lung and kidneys in neonates have been described (Bradley *et al*., 2009). To reduce the pain of intramuscular injection, ceftriaxone may be reconstituted with 1% lidocaine (Bradley *et al*., 2009). Ceftriaxone has also been investigated for efficacy in preventing relapse to cocaine addiction (Fey *et al*., 2000).

Ceftriaxone is a white crystalline powder readily soluble in water, sparingly soluble in methanol and very slightly soluble in ethanol. The pH of a 1% aqueous solution is approximately 6.7 (Barclay, 2007). The *syn*-configuration of the methoxyimino moiety confers stability to β-lactamase enzymes produced by many Gram-negative bacteria. Such stability to β-lactamases increases the activity of ceftriaxone against otherwise resistant Gram-negative bacteria. In place of the easily hydrolysed acetyl group of cefotaxime, ceftriaxone has a metabolically stable thiotriazinedione moiety (Barclay, 2007; Bradley *et al.,* 2009). The emergence of resistance to antimicrobial agents within the salmonellae is a worldwide problem that has been associated with the use of antibiotics in livestock. Resistance to ceftriaxone and the fluoroquinolones, which are used to treat invasive *Salmonella* infections, was rare in the United States (Fey *et al*., 2000). According to Fey *et al.* (2000), they analyzed the molecular characteristics of a ceftriaxone-resistant strain of *Salmonella enterica* serotype typhimurium isolated from a 12-year-old boy with fever, abdominal pain, and diarrhoea. The ceftriaxone-resistant isolate from the child was indistinguishable from one of the isolates from cattle, which was also resistant to ceftriaxone. Both ceftriaxone-resistant isolates were resistant to 13

antimicrobial agents; all but one of the resistance determinants were on a conjugative plasmid of 160 kpb that encoded the functional group 1 (Beta-lactamase CMY-2). Both ceftriaxone-resistant isolates were closely related to the three other salmonella isolates obtained from cattle, all of which were susceptible to ceftriaxone (Fey *et al*., 2000). Also their study provides additional evidence that antibiotic-resistant strains of *Salmonella* in the United States evolve primarily in livestock (Fey *et al*., 2000).

2.13 Ethidium bromide

The IUPAC name is 3, 8-Diamino-5-ethyl-6-phenylphenanthridinium bromide with the Molecular formular is $C_{21}H_{20}BrN_3$. The molar mass of the compound is 394.294 g/mol with the appearance of Purple-red solid, and a melting point of 260 - 262 °C. Its solubility in water is approximately 40 g/l (Diaz *et al*., 2002; Von Wurmb-Schwark *et al*., 2006). Ethidium bromide is an intercalating agent commonly used as a fluorescent tag (nucleic acid stain) in molecular biology laboratories for techniques such as agarose gel electrophoresis (NTP, 2005). It is commonly abbreviated as "EtBr", which is also an abbreviation for bromoethane (Huang and Fu, 2005).

When exposed to ultraviolet light, it fluoresces with an orange colour, intensifying almost 20-fold after binding to DNA (NTP, 2005). Ethidium bromide may be a mutagen, carcinogen or teratogen although this depends on the organism and the conditions (Huang and Fu, 2005; NTP, 2005). Ethidium bromide is commonly used to detect nucleic acids in molecular biology laboratories when viewed under ultra violet light (Huang and Fu, 2005; Von Wurmb-Schwark *et al*., 2006).

Where direct viewing is needed, the viewer's eyes and exposed skin should be protected. In the laboratory the intercalating properties have long been utilized to minimize chromosomal condensation when a culture is exposed to mitotic arresting agents during harvest. The resulting slide preparations permit a higher degree of resolution, and thus more confidence in determining structural integrity of chromosomes upon microscopic analysis (Von Wurmb-Schwark *et al*., 2006). Ethidium bromide has also been used extensively to reduce mitochondrial DNA copy number in proliferating cells (Diaz *et al*., 2002). Ethidium bromide is thought to act as a mutagen because it intercalates double

stranded DNA, thereby deforming the molecule (Von Wurmb-Schwark *et al*., 2006). This can affect DNA biological processes, like DNA replication and transcription. Ethidium bromide has been shown to be mutagenic to bacteria via the Ames test (with liver homogenate) (Von Wurmb-Schwark *et al.,* 2006). The requirement of S9 liver homogenate indicates that Ethidium bromide isn't mutagenic, but its metabolites are. The identities of these mutagenic metabolites are unknown. The National Toxicology Program states it is nonmutagenic in rats and mice (NTP, 2005). Ethidium bromide (Homidium) use in animals to treat trypanosome infection suggests that toxicity and mutagenicity are not high. Studies have been conducted in animals to evaluate EtBr as a potential antitumorigenic chemotherapeutic agent (NTP, 2005). Its chemotherapeutic use is due to its toxicity to mitochondria (Von Wurmb-Schwark *et al*., 2006). The above studies do not support the commonly held idea that Ethidium bromide is a potent mutagen in humans, but they do indicate that it can be toxic at high concentrations. Most use of Ethidium bromide in the laboratory (0.25 - 1 microgram/ml) is below the level required for toxicity (NTP, 2005). The level is high enough that exposure may interfere with replication of mitochondrial DNA in some human cell lines, although the implications of that are not clear. Testing in humans and longer studies in any mammalian system are required (NTP, 2005). R-plasmids may be classified as transferrable or non transferable. Transferable R-plasmid is detected by conjugation where as-non transferrable R-plasmids are detected by their loss from host cell spontaneously due to some errors in replication or segregation. Ethidium bromide is not technically hazardous waste at low concentrations (NTP, 2005; Von Wurmb-Schwark *et al*., 2006), but is treated as hazardous waste by many organizations. Waste should be treated in accordance with federal, state and local guidelines (NTP, 2005). Ethidium bromide can be degraded chemically, or collected and incinerated. It is common for ethidium bromide waste below a mandated concentration to be disposed of normally. A common practice is to treat ethidium bromide with sodium hypochlorite (bleach) before disposal (NTP, 2005). Chemical degradation using bleach yields compounds which are mutagenic by the Ames test (NTP, 2005; Von Wurmb-Schwark *et al*., 2006). Data is lacking on the mutagenic effects of degradation products. EtBr can be removed from

solutions with activated charcoal or amberlite ion exchange resin. Various commercial products are available for this use (NTP, 2005).

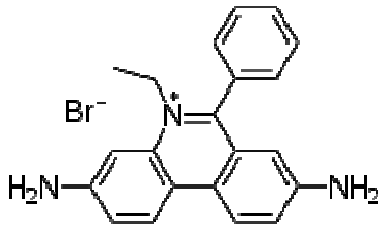

Figure 2.7: Ethidium bromide (Sanofi-aventis, 2008).

2.14 Acridine

The molecular formula of acridine is $C_{13}H_9N$ with Molar mass of 179.22 g mol^{-1}. Its melting point is 107 °C and boiling point is 346 °C , Acidity (pK_a) is 5.60. Acridine is an organic compound and a nitrogen heterocycle. Acridine is also used to describe compounds containing the $C_{13}N$ tricycle. Acridine, a colorless solid, was first isolated from coal tar. It is separated from coal tar by extracting with dilute sulfuric acid; addition of potassium dichromate to this solution precipitates acridine bichromate. It is a raw material used for the production of dyes and some valuable drugs. Many acridines, such as proflavine, also have antiseptic properties. Acridine and related derivatives bind to DNA and RNA due to their abilities to intercalate. Many synthetic processes are known for the production of acridine and its derivatives. Bernthsen condensed diphenylamine with carboxylic acids, in the presence of zinc chloride in the Bernthsen acridine. With formic acid as the carboxylic acid the reaction yields acridine itself, and with the higher homologues the derivatives substituted at the meso carbon atom are generated (Moloney *et al*., 2001). A classic method for the synthesis of acridones is the Lehmstedt-Tanasescu reaction (Moloney *et al*., 2001).

Acridine orange (3, 6-dimethylaminoacridine) is a nucleic acid-selective metachromatic stain useful for cell cycle determination. Acridarsine is formally derived from Acridine

by replacing the nitrogen atom with one of arsenic, and acridophosphine by replacing it with one of phosphorus. Acridine is a known human carcinogen. It causes mutations in incorporating into the DNA, and doing so creating an additional base on the opposite strand. If that mutation occurs in a coding sequence, it almost always leads to inactivation of the protein it encoded. Increased tolerance of disinfecting agents can be caused by energy – dependent efflux pumps located in the cell membrane (Surolia and Surolia, 2001). The use of acridine dyes such as acridine orange for curing and recognizing resistant plasmid in resistant *S. aureus* had been reported by Adeleke *et al.* (2002). Apart from acridine, other agents used for curing were ethidium bromide and mepacrine (Onyenwe *et al*., 2011). The genetic effects of acriflavine (an example of acridine dyes) have been studied on two different strains of *Fusarium* with regard to photodynamic inactivation and reversion of genetic markers (Surolia and Surolia, 2001). In the presence of acriflavine photodynamic inactivation was observed and did not result in a change in the shapes of the survival curves, but induces reversion of the genetic markers in each strain of organisms used (Surolia and Surolia, 2001). Since the report of elimination of resistance cytoplasmic factors of yeast with acriflavine and acridine orange was made by Surolia and Surolia (2001) and Adeleke *et al.* (2002) respectively, loss of antibiotic resistance after exposure to a curing agent has been widely accepted as an evidence of Plasmid mediated resistance to some extent.

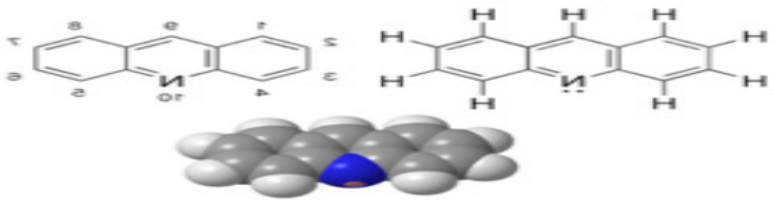

Figure 2.8: Acridine orange (Sanofi-aventis, 2008).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment and glassware

The items used in this study were:

Dry-block heater (Model FDB03AP-Techne, Barloworld scientific Ltd. USA): It was used for the boiling of microbial cells during DNA extraction.

Vortex mixer (Denville scientific Inc.England): It was used for mixing and homogenising cell pellets, DNA and Primers before centrifuging.

Centrifuge (Spectrafuge-7M. Labnet international Edison,USA.): Separation of cells and their products was done using this equipment

Hot-air oven (gallenkamp England): It was used for the sterilization of all equipment made of glassware at 160^{o}C for 1hour.

Incubator (Gallenkamp England and Uniscope-SM9082, Surgifield medicals England): It was used for the purpose of incubating culture media used at 37^{o}C.

Gel tank (Bioneer Agarose-power TM, Germany): It was used for the separation of the plasmid DNA extracts and PCR products after amplification.

Autoclave (Gallenkamp, England): It was used for the sterilization of all the relevant culture media used at 121^{o}C for 15 mins.

Transilluminator (an ultraviolet lightbox, CBS.Scientific Company Inc., Germany): Ethidium bromide-stained DNA in gels was visualized on this equipment.

Weighing balance (Mattler PC 400 and Bartonus BS2005, England): It was used for the purpose of weighing appropriate antibiotic powder samples and culture media and other relevant material.

Eppendorf pipette (Uniscope, Labnet and Oxford research pipette, Germany): It was used for incubating microbial cells, centrifuging of cell pellets and during PCR analysis.

Water bath (Electrothermal, England): It was used for dissolving powdered culture media to suspension before sterilization and also for heating some powdered reagents mixed with water to solution.

PCR Machine (Models; Eppendorf vapo-protect, Master cycler gradient and Eppendorf thermomixer comfort, Germany): They were used for DNA amplification

Spectrophotometer (Nano drop ND1000, Germany): It was used for testing DNA purification level and quantification.

Glassware (Conical flask, measuring cylinder, Petri dishes, glass beakers, pipettes, and test tubes) (Pyrex, England): They were used for measuring solutions.

Agarose voltameter or Electrophoretic machine (model EPS-300,11v, size 1-34 well,CBS Scientific company Inc. Germany): The meter was used to convey electric current to the agarose gel during electrophoresis.

Hot plate (Jenway 1000-Burboword scientific Ltd., England): It was used in heating or boiling liquid substances.

Sanger sequencing machine (ABI 3730 x l of Applied Biosystems, Germany): It was used for DNA sequencing).

3.1.2 Chemicals and culture media

The lists and compositions of the culture media and chemicals used are given in the Appendix 1.

3.1.3 Antibiotics and primers

The five selected antibiotic referenced standards used for MIC determination were;

Cefotaxime powder (Aventis Pharma)

Ceftriaxone powder (Aventis Pharma)

Amoxicillin-clavulanic acid powder (Merck Sharpe and Dohme Ltd)

Ciprofloxacin powder (Merck Sharpe and Dohme Ltd)

Levofloxacin (Merck- Sharpe and Dohme, Ltd.).

The other antibiotic impregnated discs used for the antibiogram were from Oxoid, England and are as follows;

Septrin® (co-trimoxazole) (30µg), chloramphenicol (30µg), sparfloxacin (10µg), amoxicillin (30µg), gentamycin (10µg), pefloxacin (30µg), ofloxacin (10µg), streptomycin (30µg), amoxicillin/clavulanic acid (30µg), cefotaxime (30µg), ceftazidime (30µg), ciprofloxacine (10µg), ceftriaxone (30µg), and levofloxacin (5µg).

The primers used were as shown in appendix I.

3.1.4 Test microorganism

Clinical human isolates of the *Salmonella enterica* from stool were obtained from the Routine Section of the Medical Microbiology Laboratory in the South-eastern part of Nigeria, namely; Federal Medical Centre Owerri, Imo State; University of Nigeria Teaching Hospital Enugu, Enugu State; Federal Medical Centre Umuahia, Abia State; Federal Medical Centre Abakiliki, Ebonyi State. Thus the codes were designated as 'O' for isolates from Owerri, 'U' (Umuahia), 'E' (Enugu) and 'A' (Abakaliki) accordingly.

3.2 Methods

3.2.1 Maintainance of pure isolates

All purified isolates were maintained as stock cultures on nutrient agar slants and stored at 4^{o}C. Sub culturing was carried out fortnightly into Salmonella Shigella Agar (SSA) to ensure viability of the isolates.

3.2.2 Collection of bacteria isolates

The bacteria isolates from stool samples of patients diagnosed with typhoid fever were obtained from one teaching hospital and three federal medical centres in South-east Nigeria between July, 2009 and September, 2010. Out of which a total of one hundred (100) bacteria isolate of *S. enterica* from the different hospitals (both the teaching hospitals and federal medical centres) in the different States of the South east Nigeria namely; Federal Medical Centre Owerri, University of Nigeria Teaching Hospital Enugu, Federal Medical Centre Umuahia, and the Federal Medical Centre Abakaliki. A total of twenty- five isolates each were collected from the hospitals. The isolates were recovered from different Departments such as General out Patients Department (GOPD), In pataients Unit (IPU), National Health Insurance Scheme (NHIS), Children Out Patience (CHOP), and Emergency Patient Unit (EPU) as stated in their record ledger in each Microbiology unit visited. Each isolate was transferred into a sterile media slant for further confirmatory test and biochemical characterization. They were then further authenticated as *S. enterica* using Microbact® 12E kit.

3.2.3 Culturing and identification of the *Salmonella enterica*

All the isolates were appropriately labeled for the purpose of clearity and identification. The isolates from FMC Owerri were designated SO1-SO25, FMC Umuahia SU26-SU50 and UNTH Enugu SE51-SE75, while FMC Abakaliki SA76-SA100 accordingly. All the colonies of *Salmonella enterica* previously isolated from these hospitals were subcultured into a selective medium such as Salmonella shigella agar (SSA), to further verify their H_2S production through exhibiting tiny black spots on the culture plate. They were later transferred into Mueller Hinton agar (MHA) for Grams staining and biochemical characterization using microbact identification kit (oxoid-England) as specified by the manufacturer, to be certain of the right isolates as described by Cheesbrough (2006).

3.2.4. Identification of *Salmonella* species using Microbact® kit GNB12E

Procedures:

For the test, a single colony from an 18-24 hour plate culture was inoculated aseptically into 5ml sterile normal saline solution (0.9% NaCl) and the suspension was homogenized to a cell suspension with turbidity equivalent to that of 0.5 McFarland standard (1.5×10^9 CFU/mL). Using a sterile pipette, few drops of the bacterial suspension were distributed into 12 wells of the Microbact® 12E (Oxoid Ltd. Australia) and sealed after the addition of two drops of mineral oil (Oxoid reagent) into 1 - 3 of the wells and reseal the well for incubation. The microbact 12E kit consist of 12 different biochemical test specific for the identification of Enterobacteriaceae. In the absence of colour change in the wells as suggested by the manufacturer. The kit was incubated at 35 $\pm 2^0$C for 18-24 hours. After incubation, colour changes were read against colour chart as provided by the manufacturer. Further test were performed on the unchanged wells or tubes. Then one to two drops of the reagent were introduced in each well 8 and 10 to check for indole and vogues proskauer reaction respectively. The reaction colour change or values corresponding to each group were added to get a profile (A four digit number). Then the numbers are entered into the softwasre of Microbact® 12E for final identification. The Microbact® API 20E could also be used for this purpose.

The slide agglutination antigen kit (Lab-Care Diagnostics, India) was used for this analysis. A bit of discrete colony from nutrient agar culture plate was put on the grease free slide and mixed with a drop of physiological saline solution using a mixing stick. The results were observed macroscopically after one minute, for auto-agglutinating strains after rocking the slide for 30–60 seconds. This procedure was repeated simultaneously with a drop of the antiserum (Lab-care diagnostics, India). The O antigen suspensions were tested first. If they were positive, then the monovalent O antisera belonging to this group will also be tested. A positive result shows that the isolated bacterium possesses the antigen corresponding to the antiserum. The same procedures were subsequently carried out on the H antigen suspension

3.3 Antibiogram of the clinical isolate *Salmonella enterica*

The antibiogram was carried out by the multidisc agar- diffusion method. Using sterile pipette, 0.1ml of 10^{-2} dilution of 18- 24 hours culture of the test isolates was added to 20ml molten mueller Hinton Agar cooled at 45^{o}C, the contents were gently swirled to mix before pouring into sterile petri dishes. The seeded culture plates were allowed to set and subsequently dried for 20mins, in the incubator at 37^{o}C. After drying, the antibiotic discs were aseptically introduced on the surface of the medium using apair of sterile forceps and allowed for 10-15 minutes. before incubation at 37^{o}C for 24 hours. Thereafter, the zones of growth inhibition were interpreted using CLSI breakpoint as standard zones depending on the antibiotics used (CLSI, 2007; CLSI, 2011), as sensitive / intermediate sensitive/ enzyme inactivation / resistant. Statistics analysis by Analysis of Variance (ANOVA) was used to determine the level of significance among the zone of growth inhition produced by the five selected antibiotic against the clinical isolate of *S.enterica.*

3.4 Determination of the Minimum Inhibitory Concentration (MIC) of five selected antibiotics

Using the tube broth-dilution method the MIC of Five selected antibiotics; ceftriaxone, cefotaxime, augmentine® (amoxicillin/ clavulanic acid), levofloxacin and ciprofloxacin)

against the standard and clinical strains of *S. enterica* was determined. Two sets of graded drug concentrations were prepared in duplicates in nutrient broth. For each test antibiotic, a stock concentration of 100 µg/ml was prepared by dissolving 1mg in 10ml of sterile water. From the stock, 100 µg/ml was prepared in duplicates each of which was diluted down serially by doubling fold dilution to 0.39 µg/ml. Using 2- fold serial dilution (by introducing 5mls of the stock into another 5mls nutrient broth and diluting serially). The serially diluted test drugs were then inoculated each with 0.1ml of 10^{-2} dilutions of the respective overnight broth cultures and equivalent of x 10^7 cell/ml.

Control tubes were also set up consisting of nutrient broth incubated with the same organism, but drug free as positive control at 37^{o}C for 24hours, and then examined for growth. Also, 5ml from the last tube in each set of diluted concentrations was pipetted into a clean sterile test tube, was similarly incubated without inoculum as a transference negative control. Another negative control was a mixture of 0.1ml from the stock concentration and 5ml sterile broth, similarly were incubated and examined. The least drug concentration showing no visible growth was chosen as the MIC for the particular antibiotic. Such concentration and the next two higher concentrations were subsequently plated using 0.1ml on drug- free nutrient agar plates and incubated at 37^{o}C for 24hours-48hours. Observation of scanty growth on the plate of MIC broth dilution confirms the chosen concentration as MIC while absence of growth indicates a bactericidal concentration; hence, the Minimum Bactericidal Concentration (MBC). A standard strain of *S.enterica serovar Typhimurium* (ATCC14028) was also used as control organism and included with each batch of the isolates tested.

3.5 Beta-Lactamase production test using nitrocefin sticks

Nitrocefin (Oxoid) is the chromogenic cephalosporin (Yah, 2010), developed by Glaxo Research Limited coded 87/312; 3-(2,4 dinitrostyrl) –6R, 7R) –7-(2 thienylacetamido)-ceph-3-em-4 carboxylic acid, E-isomer. This compound exhibits a rapid distinctive colour change from yellow to red as the amide bond in the beta-lactam ring is hydrolysed by a Beta-lactamase. It is sensitive to hydrolysis by all known Beta-lactamases produced by Gram-positive and Gram-negative bacteria.

Procedure;

The container was removed from the freezer and allowed to reach room temperature. Then a well separated representative colony from the primary isolation medium was selected. The colony was touched with the impregnated tip of the stick, which was rotated to pick up a small mass of cells. The inoculated tip of the stick was placed between the lid and the base of the inverted plate. The reaction requires moisture, so the inoculated tip of the stick was placed in the moisture condensate in the lid or in absence of condensate in the inverted lid; one or two drops of distilled water were added to the lid to moisten the tip of the stick. The reagent end of the stick was examined for up to five minutes or up to the end of fifteen minutes. A positive reaction was shown by the development of a pink/red colour. No colour change shows absence of Beta-lactamase. In every experiment an unused nitrocephine stick serves as a control.

3.6. Plasmid DNA isolation

The alkaline Lysis method was adopted for the extraction. Buffer 1A, 200 µl, (see appendix 1) was added to the cell pellets and vortexed. Then, 400 µl of lysing solution was added and the tubes were inverted 20 times gently at room temperature bet (25-30 ^{o}C) and 300 µl ice cold buffers 2B (see appendix1) was added followed by vortexing and the mixture was placed on ice for 30 minutes. Centrifuge followed at 3,000 xg for 15 mins. To the supernatant was added 700 µl of chloroform and mixture was vortexed. Centrifugation again followed at 3,000 xg for 10 minutes. Then 500 µl of the aqueous layer was transfered into a fresh tube, and 1ml of absolute ethanol was added. The mixture was kept on ice for 1 hour. followed by centrifugation at 3,000 xg for 30minutes and then washing of pellets with 70% ethanol. By decantation, dry pellets were obtained, to which 50 µl of buffer 3C (appendix I) was added and then kept for electrophoresis on ice. The Buffer constituents are as shown in appendix I.

3.7. Curing of antibiotic resistance in *Salmonella* strains

Twenty-five resistant clinical strains of *Salmonella enterica* (Human) were randomly selected from among the identified antibiotic resistant groups. The method used for this

test was the modified method as described by Onyenwe *et al*. (2011) with further modification in the use of more than one subinhibitory concentration of the curing agents. Overnight broth culture of each resistant strain and the control strain were obtained each in 10ml nutrient broth (5 test tubes per strain) containing 5, 2.5, 1.25, 0. 625, 0.3125 µg/ml, of the mutagens (acridine orange and ethidium bromide). The mixtures were incubated at 37^{o}C for 24hours. After the incubation, each mutagen-exposed culture was plated on Mueller Hinton agar medium and incubated. Colonies were subcultured each into 5ml Mueller Hinton broth and incubated at 37^{o}C for 24 hours. Then following incubation, a 10^{-2} in sterile distilled water was made and after shaking, 0.1ml of the dilution (10^{-2}) was seeded into a molten Mueller Hinton agar (10-15ml), swirled to mix and then poured on a sterile culture plate and allowed to solidify. Wells of 6.5mm diameter each were later made on the set agar medium. The wells were then filled each with referenced MIC and resistant MIC (before curing) of each test antibiotic against each strain of *Salmonella enterica*, for cefotaxime, ceftriaxone, levofloxacin, augmentin®, and ciprofloxacin. After pre-incubation diffusion period of 2hours, plates were incubated at 37^{o}C for 24hours, and then examined for zones of growth inhibition measured in millimeter (mm).

3.8. Detection of ESBL's using double disc synergy test (DDST).

Procedure;

The test inoculum, (0.2 ml of 10^{2} of over night broth culture) was spread evenly onto Mueller-Hinton agar (MHA-Oxoid, India) using a sterile cotton wool swab. A disc of augmentin® (20 µg amoxycillin + 10 µg Clavunalic acid) was placed on the surface of MHA; then discs of ceftriaxone CRO (30 µg), Ceftazidime CAZ (30 µg) and Cefotaxime CTX (30 µg) were kept around the disc of augumentin ® in such a way that each disc was at a distance ranging between 16 and 20 mm from the augmentin® disc (centre to centre). The plate was incubated at 37 °C 18- 24hours. The organisms were considered to be producing ESBL when the zone of growth inhibition around any of the Broad-spectrum cephalosporin discs showed a clear-cut increase towards the augmentin® (amoxycillin + clavunalic acid) disc (Yah, 2010).

3.9. Preparation of purified chromosomal DNA for PCR analysis using boiling method.

Procedures;

An overnight broth culture of 1.5ml was pipetted into an eppendorf tube and centrifuged at 5r.p.m. The supernatant was discarded and sterile water was introduced into the tube and the mixture was shaken vigorously by a vortexing machine and the supernatant was decanted. This process was repeated twice. Then the mixture was resuspended in 100µl sterile distilled water, vortexed and placed in a water block to boil for 10-20mins at 100^{o}C. After boiling, the mixture was then centrifuged for 10 mins, and the purified chromosomal DNA pellets were transferred into a new tube and stored on ice.

3.10. Agarose gel electrophoresis of DNA products

The agarose powder was mixed with electrophoresis buffer to the concentration of 0.5µg/ml, and then heated in a microwave oven until completely melted. Then, ethidium bromide was added to the gel (0.5µg/ml concentration of agarose gel) at this point to facilitate visualization of plasmid DNA and PCR products after electrophoresis. After cooling the solution to about 60^{o}C, it was poured into a casting tray containing a sample comb and allowed to solidify. Then the comb was carefully removed. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and covered with buffer solution. Samples containing DNA mixed with loading buffer were then pipetted into the sample wells; the lid and power leads were placed on the apparatus, and current was applied. The current was confirmed to be flowing by observing bubbles coming off the electrodes and movement of the tracking dyes.

3.11. Spectrophotometric quantification and purity test of chromosomal DNA

Procedures:

The spectrophotometer lens was cleaned with sterile distilled water. Then the screen of the computer to which it was connected to was adjusted, after blanking the spectrophotometer with sterile water. DNA sample of 0.2 µl was added on the lens, closed and the measure on the computer screen was clicked waiting for the readings. After each sample the lens was cleaned with sterile water and dried with dry cotton wool.

Note: The normal quantity required in DNA analysis is between 10 - 200 µl, while the purity level is detectable within the volume 1.5 - 2 µl. Results are shown on Appendix 3.

3.12. PCR analysis and gene sequencing

The Polymerase Chain Reaction (PCR) was performed under the following conditions with the Solis biodyne 5x FIREPol Master mix Ready to load. The thermocycling condition for *Bla*- SHV-1, *Bla*-TEM, and Bla_{CTX-M} were 30 – 35 cycles at 95^{o}C for 30secs, 72^{o}C for 1min.,66.2 for1 min., 72^{o}C for 1min, 95^{o}C for 30 sec., (PCR timing 1.38- 2.58 hrs). The cycling condition for *qnrB, GyrA* and *ParC* were 30 cycles of 95^{o}C for 30 secs., 42^{o}C for 1 mins, 72^{o}Cfor 1 mins, and 95^{o}C for 30 sec., (PCR timing 12.38 - 2.34 hrs.). The PCR primers to amplify the *Bla*- SHV-1, *Bla*-TEM, Bla_{CTX-M} (universal primer) genes, Quinolone Resistant Regions QRDR's of the *GyrA* and *ParC* genes and quinolone plasmid-mediating resistant *qnrB* type respectively were;

Bla-SHV-1; F (5′-GTA TTG AAT TCA TGC GTT ATA TTC GCC TGT GTA-3′), R (5′-CAG AAT TCG GCT AGC GTT GCC AGT GCT CGA T-3')oligonumber; 80303Y1185C02).

Bla-TEM-1; F (5'-ATGAGTATTCAACATTTCCG-3'), R-(5'-ACC AAT GCT TAA TCA GTG AG -3') (oligo number; 80303Y1185C01).

Bla_{CTX-M} ; F (5'ATG TGC AGY ACC AGT AAR GTK ATG GC-3') (oligo number; 90303X1185C03), R (5'- TGG GTR AAR TAR GTS ACC AGA AYC AGC GG- 3') (oligo number; 90303X1185C04) where R in the sequence is purine,Y is pyrimidine, and S is G or C.

GyrA; F (5'CGT TGG TGA CGT AAT CGG- 3') (oligo number; 00123839_3),
R (5'CCG TAC CGT CAT AGT TAT- 3') (oligo number; 00123839_4).

ParC ; F (5'CTA TGC GAT GTC AGA GCT GG-3') (oligo number;00123839_5), R(5'TAA CAG CAG CTC GGC GTA TT- 3') (oligo number; 00123839_6), while

QnrB is F-(*5'*-GAT CGT GAA AGC CAG AAA GG- 3') (oligo number; 00123839_7), R- (5'-ATG AGC AAC GAT GCC TGG TA-3') (oligo number; 00123839_8).

Procedure;

The purified DNA template in PCR eppendorf tubes (2.0µl DNA) was mixed with the Master mix ready to load containing all the required contents as stated in the Appendix including the primers for the amplification. The mixture was vortexed to mix and then centrifuged before introducing it into the PCR machine (Eppendorf-Germany).Then the machine was adjusted to annealing temperature and switched to start. After the time for the amplification was completed, the PCR products were then taken for electrophoresis on the agarose gel as described earlier and finally viewed on the UV light for amplified image of the genes. The PCR products were now selected for gene sequencing, if the gene of interest is amplified and recovered.

The DNA (amplified gene of the PCR products) was packed and sent to Gatc Biotech Laboratory, Germany, through correspondence in an Eppendorf tubes for sequencing. The gene sequencing was carried out at Gatc Biotech Germany using Sanger sequencing machine (ABI 3730 x l of Applied Biosystems, Germany) automatically edited with PhredPhrap. The Mega-5 soft ware was used for the alignment of the gene and the blasting was done using the NCBI database BLAST.

CHAPTER FOUR
RESULTS

4.1 Authentication of *Salmonella enterica* Serovar.Typhi

A total of 100 isolates of *Salmonella enterica* serovar.*typhi* were obtained from the Federal Medical Centres and University Teaching Hospital in the Southeast region of Nigeria between July, 2009 and September, 2010. The isolates were identified by cultural and biochemical characteristics using Microbact identification kit® 2E. The slide agglutination test identified the antigenic property of the organism into O and H antigen, thereby identifying the isolate to be *S. enterica serovar. typhi*. All the isolates were Gram negative rods, oxidase negative, catalase positive, indole and Voges Proskauer (VP) negative, methyl red and Simmons citrate positive, H_2S producing and urea negative. Some of these characteristics were used for the biochemical confirmation of *Salmonella* in this study as shown in Table 4.1.

4.2 Antibiogram screening and resistance pattern of the *S.enterica*

Table 4.2 a- d shows the susceptibility pattern of 100 isolates out of which each twenty-five clinical strains of *S. enterica* serovar.*Typhi* obtained were from Federal Medical Centre Owerri (FMC), Federal Medical Centre (FMC) Umuahia (Table 4.2 b), University of Nigeria Teaching hospital (UNTH) Enugu (Table 4.2 c) and Federal Medical Centre (FMC) Abakaliki (Table 4.2 d). Some of the *S. enterica* strains were sensitive, but exhibited some forms of resistant on which 1or 2 colonies where found to be resistant (Resistant mutants- appendix II, plate 2) (SO10, 11, 13 in Table 4.2 a) in the area of zone of growth inhibition. These types of resistant were classified as resistant mutant (R.M), while some were intermediately sensitive and still exhibited the form of resistance. Some of the *S.enterica* strain on screening exhibited some forms of enzyme inhibition other than the conventional Beta-lactamase (SU38 in Table 4.2 b) which was responsible for their reduced susceptibility i.e Enzyme inactivation (E.I) of the antibiotics tested. Other strains though, completely showed total resistance (R) to some group of antibiotic tested

(SE69 and SE75 in Table 4.2 c), and isolates from SA77 and SA88 (Table 4.2 d). Similarly, analysis showed that the same trend of results (resistant pattern) was observed when the fluoroquinolone and cephalosporins groups of antibiotics were used on *S. enterica* serovar isolates, as stated on Tables 4.2 a-d of the various hospitals analysed. The resistant mutant and enzyme inhibition plates 1 and 2 are as shown in appendix II.

Table 4.1. Authentication of *Salmonella enterica* Serovar.Typhi

Biochemical test	**probability rate of Colour / reaction**
99.8 (%) based on the Microbact soft ware	
Oxidase	Yellow -
Lysine decarxylase	Yellow +
Glucose fermentation	Yellowish +
HS_2	Black deposit +
Mannose fermentation	Yellowish +
Catalase	Effervescent bubbles +
Nitrate utilization or reduction (ONPG)	Red +
Indole	Red -
Urease	Orange -
Simone Citrate utilization	Blue-green +
Voges Proskauer	Pink -
Trypton Deaminase	Yellowish brown -
Gram reactions under microscope	Tiny short negative rods

KEY: + = Positive reaction**, - =** Negative reaction

Table 4.2 a. Antibiogram of *S. enterica* from Federal medical center (FMC) Owerri, Imo state.

S/NO	SXT	CH	SP	CPX	AM	GEN	PEF	OFX	STR	LEV	CRO	CTX	CAZ	AMC
O1.	18	15	R	30	R	23	25	24	R	20^{RM}	21	23	R	R
O2.	18	11	18	22	18	23	23	23	18	25^{RM}	28^{RM}	28^{RM}	25	R
O3.	15	12	20	23	18	17	23	23	18	30	14	23	29	R
O4.	8	8	17	23	18	19	23^{EI}	23	15	25	25	24	28	18^{RM}
O5.	R	R	R	32	R	18	25	17	R	22	24	25	R	R
O6.	19	12	18	20	16	21	23	23	18	R	14	10	10	R
O7.	10	R	15	21	15	20	23	22	14	25^{RM}	23	23	19	R
O8.	10	16	18	21	15	23	23	25	15	20	29	29	R	19
O9.	R	R	R	8	R	23	17	16	8	22	21	24	R	R
O10.	R	R	R	R	R	23	16	16	10	14^{RM}	21	25	20	R
O11.	R	R	R	R	R	R	18	16	R	20^{RM}	22^{RM}	28^{RM}	R	R
O12.	R	R	R	R	R	14	15	17	10	22	21	23	R	R
O13.	R	R	10	24^{EI}	R	R	20	22	10	20	20^{RM}	23^{RM}	22^{RM}	13
O14.	R	R	R	24	R	16	20	23	10	10	R	R	R	R
O15.	R	R	11	23	R	21	21	23	10	26	20	23	24	R
O16.	R	R	10	18	R	15	23^{EI}	23	10	25	21	26	R	R
O17.	R	R	R	24	R	R	14	14	10	22	20	24	R	R
O18.	R	R	R	R	R	R	R	R	R	24	21	28	20	R
O19.	R	R	R	10	R	14	8	8	R	20	21	25	27	R
O20.	18	15	R	35	R	26	24	25	R	R	R	R	9	R
O21.	R	11	R	28	R	20	18	15	R	25	27	25	23	R
O22.	16	15	16	28	R	18	17	14	R	30	30	30^{EI}	30	12
O23.	16	16	12	30	R	16	19	15	11	25	20	24	8	9
O24.	18	15	20	30	10	18	24	18	13	14	R	31	9	R
O25	16	R	12	28	10	16	15	16	16	25	25	28	8	8
T/C.	**16**	**R**	**27**	**25**	**15**	**18**	**17**	**16**	**R**	**28**	**26**	**28**	**24**	**12**

KEY: SXT= cotrimoxazole 30µg, CH= chloramphenicol 30µg, SP = sparfloxacin 10µg, CPX= ciprofloxacin 10µg, AM= amoxicillin 30µg, GEN= gentamycin 10µg, PEF= pefloxacin 30µg, OFX= ofloxacin 10µg, STR= streptomycin 30µg, LEV= levofloxacin 5µg, CRO= ceftriaxone 30µg, CTX= cefotaxime 30µg, CAZ= ceftazidime 30µg, AMC= amoxicillin /clavulanic acid 30µg. R= Resistant, S= Sensitive, R.M = Resistant mutant, E.I=Enzyme inhibition.

Table 4.2 b. Antibiogram of *S. enterica* from Federal medical center (FMC) Umuahia, Abia State (Continue)

S/NO	SXT	CH	SP	CPX	AM	GEN	PEF	OFX	STR	LEV	CRO	CTX	CAZ	AMC
U26.	14	R	12	28	12	16	16	17	13	24	22	23	9	R
U27.	R	R	R	32	R	30	28	26	11	25	25	25	20^{RM}	8
U28.	R	R	22	36	R	R	R	R	R	23^{RM}	17^{RM}	28^{RM}	20^{RM}	15
U29.	18	15	22	28	10	14	17	17	15	23	20	29	R	R
U30.	R	R	R	29	R	16	16	15	10	25	24	23	20	8
U31.	15	14	15	32	12	18	14	15	16	25	26	30	21	15
U32.	R	R	R	32	R	23	26	26	10	27^{RM}	26	27	25	12
U33.	10	14	12	24	12	17	R	12	17	25	29	29	10	R
U34.	14	13	15	29	10	13	14^{EI}	14	11	28	21	23	24	R
U35.	R	R	R	30	R	25	22	22	14	25	21	23	27	13
U36.	R	R	R	34	15	24	19	18	17	28^{RM}	29	29	22	R
U37.	14	13	R	30	R	13	16	16	R	25	20	29^{EI}	22	R
U38.	R	14	R	28^{EI}	12	13	15	17	11	24	20	23	10	R
U39.	14	R	R	27	R	R	R	14	R	27	20	28	20	18
U40.	R	R	R	27	12	R	R	R	R	16^{RM}	10	10	20	R
U41.	10	R	R	25	14	13	16	17	14	25	20	20	26	R
U42.	17	R	R	30	14	R	16	16	10	23	24	25	21	R
U43.	R	R	R	28	15	R	16^{RM}	15^{RM}	R	22^{RM}	21	27	25	R
U44.	R	R	R	25	R	R	R	R	R	26^{RM}	25	25	21	R
U45.	16	R	R	28	17	16	16	18	14	23	24	25	19	R
U46.	R	R	R	29	18	17	15	13	10	22^{RM}	20	23^{RM}	20^{RM}	R
U47.	18	15	10	30	13	12	16	17	R	24	21	23	25	R
U48.	R	R	R	25	15	16	R	R	R	15^{RM}	21	23	15	R
U49.	14	R	10	23	14	R	R	R	11	11	8	R	R	R
U50	R	R	11	20	10	R	18	18	R	26	28	28	27	R

KEY: SXT= cotrimoxazole 30µg, CH= chloramphenicol 30µg, SP = sparfloxacin 10µg, CPX= ciprofloxacin 10µg, AM= amoxicillin 30µg, GEN= gentamycin 10µg, PEF= pefloxacin 30µg, OFX= ofloxacin 10µg, STR= streptomycin 30µg, LEV= levofloxacin 5µg, CRO= ceftriaxone 30µg, CTX= cefotaxime 30µg, CAZ= ceftazidime 30µg, AMC= amoxicillin /clavulanic acid 30µg. R= Resistant, S= Sensitive, R.M = Resistant mutant, E.I=Enzyme inhibition.

Table 4.2 c. Antibiogram of *S. enterica* from (UNTH) Enugu, Enugu State. (Continue)

S/NO	SXT	CH	SP	CPX	AM	GEN	PEF	OFX	STR	LEV	CRO	CTX	CAZ	AMC
E51.	R	R	R	18	11	R	16	16	R	28	21	23	26	R
E52.	R	R	R	19	11	R	R	R	R	20	25	25	15	R
E53.	R	R	R	R	R	R	16	16	R	18	22	20	24	R
E54.	R	R	12	22	15	12	19	19	R	25	23	25	26	R
E55.	R	R	R	24	R	R	15	14	R	28	24	23	22	R
E56.	R	R	12	20	13	R	17	16	18	23	22	23	22	R
E57.	R	R	11	22	15	R	20	20	R	25	29	28	26	R
E58.	R	13	15	22 ^{EI}	18	12	16	16	R	29	25	24	28	R
E59.	10	14	15	25	12	12	15	16	11	15	31	31	30	R
E60.	16	10	R	20	R	17	10	R	14	R	28	25 ^{RM}	26	R
E61.	R	R	R	18	R	10	R	R	R	20^{RM}	25	26	27	R
E62.	10	15	15	18	R	16	17	12	R	25^{RM}	28^{RM}	25^{RM}	22^{RM}	15^{RM}
E63.	R	14	14	19	R	15	15	R	R	11	21	26	25	R
E64.	16	16	15	30 ^{EI}	10	12	R	R	R	11	11	R	14^{RM}	R
E65.	R	R	R	R	R	12	14	15	10	14^{RM}	17^{RM}	10^{RM}	10^{RM}	14^{RM}
E66.	R	R	R	R	R	15	10	10	R	11	8	8	11	R
E67.	R	R	R	22	R	16	10	R	R	10	R	R	14^{RM}	R
E68.	R	R	R	17	R	12	10	16	R	11	R	R	14^{RM}	R
E69.	10	8	R	15	R	16	R	R	R	8	8	8	8	R
E70.	R	R	R	17	R	R	17	14	10	28	26	25	26	R
E71.	R	R	R	R	R	R	R	18	R	19	20	28	20	R
E72.	R	R	R	R	R	R	R	R	R	18^{RM}	28^{RM}	9	25	R
E73.	R	R	R	18^{RM}	R	R	14^{RM}	14^{RM}	R	20^{RM}	24^{RM}	R	12^{RM}	15^{RM}
E74.	R	R	13	20	R	12	14	14	R	18	14	24	20	R
E75	R	R	R	8	R	R	R	R	R	16	8	8	R	R

KEY: SXT= cotrimoxazole 30µg, CH= chloramphenicol 30µg, SP = sparfloxacin 10µg, CPX= ciprofloxacin 10µg, AM= amoxicillin 30µg, GEN= gentamycin 10µg, PEF= pefloxacin 30µg, OFX= ofloxacin 10µg, STR= streptomycin 30µg, LEV= levofloxacin 5µg, CRO= ceftriaxone 30µg, CTX= cefotaxime 30µg, CAZ= ceftazidime 30µg, AMC= amoxicillin /clavulanic acid 30µg. R= Resistant, S= Sensitive, R.M = Resistant mutant, E.I=Enzyme inhibition.

Table 4.2 d. Antibiogram of *S. enterica* from (Federal Medical Center (FMC) Abakiliki, Ebonyi State (Continue)

S/NO	SXT	CH	SP	CPX	AM	GEN	PEF	OFX	STR	LEV	CRO	CTX	CAZ	AMC
A76.	R	R	R	20	R	R	R	R	R	14^{RM}	21	11	23	R
A77.	R	R	R	R	R	R	R	11	R	11	R	R	23	R
A78.	R	R	R	9	R	R	R	18^{RM}	R	10	R	10	8	R
A79.	R	R	12	12	R	R	R	14^{RM}	R	18	24	28^{EI}	8	8
A80.	R	R	R	R	R	R	R	16^{RM}	R	10	22	R	R	R
A81.	R	R	R	R	R	10	14	14	R	11^{RM}	R	R	8	15^{RM}
A82.	R	R	R	14	R	R	14	10	R	10	28	28^{RM}	12	R
A83.	R	15	R	R	R	10	R	11	R	11	R	R	26	R
A84.	R	R	R	R	R	R	14	13	R	11	R	R	R	R
A85.	R	17	R	R	R	16	14	15	R	13	20	10	9	R
A86.	R	R	R	R	R	R	14	14	R	13	20	10	9	R
A87.	R	R	R	23^{EI}	R	13	14	14	R	14	R	R	13	R
A88.	R	R	R	R	R	R	14^{RM}	13^{RM}	R	R	25^{RM}	25^{RM}	R	R
A89.	R	R	14	22	R	R	18	18	11	24	22	23	20	R
A90.	R	R	R	25	R	R	16	15	R	16	24^{RM}	13^{EI}	R	R
A91.	R	R	R	R	R	R	R	R	14	17	22	27	12	14
A92.	16	12	16	20	15	16	20	21	18	21	25	25	22	11
A 93.	R	R	R	R	R	14	R	R	R	20	21	24	20	14
A 94.	R	R	R	R	R	R	R	R	R	18	26	25	8	R
A 95.	R	12	12	18^{EI}	R	14	20	20	15	14	27	25	8	R
A 96.	R	R	R	16	R	R	17	14	15	14	28^{RM}	14^{RM}	27^{RM}	R
A 97.	R	R	R	R	R	R	17	17	16	17	23	23^{RM}	24	15
A 98.	R	R	R	R	R	R	16	16	R	14	20^{RM}	29^{RM}	23	15
A 99.	R	R	R	22	R	R	14	14	11	14	28	15	9	R
A100.	R	R	R	25	R	R	15	R	R	13	23	13	R	R

KEY: SXT= cotrimoxazole 30µg, CH= chloramphenicol 30µg, SP = sparfloxacin 10µg, CPX= ciprofloxacin 10µg, AM= amoxicillin 30µg, GEN= gentamycin 10µg, PEF= pefloxacin 30µg, OFX= ofloxacin 10µg, STR= streptomycin 30µg, LEV= levofloxacin 5µg, CRO= ceftriaxone 30µg, CTX= cefotaxime 30µg, CAZ= ceftazidime 30µg, AMC= amoxicillin /clavulanic acid 30µg. R= Resistant, S= Sensitive, R.M = Resistant mutant, E.I=Enzyme inhibition.

4.3. Determination of the Minimum Inhibitory Concentration (MIC) using tube dilution method on five selected antibiotics

Table 4.3. shows the Minimum Inhibitory Concentration (MIC) and Minimum Bacteriocidal Concentration (MBC) of the isolates of *S. enterica* using the selected antibiotics as follows; levofloxacin (LEV), ciprofloxacin (CPX), amoxiclavulanic acid (AMC), cefotaxime (CTX) and ceftriaxone (CRO). From Table 4.7; having the code number SO1- SO25, levofloxacin (a fluoroquinolone) and ceftriaxone had their MIC within the range of 1.56 µg/ml - 3.125 µg/ml and 1.56 µg/ml- 6.25 µg/ml respectively, except in some cases where the isolates were very resistant wherby MIC increased to 25.0 µg/ml and 12.5 µg/ml respectively, followed by cefotaxime and ciprofloxacin having the same range (1.56 - 3.125 µg/ml) except in cases where some isolates showed high resistance, thereby increasing the MIC's of both drugs to 6.25 µg/ml and 12.5 µg/ml respectively. The typed culture used had MIC range of 1.56 µg/ml except with the antibiotic, amoxi-clavulanic acid where it had MIC's range of 12.5 µg/ml- 25 µg/ml. All the isolates showed increased MIC's for amoxi-clavulanic acid up to 50 µg/ml. The same trend of MIC results was observed in the other isolates from Umuahia (SU26-SU50), but with difference from Enugu (SE51-SE75), and Abakaliki (SA76 - SA100), which showed sharp increase in the MIC's. However, some of the antibiotics like levofloxacin, ciprofloxacin, ceftriaxone and cefotaxime showed good activity as relate to having their potency intact, but amoxi-clavulanic acid showed increased MIC in almost all the isolates of the centres as shown in Tables 4.3 a- d.

Table 4.3 a. The MICs and MBCs (µg/l) of five selected antibiotics against 25 isolates of *S. enterica* from FMC owerri

S/NO	SEROTYPE	LEV		CPX		AMC		CTX		CRO	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
O1.	*S.typhi O/H*	1.56	3.125	3.125	6.25	50.0	>50.0	3.125	6.25	1.56	3.125
O2.	,,	6.25	12.5	3.125	6.25	50.0	>50.0	3.125	6.25	1.56	3.125
O3.	,,	1.56	3.125	3.125	6.25	50.0	>50.0	6.25	12.5	6.25	12.5
O4.	,,	1.56	3.125	1.56	3.125	25.25	>50.0	1.56	3.125	1.56	3.125
O5.	,,	1.56	3.125	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
O6.	,,	25.0	>25.0	3.125	6.25	50.0	>50.0	6.25	12.5	6.25	12.5
O7.	,,	1.56	3.125	3.125	6.25	50.0	>50.0	3.125	6.25	3.125	6.25
O8.	,,	1.56	3.125	3.125	6.25	50.0	>50.0	3.125	6.25	1.56	3.125
O9.	,,	3.125	6.25	12.5	25.0	50.0	>50.0	3.125	6.25	1.56	3.125
O10.	,,	3.125	6.25	12.5	25.0	50.0	>50.0	6.25	12.5	1.56	3.125
O11.	,,	1.56	3.125	12.5	25.0	50.0	>50.0	1.56	3.125	1.56	3.125
O12.	,,	1.56	3.125	12.5	25.0	50.0	>50.0	1.56	3.125	1.56	3.125
O13.	,,	1.56	3.125	3.125	6.25	50.0	>50.0	1.56	3.125	1.56	3.125
O14.	,,	25.0	>25.0	3.125	6.25	50.0	>50.0	6.25	12.5	6.25	12.5
O15.	,,	3.125	6.25	1.56	3.125	50.0	>50.0	1.56	3.125	3.125	6.25
O16.	,,	3.125	6.25	3.125	6.25	50.0	>50.0	1.56	3.125	1.56	3.125
O17.	,,	3.125	6.25	3.125	6.25	50.0	>50.0	1.56	3.125	1.56	3.125
O18.	,,	1.56	3.125	12.5	25.0	50.0	>50.0	3.125	6.25	1.56	3.125
O19.	,,	1.56	3.125	12.5	25.0	50.0	>50.0	3.125	6.25	1.56	3.125
O20.	,,	25.0	>25.0	1.56	3.125	50.0	>50.0	12.5	25.0	12.5	25.0
O21.	,,	3.125	6.25	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
O22.	,,	3.125	6.25	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
O23.	,,	1.56	3.125	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
O24.	,,	1.56	3.125	1.56	3.125	50.0	>50.0	12.5	25.0	12.5	25.0
O25	,,	1.56	3.125	1.56	3.125	50.0	>50.0	6.25	12.5	3.125	6.25
T/C	**ATCC14028**	**1.56**	**3.125**	**1.56**	**3.125**	**25.5**	**50.0**	**1.56**	**3.125**	**1.56**	**3.125**

KEY: **CPX=** ciprofloxacin 10 µg, **LEV=** levofloxacin 5 µg, **CRO=** ceftriaxone 30 µg, **CTX=** cefotaxime 30 µg, **AMC=** amoxicillin/clavulanic acid 30 µg. **MIC =** Minimum Inhibitory Concentration**, MBC =** Minimum Bacteriocidal Concentration, **TC=** Typed culture, **O1- 25=** Isolates from Owerri.

Table 4.3 b. The MICs and MBCs (µg/l) of five selected antibiotics against 25 isolates of *S. enterica* from FMC Umuahia (continued)

S/NO	SEROTYPE	**LEV**		**CPX**		**AMC**		**CTX**		**CRO**	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
U26.	*S.typhi O/H*	3.125	6.25	3.125	6.25	50.0	>50.0	3.125	6.25	1.56	3.125
U27.	,,	6.25	12.5	6.25	12.5	50.0	>50.0	3.125	6.25	6.25	12.5
U28.	,,	12.5	25.5	6.25	12.5	50.0	>50.0	3.125	6.25	6.25	12.5
U29.	*S.typhi O*	1.56	3.125	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
U30.	,,	1.56	3.125	1.56	3.125	50.0	>50.0	3.125	6.25	1.56	3.125
U31.	,,	1.56	3.125	1.56	3.125	12.5	25.0	1.56	3.125	1.56	3.125
U32.	,,	1.56	3.125	1.56	3.125	25.0	>25.0	1.56	3.125	1.56	3.125
U33.	*S.typhi O/H*	1.56	3.125	1.56	3.125	25.0	>25.0	1.56	3.125	1.56	3.125
U34.	*S.typhi O/H*	1.56	3.125	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
U35.	,,	1.56	3.125	1.56	3.125	50.0	>50.0	3.125	6.25	1.56	3.125
U36.	,,	3.125	6.25	1.56	3.125	50.0	>50.0	1.56	3.125	3.125	6.25
U37.	,,	1.56	3.125	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
U38.	,,	3.125	6.25	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
U39.	,,	6.25	12.5	1.56	3.125	12.5	25.0	6.25	12.5	6.25	12.5
U40.	,,	6.25	12.5	1.56	3.125	50.0	>50.0	6.25	12.5	6.25	12.5
U41.	,,	1.56	3.125	1.56	3.125	25.0	>25.0	1.56	3.125	1.56	3.125
U42.	,,	1.56	3.125	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
U43.	,,	1.56	3.125	1.56	3.125	50.0	>50.0	6.25	12.5	6.25	12.5
U44.	,,	1.56	3.125	1.56	3.125	50.0	>50.0	6.25	12.5	1.56	3.125
U45.	,,	3.125	6.25	1.56	3.125	50.0	>50.0	3.125	6.25	1.56	3.125
U46.	,,	3.125	6.25	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
U47.	,,	3.125	6.25	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
U48.	,,	6.25	12.5	6.25	12.5	50.0	>50.0	6.25	12.5	3.125	6.25
U49.	,,	12.5	25.0	1.56	3.125	50.0	>50.0	12.5	25.0	12.5	25.0
U50	,,	1.56	3.125	1.56	3.125	50.0	>50.0	3.125	6.25	3.125	6.25
T/C	**ATCC14028**	**1.56**	**3.125**	**1.56**	**3.125**	**25.5**	**50.0**	**1.56**	**3.125**	**1.56**	**3.125**

KEY: **CPX=** ciprofloxacin 10 µg, **LEV=** levofloxacin 5 µg, **CRO=** ceftriaxone 30 µg, **CTX=** cefotaxime 30 µg, **AMC=** amoxicillin/clavulanic acid 30 µg. **MIC =** Minimum Inhibitory Concentration**, MBC =** Minimum Bacteriocidal Concentration, **TC=** Typed culture, **U26- 50** = Isolates from Umuahia.

Table 4.3 c. The MICs and MBCs (µg/l) of five selected antibiotics against 25 isolates of *S. enterica* from UNTH Enugu (continued)

S/NO	SEROTYPE	**LEV**		**CPX**		**AMC**		**CTX**		**CRO**	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E51.	*S.typhi O/H*	1.56	3.125	3.125	6.25	50.0	>50.0	1.56	3.125	1.56	3.125
E52.	,,	3.125	6.25	3.125	6.25	50.0	>50.0	1.56	3.125	1.56	3.125
E53.	,,	3.125	6.25	12.5	25.0	50.0	>50.0	6.25	12.5	1.56	3.125
E54.	*S.typhi H*	1.56	3.125	3.125	6.25	50.0	>50.0	6.25	12.5	3.125	6.25
E55.	*S.typhi O/H*	1.56	3.125	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
E56.	,,	1.56	3.125	1.56	3.125	25.0	>25.0	1.56	3.125	1.56	3.125
E57.	,,	1.56	3.125	1.56	3.125	25.0	>25.0	3.125	6.25	3.125	6.25
E58.	*S.typhi H*	1.56	3.125	3.125	6.25	25.0	>25.0	1.56	3.125	1.56	3.125
E59.	*S.typhi O/H*	6.25	12.5	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
E60.	,,	>12.5	>25.0	3.125	6.25	50.0	>50.0	1.56	3.125	1.56	3.125
E61.	,,	3.125	6.25	3.125	6.25	50.0	>50.0	6.25	12.5	6.25	12.5
E62.	,,	1.56	3.125	3.125	6.25	50.0	>50.0	1.56	3.125	1.56	3.125
E63.	,,	12.5	25.0	1.56	3.125	50.0	>50.0	3.125	6.25	1.56	3.125
E64.	,,	12.5	25.0	1.56	3.125	25.0	>25.0	12.5	25.0	6.25	12.5
E65.	,,	12.5	25.0	12.5	25.0	25.0	>25.0	12.5	25.0	3.125	6.25
E66.	,,	12.5	25.0	12.5	25.0	50.0	>50.0	12.5	25.0	12.5	25.0
E67.	,,	12.5	25.0	3.125	6.25	50.0	>50.0	12.5	25.0	12.5	25.0
E68.	,,	12.5	25.0	6.25	12.5	50.0	>50.0	12.5	25.0	12.5	25.0
E69.	,,	12.5	25.0	6.25	12.5	50.0	>50.0	12.5	25.0	12.5	25.0
E70.	,,	3.125	6.25	3.125	6.25	50.0	>50.0	3.125	6.25	3.125	6.25
E71.	,,	3.125	6.25	12.5	25.0	25.0	>25.0	3.125	6.25	3.125	6.25
E72.	,,	3.125	6.25	12.5	25.0	25.0	>25.0	12.5	25.0	3.125	6.25
E73.	,,	3.125	6.25	3.125	6.25	12.5	25.0	12.5	25.0	6.25	12.5
E74.	,,	3.125	6.25	1.56	3.125	25.0	>25.0	6.25	12.5	6.25	12.5
E75	,,	6.25	12.5	12.5	25.0	25.0	>25.0	25.0	>25.0	12.5	25.0
T/C	**ATCC14028**	**1.56**	**3.125**	**1.56**	**3.125**	**25.5**	**50.0**	**1.56**	**3.125**	**1.56**	**3.125**

KEY: **CPX=** ciprofloxacin 10 µg, **LEV=** levofloxacin 5 µg, **CRO=** ceftriaxone 30 µg, **CTX=** cefotaxime 30 µg, **AMC=** amoxicillin/clavulanic acid 30 µg. **MIC =** Minimum Inhibitory Concentration**, MBC =** Minimum Bacteriocidal Concentration, **TC=** Typed culture, **E51- 75** = Isolates from Enugu.

Table 4.3 d. The MICs and MBCs (µg/l) of five selected antibiotics against 25 isolates of *S. enterica* from FMC, Abakaliki (continued).

S/NO	SEROTYPE	**LEV**		**CPX**		**AMC**		**CTX**		**CRO**	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
A76.	*S.typhi O/H*	12.5	25.0	1.56	3.125	50.0	>50.0	12.5	25.0	12.5	25.0
A77.	‘’	12.5	25.0	12.5	25.0	50.0	>50.0	12.5	25.0	12.5	25.0
A78.	‘’	12.5	25.0	12.5	25.0	50.0	>50.0	12.5	25.0	12.5	25.0
A79.	‘’	3.125	6.25	12.5	25.0	50.0	>50.0	12.5	25.0	3.125	6.25
A80.	*S.typhi H*	12.5	25.0	12.5	25.0	50.0	>50.0	12.5	25.0	12.5	25.0
A81.	*S.typhi O/H*	12.5	25.0	12.5	25.0	25.0	>25.0	12.5	25.0	12.5	25.0
A82.	,,	12.5	25.0	6.25	12.5	25.0	>25.0	3.125	6.25	3.125	6.25
A83.	,,	12.5	25.0	12.5	25.0	50.0	>50.0	12.5	25.0	12.5	25.0
A84.	,,	12.5	25.0	12.5	25.0	25.0	>25.0	12.5	25.0	12.5	25.0
A85.	,,	12.5	25.0	12.5	25.0	25.0	>25.0	12.5	25.0	12.5	25.0
A86.	,,	12.5	25.0	12.5	25.0	50.0	>50.0	12.5	25.0	12.5	25.0
A87.	,,	12.5	25.0	12.5	25.0	50.0	>50.0	12.5	25.0	12.5	25.0
A88.	,,	12.5	25.0	12.5	25.0	50.0	>50.0	12.5	25.0	12.5	25.0
A89.	,,	1.56	3.125	1.56	3.125	50.0	>50.0	1.56	3.125	1.56	3.125
A90.	*S,typhi O*	6.25	12.5	6.25	12.5	50.0	>50.0	6.25	12.5	6.25	12.5
A91.	*S.typhi O/H*	3.125	6.25	12.5	25.0	25.0	>25.0	3.125	6.25	1.56	3.125
A92.	,,	1.56	3.125	1.56	3.125	25.0	>25.0	1.56	3.125	1.56	3.125
A93.	,,	1.56	3.125	12.5	25.0	25.0	>25.0	1.56	3.125	1.56	3.125
A94.	,,	3.125	6.25	12.5	25.0	50.0	>50.0	6.25	12.5	6.25	12.5
A95.	,,	12.5	25.0	3.125	6.25	50.0	>50.0	6.25	12.5	3.125	6.25
A96.	,,	12.5	25.0	3.125	6.25	50.0	>50.0	12.5	25.0	3.125	6.25
A97.	,,	3.125	6.25	12.5	25.0	50.0	>50.0	1.56	3.125	1.56	3.125
A98.	,,	12.5	25.0	12.5	25.0	50.0	>50.0	3.125	6.25	1.56	3.125
A99.	,,	12.5	25.0	12.5	25.0	25.0	>25.0	6.25	12.5	3.125	6.25
A100.	,,	12.5	25.0	6.25	12.5	25.0	>25.0	12.5	25.0	12.5	12.5
T/C	**ATCC14028**	**1.56**	**3.125**	**1.56**	**3.125**	**25.5**	**50.0**	**1.56**	**3.125**	**1.56**	**3.125**

KEY: **CPX=** ciprofloxacin 10 µg, **LEV=** levofloxacin 5 µg, **CRO=** ceftriaxone 30 µg, **CTX=** cefotaxime 30 µg, **AMC=** amoxicillin/clavulanic acid 30 µg. **MIC =** Minimum Inhibitory Concentration**, MBC =** Minimum Bacteriocidal Concentration, **TC=** Typed culture, **A76 – 100** = Isolates from Abakaliki.

4.4. Prevalence of *S. enterica* in various Departmental units and hospitals in the Southeast region of Nigeria.

Table 4.4 shows the percentage prevalence rate of *S. enterica* recovered from various units in the hospitals from the Southeast region investigated. Analysis revealed that the hospital in Owerri had a total of Twenty-five isolates of *S. enterica* (the same number of isolates applies to other hospitals in the region) 14 (56%) of the isolates were recovered from the general outpatient department (GOPD). Other departmental units were National Health insurance scheme (NHIS) 5 (20%), In-patient units (IPU) 4 (16%), Children out Patient units and Emergency Patient units were found to have only 1 (4%) of the isolates each. In the hospital in Umuahia, IPU had the highest number of *S. enterica* isolated 23 (92%) and GOPD 2 (8%). No isolates were recovered from other units from the hospital in Umuahia during the period of investigation (i.e other units like NHIS, CHOP, EPU and the SKIN CLINIC recorded 0 (0%) of isolates. From other hospitals in Enugu and Abakaliki, the percentage recovery of isolates were; NHIS 0 (0%) and 3 (12%), IPU 8 (35%) and 13 (52%), GOPD 15 (60%) and 8 (32%), CHOP 1 (4%) and 0 (0%), EPU 0 (0%) and1 (4%), while the SKIN CLINIC recorded 1 (4%) and 0 (0%) respectively.

Table 4.4. Prevalence of *S. enterica* in various Departmental unit and hospitals in the South-east region of Nigeria

S/NO	Unit/Department	(Location) Owerri	(Location) Umuahia	(Location) Enugu	(Location) Abia	
		S.enterica	*S.enterica*	*S.enterica*	*S.enterica*	
		1-25	**26-50**	**51-75**	**76-100**	**Total**
1.	**NHIS**	5 (20%)	0 (0%)	0 (0%)	3 (12%) =	**8**
2.	IPU	4 (16%)	23 (92%)	8 (32%)	13 (52%) =	**48**
3.	GOPD	14 (56%)	2 (8%)	15 (60%)	8 (32%) =	**39**
4.	CHOP	1 (4%)	0 (0%)	1 (4%)	0 (0%) =	**2**
5.	EPU	1 (4%)	0 (0%)	0 (0%)	1 (4%) =	**2**
6.	SKIN CLINIC	0 (0%)	0 (0%)	1 (4%)	0 (0%) =	**1**
TOTAL		**25**	**25**	**25**	**25 = 100**	

KEY: NHIS = National Health Insurance Scheme, IPU= In - Patient Unit , GOPD= General Out Patient Department , CHOP= Children Out Patient , EPU= Emergency Patient Unit, SKIN= Skin Clinic.

4.5 Beta-lactamase production and plasmid profiling of *S. enterica* isolates in relation to gender distribution

Tables 4.5 show Beta-lactamase production and plasmid profiling of *S. enterica* isolates in relation to gender distribution**.** Results (Table 4.5 a) **s**how that only 2 (8%) isolates out of the twenty-five isolates recovered in GOPD unit from Federal Medical Center Owerri harboured plasmids, while 20 (80%) isolates produced the conventional Beta- lactamase. It was also observed that isolates recovered from a female child of 2years in the CHOP unit produced the conventional Beta-lactamase enzyme but was resistant to only 4 (28.6%) antibiotics used in this study, while an isolate recovered in the EPU unit from a female child of 1 year old was resistant to the 4 (28.6%) of the antibiotics used but produced no conventional Beta-lactamase and harboured no plasmid. From the results obtained from the *S. enterica* isolates (Table 4.5b) from Federal Medical Centre (FMC) Umuahia, only 3 (12%) harboured plasmid, while 18 (72%) of the isolates recovered produced the conventional Beta-lactamase enzyme. It was also observed that isolates recovered from 2 (8%) female children in the IPU produced Beta-lactamase, though no plasmids were found on them, they were resistant to only 5 (35.7) of the 14 antibiotic used.

Results from University of Nigeria Teaching Hospital (UNTH) Enugu (Table 4.5c) showed that there were no plasmid recovered from the twenty-five isolates but 20 (80%) of the isolates produced the conventional Beta-lactamase enzyme. It was also observed from this study that an isolate recovered from a child of 10years from CHOP unit produced no conventional Beta-lactamase, harboured no plasmid but was resistant to 9 (64.3%) of the antibiotics used for the study. Results on the *S. enterica* recovered from Federal Medical Centre (FMC) Abakaliki (Table 4.5 d) showed that 4 (16%) of the isolates harboured plasmids, with one of isolates habouring the plasmid coming from a female child of 6 years old in the NHIS unit. Analysis showed that the isolates recovered from the female child of 6 years as described above produced the conventional ßeta-lactamse and was resistant to 10 (71.4%) of the 14 antibiotics used in this study. Almost all the isolates recovered from this part of Southeast (Abakaliki) in this study, produced the conventional ßeta-lactamase. This could be as a result of the isolates been recovered

from IPU and GOPD unit of the hospital. It was also observed that one isolate recovered from a female patient in the IPU unit harboured no plasmid, but produced the Beta-lactamase enzyme and was resistant to only one antibiotic used in this study (Table 4.5 d). The resistance by *S. enterica* on the number of antibiotics tested was found in FMC Abakaliki to be the highest in the region (Southeast Nigeria).

Plate 4.1 shows the Agarose gel Electrophoresis pattern revealing single PCR amplification of Bla_{CTX-M} gene on *S. enterica* from Federal Medical Centre (FMC) Owerri. The PCR amplicons size of 593 bp's were as shown on lane numbers 3 (SO3), 14 (SO14), 15 (SO15) and 24 (SO24) and were positive for Bla_{CTX-M} gene (A class of CTX-M) type enzyme of the extended spectrum B-lactamases (ESBLs); the other *bla*SHV, *bla*TEM and quinolone resistance protein were not detected on the isolates. The marker used here was DNA molecular marker of 100 bp ladder (see Fig. 4.1, lane M), while lane T was a type culture strain (ATCC14028) negative for Bla_{CTX-M} gene used as positive control (though no amplicon was seen) and lane N was the negative control containing sterile water.

Plate 4.2 shows the agarose gel electrophoresis pattern, revealing single PCR amplification of Bla_{CTX-M} gene in *S. enterica* from Umuahia and Enugu. The PCR amplicons size of 593bp's were as shown on lanes 28 (SU28), 39 (SU39), 40 (SU40), 49 (SU49) and 50 (SU50) and were positive for Bla_{CTX-M} gene. Lane numbers 53 (SE53) and 54 (SE54) having the same amplicons size of 593 bp's were from UNTH Enugu. The lanes labeled M, T, and N were for DNA molecular marker, typed culture for positive control and sterile water for negative control.

Plate 4.3 shows the agarose gel electrophoresis pattern, revealing single PCR amplification of Bla_{CTX-M} gene amplified at different annealing temperature of 62.5^{o}C at the same cycling condition. The results of the PCR amplicons size were still seen at 593bp's on lane number 15 (SO15) at different annealing temperature (Plate 4.1, lane 15 the PCR amplicons sizes for the Bla_{CTX-M} gene was almost faded, while at the same annealing temperature it was prominent). Also on lane number 27 (SU27), there were no

amplicons, while at this temperature, the PCR amplicon size 593bp's were amplified. Note: The primer used here is a universal primer.

Plate 4.4 shows the agarose gel electrophoresis pattern of single PCR amplification of *S. enterica* from Umuahia and Enugu. The PCR amplicons sizes of 593 were as shown on only Bla_{CTX-M} gene on lane number 44 (SU44). Note also, that there were no amplicons on lane number 44 (Plate 4.2). Lane M shows DNA molecular marker of 100bp's ladder.

Plate 4.5 **s**hows the plasmid pattern of *S. enterica* serovar typhi on the agarose gel electrophoresis. It could be deduced that the molecular weights of the plasmid were considerably low. Lane numbers 2 and 9 (SO2 and SO9) show molecular weight of 1.39 kbs and 1.37 kbs (SO2) and 1.37 kbs (SO9). The isolates on lane number 28, 40, and 43 (SU28, SU40 and SU43) had the molecular weight of 1.37kbs respectively, just as M.wt of 1.37 kbs was obtained for each of the isolates SA76, SA81, SA83, and SA85. Thus, the plasmid profile revealed that most isolate of *S. enterica* screened harbored plasmids of low molecular weight.

Table 4.5 a. Beta-lactamase production and plasmid profiling of isolates of *S. enterica* from Federal Medical Center Owerri, in relation to gender distribution.

Org. Code S/NO	Unit/ Dept.	Sex	Age Range (yrs)	Beta Lactamase Production	Plasmid /Mwt.	Antibiotics Resistant (%)
O1.	IPU	M	Adult	+	-	5 (35.7%)
O2.	GOPD	F	20	+	+	2 (14.3%)
O3.	GOPD	F	Adult	+	-	2 (14.3%)
O4.	GOPD	F	26	-	-	3 (21.4%)
O5.	GOPD	F	22	+	-	7 (50.0%)
O6.	GOPD	F	Adult	+	-	4 (28.6%)
O7.	GOPD	F	Adult	+	-	3 (21.4%)
O8.	GOPD	F	Adult	+	-	2 (14.3%)
O9.	GOPD	F	Adult	+	+	8 (57.1%)
O10.	GOPD	M	Adult	+	-	7 (50.0%)
O11.	NHIS	F	Adult	+	-	9 (64.3%)
O12.	GOPD	M	Adult	+	-	9 (64.3%)
O13.	NHIS	F	Adult	+	-	7 (50.0%)
O14.	GOPD	M	Adult	+	-	10 (71.4%)
O15.	GOPD	F	50	+	-	6 (42.9%)
O16.	GOPD	F	50	+	-	7 (50.0%)
O17.	NHIS	M	Adult	-	-	8 (57.1%)
O18.	GOPD	F	65	-	-	10 (71.4%)
O19.	NHIS	F	Adult	+	-	10 (71.4%)
O20.	IPU	M	Adult	+	-	8 (57.1%)
O21.	IPU	M	Adult	+	-	5 (35.7%)
O22.	IPU	F	Adult	-	-	3 (21.4%)
O23.	EPU	F	1	-	-	4 (28.6%)
O24.	NHIS	M	Adult	+	-	6 (42.9%)
O25	CHOP	F	2.5	+	-	4 (28.6%)

KEY: F= Female, M= Male , NHIS = National Health Insurance Scheme , IPU= In - Patient Unit, GOPD = General Out Patient Department , CHOP = Children Out Patient, EPU= Emergency Patient Unit. **Note:** Adult (includes male and female between 18 and above, whose actual age was not determined), Nil= Negative

Table 4.5 b. Beta-lactamase production and plasmid profiling of isolates of *S. enterica* from Federal Medical Center Umuahia, in relation to gender distribution (continue).

Org. Code S/NO	Unit/ Dept.	Sex	Age Range (yrs)	Beta-Lactamase Production	Plasmid/ Mwt.	Antibiotic Resistant (%)
U26.	IPU	F	Adult	-	-	5 (35.7%)
U27.	IPU	F	Adult	+	-	6 (42.9%)
U28.	IPU	M	Adult	+	+	7 (50.0%)
U29.	IPU	F	Adult	-	-	3 (21.4%)
U30.	IPU	F	Adult	-	-	6 (42.9%)
U31.	IPU	F	28	+	-	1 (71.0%)
U32.	IPU	F	Adult	+	-	6 (42.9%)
U33.	IPU	M	Adult	+	-	7 (50.0%)
U34.	IPU	F	Adult	-	-	4 (28.6%)
U35.	IPU	M	19	+	-	5 (35.7%)
U36.	IPU	M	Adult	+	-	4 (28.6%)
U37.	IPU	F	13	-	-	5 (35.7%)
U38.	IPU	F	Adult	-	-	6 (42.9%)
U39.	IPU	M	24	+		6 (42.9%)
U40.	IPU	F	Adult	+	+	11 (78.5%)
U41.	IPU	F	3	+	-	5 (35.7%)
U42.	IPU	F	Adult	-	-	4 (28.6%)
U43.	IPU	M	Adult	+	+	5 (35.7%)
U44.	IPU	F	Adult	+	-	9 (64.3%)
U45.	IPU	M	Adult	+	-	3 (21.4%)
U46.	IPU	F	4	+	-	5 (35.7%)
U47.	GOPD	F	Adult	+	-	4 (28.6%)
U48.	GOPD	M	44	+	-	7 (50.0%)
U49.	IPU	F	Adult	+	-	11 (78.5%)
U50	IPU	M	Adult	+	-	7 (50.0%)

KEY: F= Female , M= Male , NHIS = National Health Insurance Scheme, IPU = In - Patient Unit, GOPD = General Out Patient Department, CHOP = Children Out Patient, EPU = Emergency Patient Unit. **Note:** Adult (includes male and female between 18 and above, whose actual age was not determined), Nil= Negative

Table 4.5 c. Beta-lactamase production and plasmid profiling of isolates of *S. enterica* from University of Nigeria Teaching Hospital (UNTH) Enugu, in relation to gender distribution (continue).

Org. Code S/NO	Unit/ Dept.	Sex	Age Range (yrs)	Beta Lactamase Production	Plasmid/Mwt.	Antibiotic Resistant (%)
E51.	IPU	F	60	+	-	7 (50.0%)
E52.	GOPD	F	56	+	-	9(64.3%)
E53.	GOPD	M	Adult	+	-	8 (57.1%)
E54.	IPU	M	Adult	+	-	5 (35.7%)
E55.	IPU	F	Adult	+	-	7 (50.0%)
E56.	SKIN	M	80	+	-	6 (42.9%)
E57.	GOPD	F	56	+	-	6 (42.9%)
E58.	IPU	M	21	+	-	5 (35.7%)
E59.	IPU	M	Adult	+	-	4 (28.6%)
E60.	IPU	F	Adult	-	-	7 (50.0%)
E61.	CHOP	M	10	-	-	9 (64.3%)
E62.	GOPD	M	Adult	-	-	3 (21.4%)
E63.	GOPD	M	Adult	+	-	6 (42.9%)
E64.	GOPD	M	Adult	+	-	8 (57.1%)
E65.	GOPD	F	Adult	-	-	8 (57.1%)
E66.	GOPD	F	40	-	-	13 (92.9%)
E67.	GOPD	F	60	+	-	11 (78.5%)
E68.	GOPD	F	101	+	-	10 (71.4%)
E69.	GOPD	M	21	+	-	12 (85.7%)
E70.	GOPD	F	27	+	-	7 (50.0%)
E71.	IPU	F	76	+	-	9 (64.3%)
E72.	IPU	M	45	+	-	11 (78.5%)
E73.	GOPD	M	33	+	-	8 (57.1%)
E74.	GOPD	F	Adult	+	-	6 (42.9%)
E75	GOPD	M	60	+	-	13 (92.9%)

KEY: F= Female, M= Male , NHIS = National Health Insurance Scheme , IPU = In - Patient Unit, GOPD = General Out Patient Department , CHOP = Children Out Patient, EPU = Emergency Patient Unit. **Note:** Adult (includes male and female between 18 and above, whose actual age was not determined), Nil= Negative

Table 4.5 d. Beta-lactamase production and plasmid profiling of isolates of *S. enterica* from Federal Medical Center (FMC) Abakiliki, in relation to gender distribution (Continue).

Org. Code S/NO	Unit/ Dept.	Sex	Age Range (yrs)	Beta Lactamase Production	Plasmid/Mwt.	Antibiotics Resistant (%)
A76.	GOPD	M	Adult	+ve	+ve	12 (85.7%)
A77.	GOPD	M	20	+	-	14 (100%)
A78.	EPU	F	59	+	-	13 (92.7%)
A79.	IPU	M	24	+	-	10 (71.4%)
A80.	IPU	M	Adult	+	-	13 (92.7%)
A81.	NHIS	F	6	+	+	10 (71.4%)
A82.	GOPD	M	Adult	+	-	10 (71.4%)
A83.	GOPD	F	58	+	+	12 (85.7%)
A84.	IPU	F	68	+	-	13 (92.7%)
A85.	IPU	F	Adult	+	+	10 (71.4%)
A86.	GOPD	M	34	+	-	12 (85.7%)
A87.	GOPD	F	Adult	+	-	9 (64.3%)
A88.	IPU	M	Adult	+	-	11 (78.5%)
A89.	GOPD	F	29	+	-	6 (42.9%)
A90.	HNIS	M	39	+	-	9 (64.3%)
A91.	IPU	F	39	-	-	8 (57.1%)
A92.	IPU	F	20	+	-	1 (7.1%)
A 93.	IPU	M	34	-	-	9 (64.3%)
A 94.	GOPD	F	39	+	-	11 (78.5%)
A 95.	IPU	M	25	+	-	7 (50.0%)
A 96.	IPU	F	Adult	+	-	6 (42.9%)
A 97.	IPU	F	Adult	+	-	6 (42.9%)
A 98.	NHIS	F	Adult	+	-	8 (57.1%)
A 99.	IPU	F	Adult	+	-	9 (64.3%)
A100.	IPU	F	Adult	-	-	12 (85.7%)

KEY: F= Female , M= Male , NHIS = National Health Insurance Scheme , IPU= In - Patient Unit, GOPD = General Out Patient Department, CHOP= Children Out Patient, EPU = Emergency Patient Unit. **Note:** Adult (includes male and female between 18 and above, whose actual age was not determined), Nil= Negative

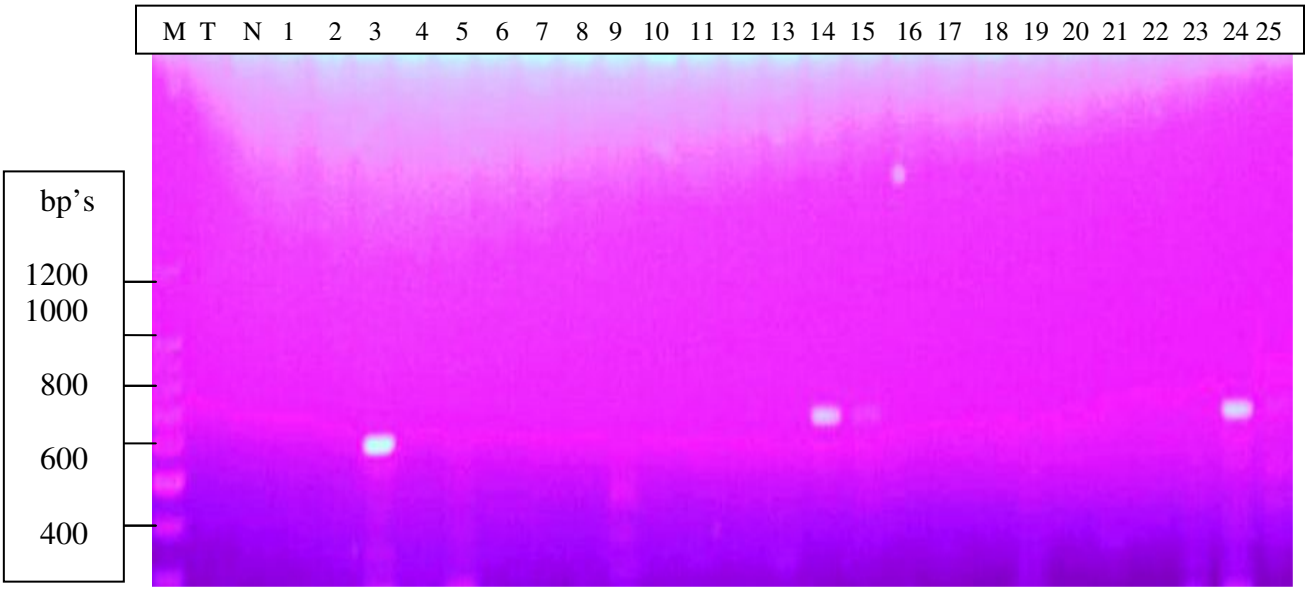

Plate 4.1. Agarose gel electrophoresis pattern of PCR amplification products of Bla_{CTX-M} genes from *S. enterica* serovar. typhi.

KEY: Lanes 1- 25 show isolates from FMC Owerri. The amplicons size (59 3bp) on lane 3, 14, 15 and24 shows positive Bla_{CTX-M} genes present in the DNA of *S.enterica* serovar. typhi. Lane M shows DNA molecular marker (100- bp ladder). Lane T is the typed isolate of *S. typhi* ATCC (negative for Bla_{CTX-M}). Lane N contains only sterile water for control.

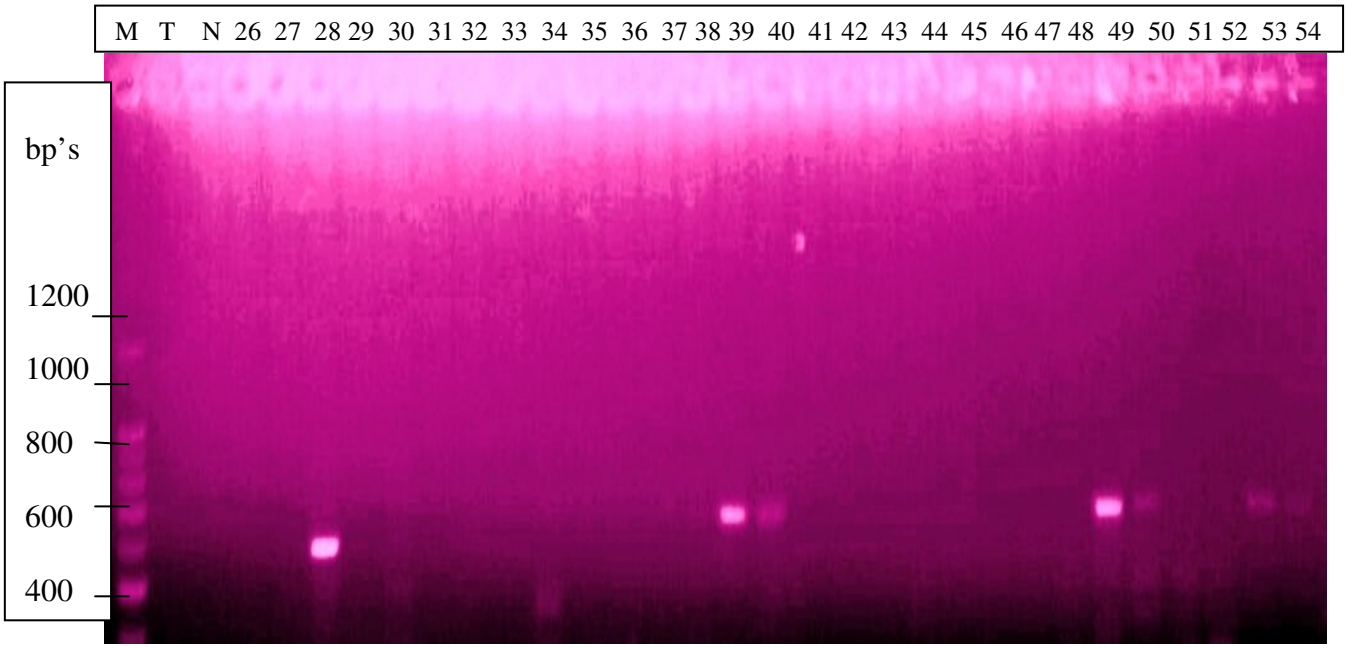

Plate 4.2. Agarose gel electrophoresis pattern of PCR amplification products of Bla_{CTX-M} genes from *S.enterica* serovar. Typhi.

KEY: Lines 26-50 show isolates from FMC Umuahia. The amplicon size (593bp) on lane 28,39,40,49 and 50 shows positive Bla_{CTX-M} genes. Lanes 51-54 are from UNTH Enugu. Amplicons on lane 53 and 54 shows positive Bla_{CTX-M} genes present in the DNA of *S. enterica* serovar. typhi respectively. Lane M shows DNA molecular marker (100- bp ladder).

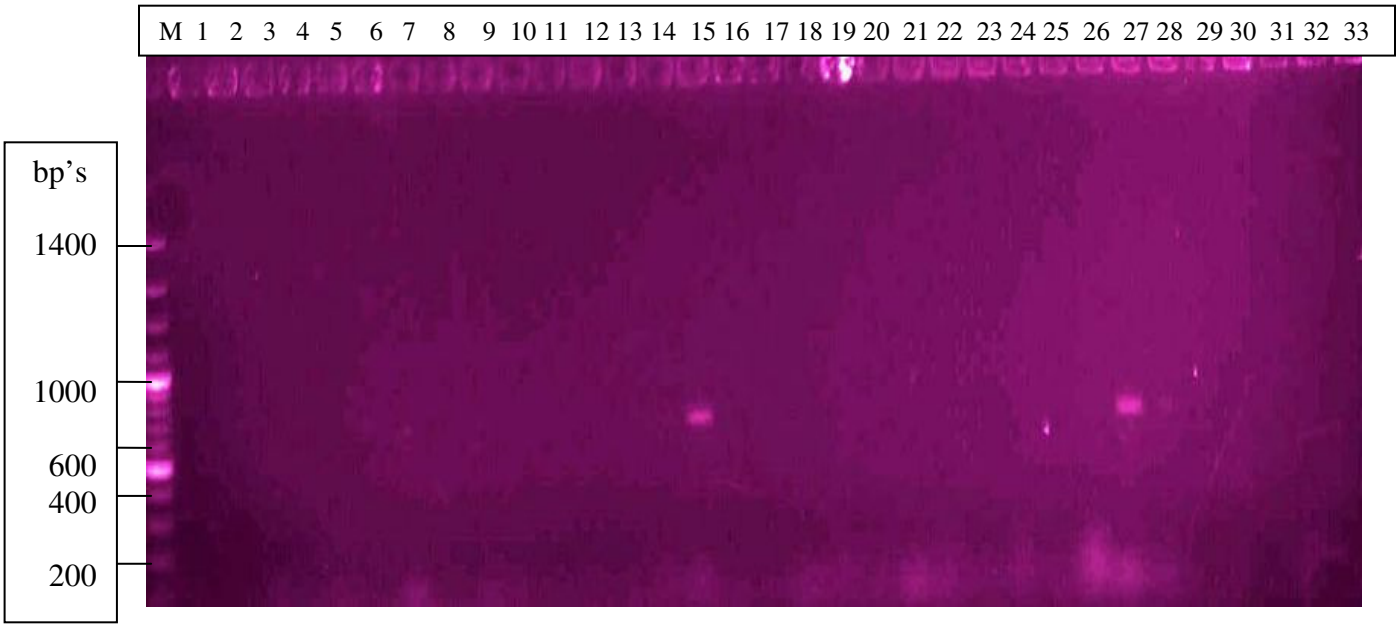

Plate 4.3. Agarose gel electrophoresis pattern of PCR amplification products of $Bla_{CTX\text{-}M}$ genes from *S.enterica* serovar. Typhi.

KEY: Lanes 1-25 show isolates from FMC Owerri while lanes 26-33 are from FMC Umuahia. The amplicon size (593bp) on lane 15 and 27 shows positive $Bla_{CTX\text{-}M}$ genes present in the DNA of *S. enterica* serovar. typhi from Owerri and Umuahia respectively. Lane M shows DNA molecular marker (100- bp ladder).

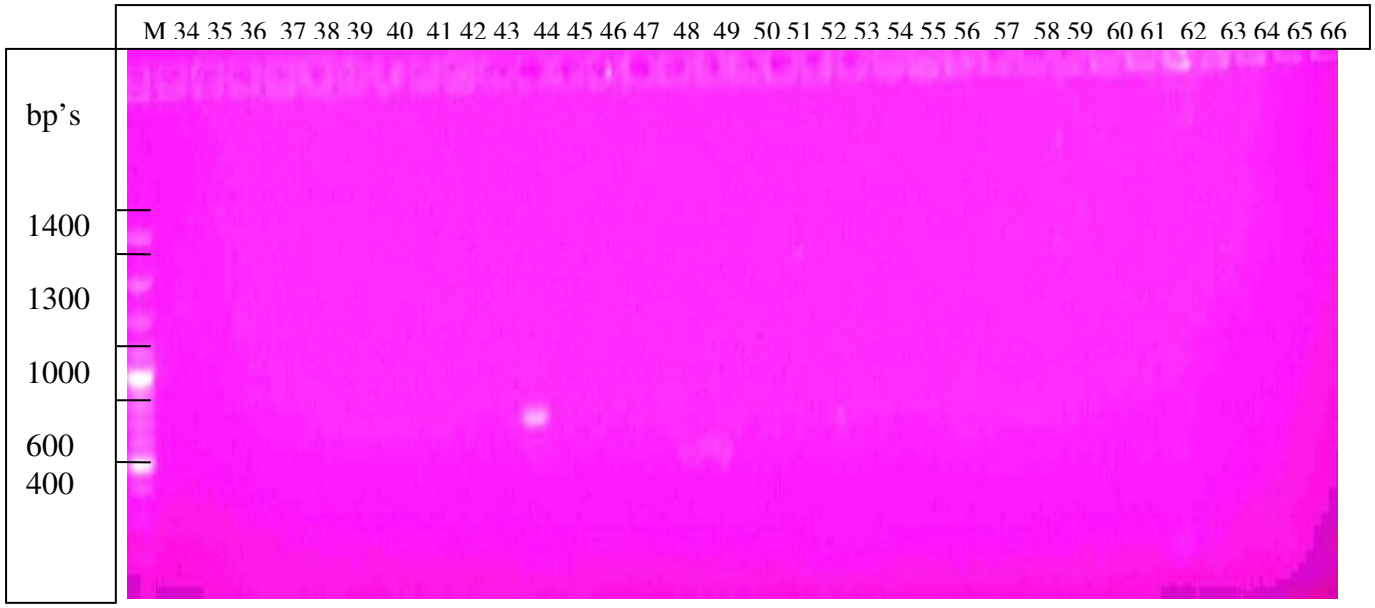

Plate 4.4. Agarose gel electrophoresis pattern of PCR amplification products of *Bla*$_{CTX-M}$ genes from *S.enterica* serovar. typhi at 62.5^{o}C.

KEY: Lanes 34-50 show isolates from FMC Umuahia while lanes 50-66 are isolates from UNTH Enugu. The amplicons size (593bp) on lane 44 shows positive Bla$_{CTX-M}$ genes present in the DNA of *S. enterica* serovar.Typhi from Umuahias. Lane M shows DNA molecular marker (100- bp ladder).

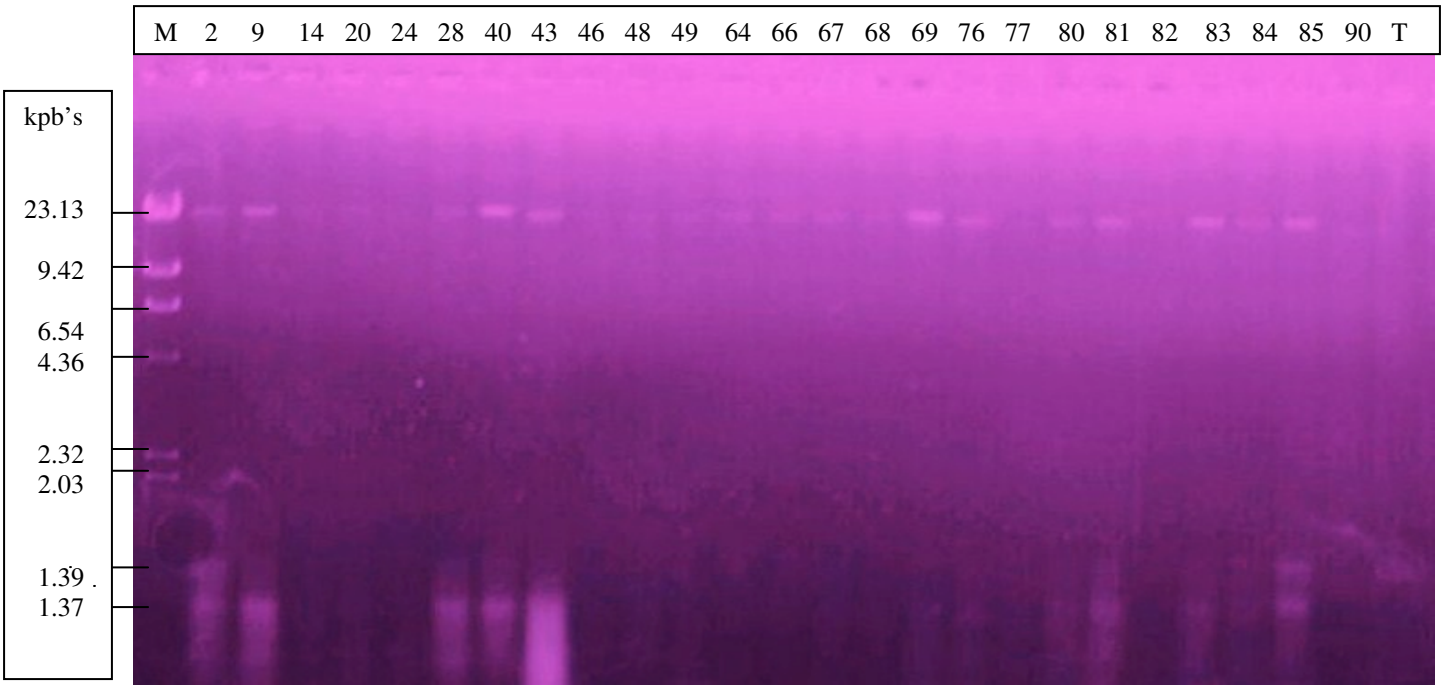

Plate 4.5. Agarose gel electrophoresis Pattern showing plasmid of *S. enterica* serovar before treatment with dyes.

KEY: Lane numbers 2, 9, 14, 20 and 24 from FMC Owerri, only lane 9 have plasmids with the Mwt. of 1.37 kb and lane 2 with two plasmids of Mwt. of 1.39 kb and 1.37 kb. Lane 28, 40,43,46,48 and 49 from FMC Umuahia with only lane number 28, 40 and 43 having plasmids of Mwt. of 1.37 kbs. Lane 64, 66, 67, 68 and 69 are from UNTH Enugu without plasmid. Lane 76, 77, 80, 81, 82, 83, 84, 85, and 90 are from FMC Abakaliki, with only lane numbers 76, 81 and 83 having plasmids of the same Mwt. of 1.37 kbs respectively, except lane number 85 with two plasmids of 1.39 and 1.37kbs. Lane T (positive control) is the *S. enterica* typed culture ATCC14028. Lane M is the lambda Hind III marker (0.12-23.1 kpb).

4.6. Distribution of resistant determinants of *S. enterica* in relation to patient's age from the Southeast region of Nigeria.

From Table 4.6 analysis shows the hospital record of the age range of individuals (patients) from whom *S. enterica* isolates were collected, the presence of plasmids harbored by the isolates, the presence of *GyrA* (Point mutation) and *ParC* (Double mutation) that codes for the quinolone resistant determining region (QRDR), *bla*-SHV, *bla*-TEM, and Bla_{CTX-M} type which codes for the presence of Extended Spectrum ß-lactamase cefotaxime type (CTX-M type) enzyme on the genomic DNA of the isolates *S.enterica*. The age range were classified from; 0-10, 11-20, 21-30, 31- 40, 41-50, 51- 60, 61-70, 71years and above. Patients who refused to declare their age on the record sheet but specified to be Adult were grouped under 18 and above as shown on the Table 4.5. The analysis revealed the number of patients from whom the isolates were recovered their age range and whether male or female. A total of 9 (9%) of the patients as shown on the record sheet of the hospital had plasmid DNA harboured in the isolates recovered from them (both males and females). While 55 (55%) of the total isolates showed positive *GryA* (male and female), 14 (14%) of the isolates had *ParC* (male and female), and 13 (13%) positive Bla_{CTX-M} type gene (male and female), and non harboured *bla*-SHV and *bla*-TEM, in the genomic DNA of the isolates *S. enterica* recovered from various age groups as shown on Table 4.6. Analysis revealed that the highest number of *S. enterica* isolates were recovered from patients between 18 and above, followed by the ages between 21- 30 and ages between 0- 10 years.

Plates 4.6, 4.7, 4.8 and 4.9 show the agarose gel electrophoresis pattern, revealing the single PCR amplification of *GyrA* gene (gyrase enzyme) in *S.enterica* serovar. typhi from the hospitals in the Southeast region of Nigeria that is FMC Owerri (SO1-SO25), FMC Umuahia (SU26-SU50), UNTH Enugu (SE51-SE75) and FMC Abakaliki (SA76-SA100). Analysis revealed the PCR amplicons size 251 bp's of *GyrA* gene mutation that codes for point mutation to fluoroquinolone, as recorded in isolates from Owerri, Umuahia, Enugu, and Abakaliki. The presence of this mutation in *GyrA* gene revealed the Quinolone Resistant Determining Region (QRDR) on the *S. enterica* serovar.typhi. This mutation in *GyrA* gene also codes for point mutation in the amino acid sequence of the *S.enterica*

chromosomal DNA sequence. Though this mutant genes (*GyrA*) alone does not rule complete resistance to fluoroquinolones (quinolones), without the presence of mutation in *ParC* (Topoisomerase IV enzyme) gene in the same isolate.

Plates 4.10 and 4.11 show the agarose gel electrophoresis pattern, revealing the single PCR amplification of double mutation in *ParC* (Topoisomerase iv enzyme) on *S.enterica* from the hospitals in the Southeast region of Nigeria. From the result the isolates from FMC Owerri (Plate 4.10) on lane number 3, 18 and 19 having amplicons size (251 bp's) in *GyrA* were also positive for mutation in *ParC* (Double mutation) genes in the DNA of *S.enterica* serovar.typhi (260 bp's) in Plate 4.10. The isolates from UNTH Enugu had only Lane number 58 and 60 having positive amplicons for double mutation in *ParC* (Plate 4.10). Also from Plate 4.11 isolates from UNTH Enugu with lane numbers 72,73,74, 75 had amplicons size of 260 bp's positive for mutation in *ParC* (Double mutation). Lane numbers 76, 77, 97, 98 and 99 are isolates from FMC Abakaliki also having amplicons positive for mutation in *ParC* (Plate 4.11).

Fig. 4.1 shows the incidence rate of the resistance gene production and plasmid profile of *S.enterica* serovars in relation to gender. Isolates from the male patients (42) irrespective of their age produced more resistance genes of *GyrA* 24 (57.14%), *ParC* 6 (14.3%) and Bla_{CTX-M} genes 7(16.7%), as against those from 58 female patients had *GyrA* 31 (53.4%), *ParC* 8(13.7%), and Bla_{CTX-M} genes 6 (10.3%), while none of these genes was detected in *bla*-SHV, *bla*-TEM, and *qnr*B. However, the production of R-plasmids profilewas higher in the females (10.3%) than males (7.14%) from their respective *S. enterica* isolates.

Table 4.6. Distribution of resistant determinants of *S. enterica* in relation to patient's age from the Southeast region of Nigeria.

S/NO	Age Limits (yrs)	No of patients (%)	Presence of Plasmids (M)	(F)	Presence of Gyr A (M)	(F)	Presence of Par C (M)	(F)	Presence of Bla_{CTX-M} (M)	(F)	*Bla*TEM/BlaSHV/Q*nrB*
1.	0-10	6	0	1	1	3	0	0	0	0	Not Detected
2.	11-20	5	0	1	2	2	1	0	0	0	Not Detected
3.	21-30	11	0	0	5	1	1	0	1	0	Not Detected
4.	31-40	6	0	0	1	2	1	0	0	0	Not Detected
5.	41-50	4	0	0	0	2	1	0	1	0	Not Detected
6.	51-60	6	0	1	0	1	1	0	0	0	Not Detected
7.	61-70	2	0	0	0	0	0	1	0	0	Not Detected
8.	71-Above	3	0	0	0	0	0	0	0	0	Not Detected
9.	Adult (18-Above)	57	3	3	15	19	1	7	6	5	Not Detected
Total		100	3	6	24	31	6	8	7	6	Nil in all
Total Percentages			9 (9%)		55 (55%)		14 (14%)		13 (13%)		

KEY: M= Male Patients, F = Female Patients , Adult (18- Above) = Patients that are up to 18 years and above and refuse to disclose their actual age., *GyrA* = Gyrase A enzyme, Bla_{CTX-M} = Beta lactamase cefotaxime-M class enzyme, *bla*-SHV = Beta- lactmase Sulfhydryl variable, *bla*-TEM = Beta-lactamase Temoniera, *ParC*= Topoisomerase iv enzyme,.

Note; The *QnrB* and other ESBL checked were not detected on all the isolates (*bla*-SHV, *bla*-TEM)

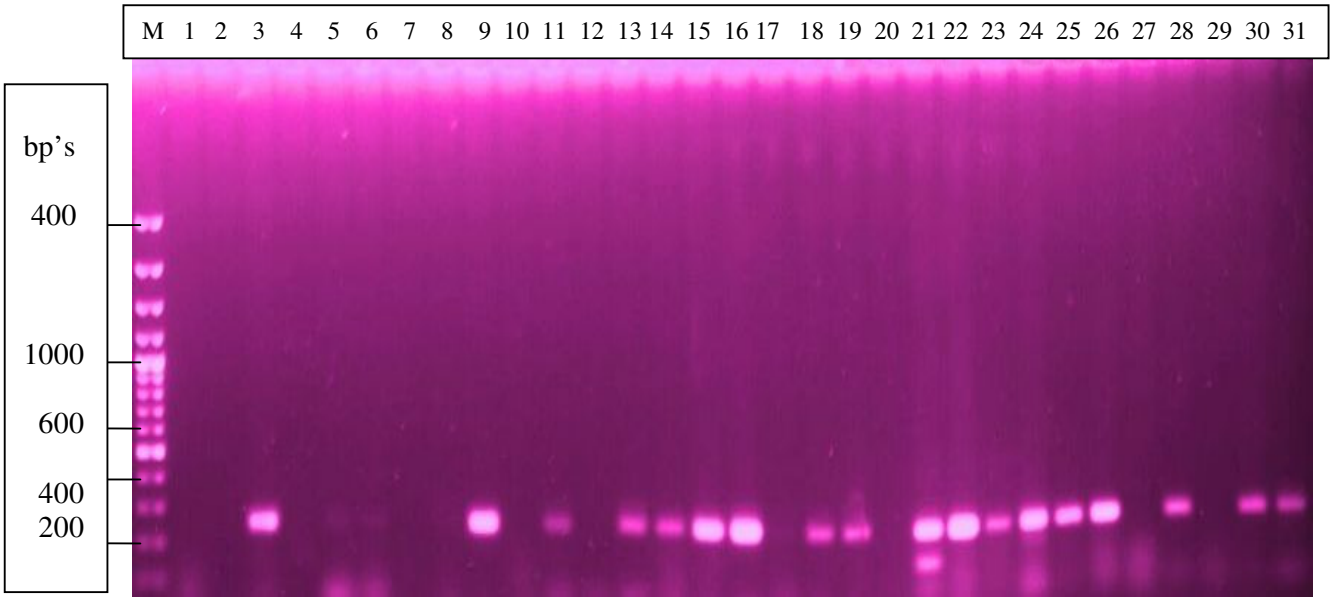

Plate 4.6. Agarose gel electrophoresis pattern of PCR amplification products of *GyrA* genes from *S. enterica*.

KEY: Lanes 1- 25 shows isolates from FMC Owerri. The amplicons size (251 bp's) on lanes 3,9,11,13,14,15,16,18,19,21,22,23,24,25 shows positive *GyrA*(point mutagen) genes present in the DNA of *S. enterica* serovar.typhi. Lanes 26-31 show the isolates from FMC Umuahia having PCR amplicons on lane 26,28,30 and 31 positive for *GyrA* (point mutation) genes present in DNA of *S. enterica* serovar typhi. Lane M shows DNA molecular marker (100- bp ladder).

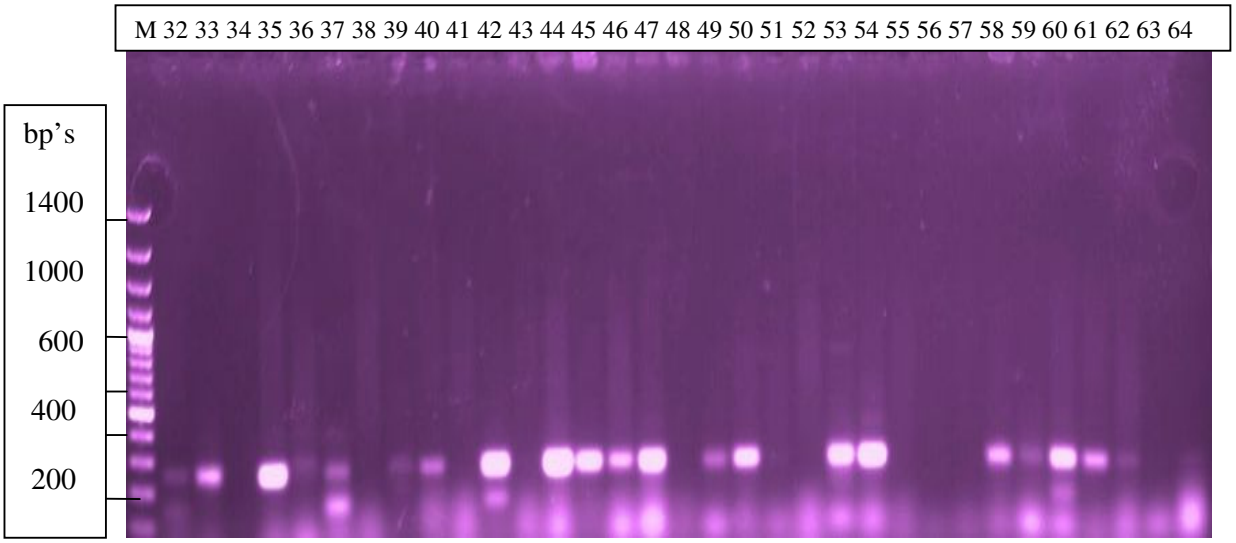

Plate 4.7. Agarose gel electrophoresis pattern of PCR amplification products of *GyrA* genes from *S. enterica* serovar. typhi.

KEY: Lanes 32- 50 shows isolates from FMC Umuahia. The amplicon size (251bp's) on lanes 32,33,35,36,37,39,40,42,44,45,46,47,49 and 50 shows positive *GyrA* (point mutation) genes present in the DNA of *S. enterica* serovar.typhi. Lanes 51-64 show the isolates from UNTH Enugu and having PCR amplicons positive for *GyrA* (point mutation) genes on lanes 53, 54, 58, 59, 60, 61 and 62. Lane M shows DNA molecular marker (100- bp ladder).

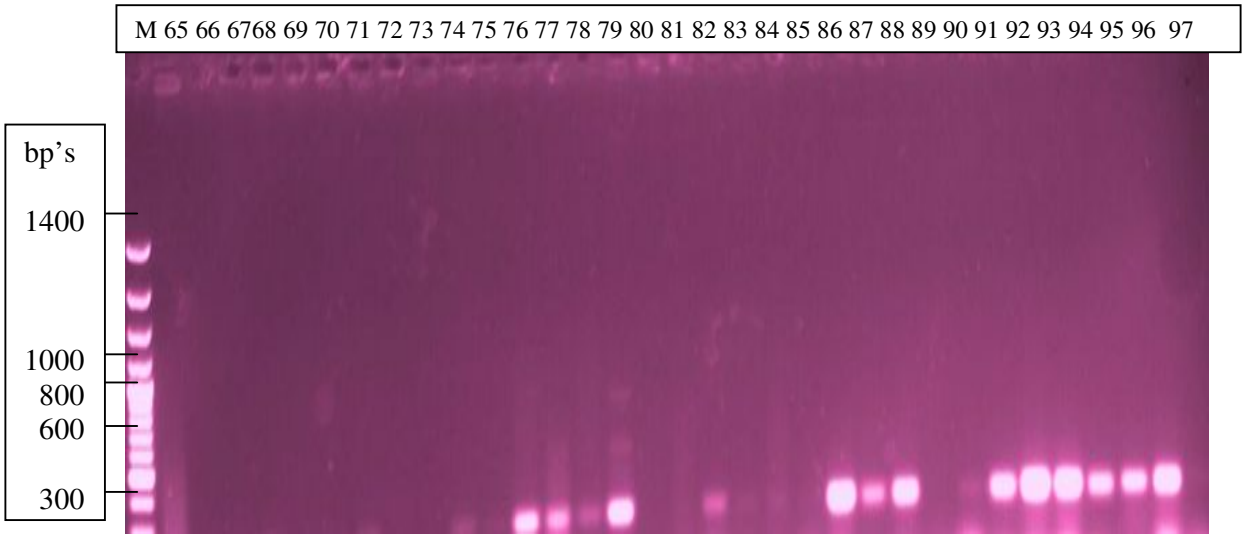

Plate 4.8. Agarose gel electrophoresis pattern of PCR amplification products of *GyrA* genes from *S. enterica*.

KEY: Lanes 65- 75 show isolates from UNTH Enugu with only lane 74 having amplicons size (251bp's) positive for *GyrA* (point mutagen) genes in the DNA of *S. enterica* serovar.typhi. Lanes 76-97 show the isolates from FMC Abakaliki, having PCR amplicons positive for *GyrA* (point mutation) genes in DNA of *S. enterica* serovar. Typhi on lane 76, 77, 78, 79, 82, 86, 87, 88, 91, 92, 93, 94, 95 and 96. Lane M shows DNA molecular marker (100- bp ladder).

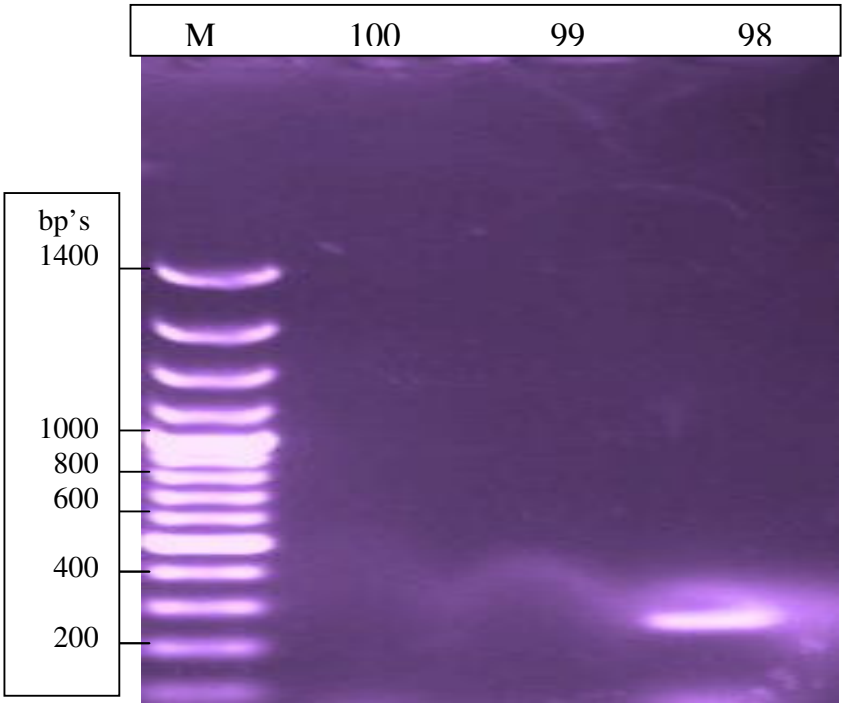

Plate 4.9. Agarose gel electrophoresis pattern of PCR amplification products of *GyrA* genes from *S. enterica*.

KEY: Lanes 98-100 show isolates from FMC Abakaliki with only lane 98 having amplicon size (251 bp's) positive for *GyrA* (point mutation) genes in the DNA of *S. enterica* serovar.typhi. Lane M shows DNA molecular marker (100- bp ladder).

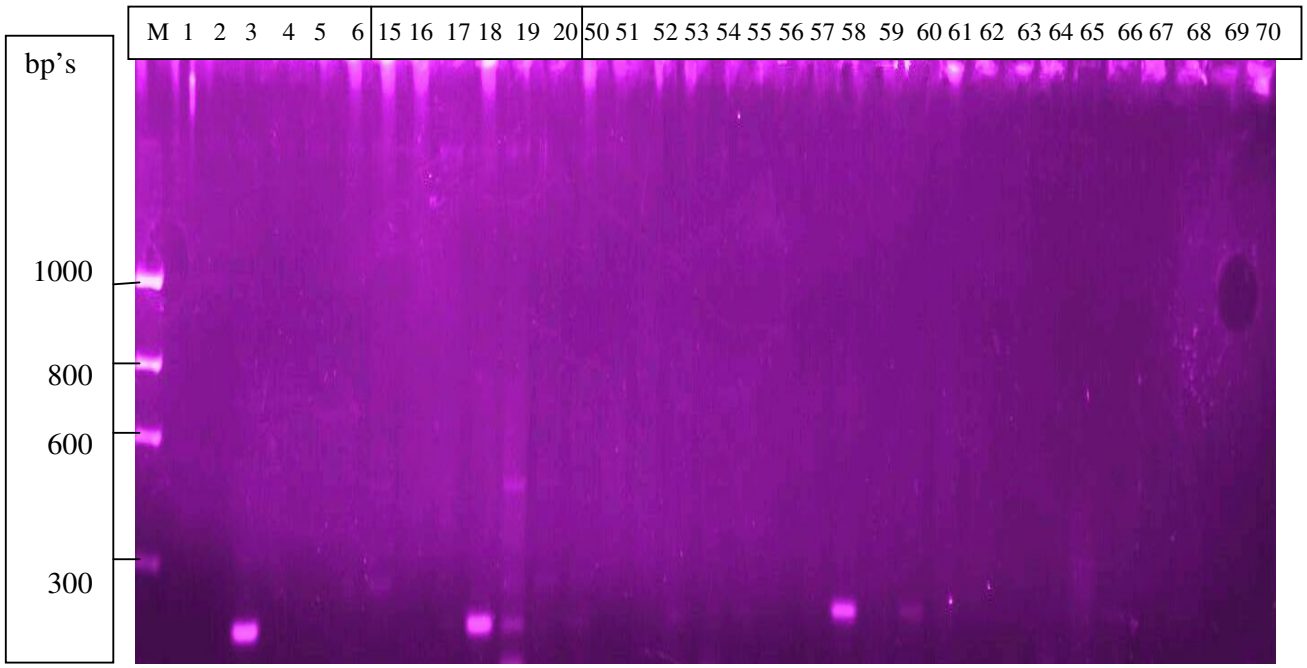

Plate 4.10. Agarose gel electrophoresis pattern of PCR amplification products of *ParC* genes from *S. enterica.*

KEY: Lanes 1-6 and lane numbers 15-20 show isolates from FMC Owerri with only lane number 3, 18 and 19 having amplicon size (260bp's) positive for *ParC* (Double mutation) genes in the DNA of *S.enterica* serovar.typhi. Lane number 50 shows only isolates from FMC Umuahia and was negative to the *ParC* genes. Lanes 51-70 show the isolates from UNTH Enugu with only Lane numbers 58 and 60 having positive amplicon for ParC genes in *S.enterica*. Lane M shows DNA molecular marker (100- bp ladder).

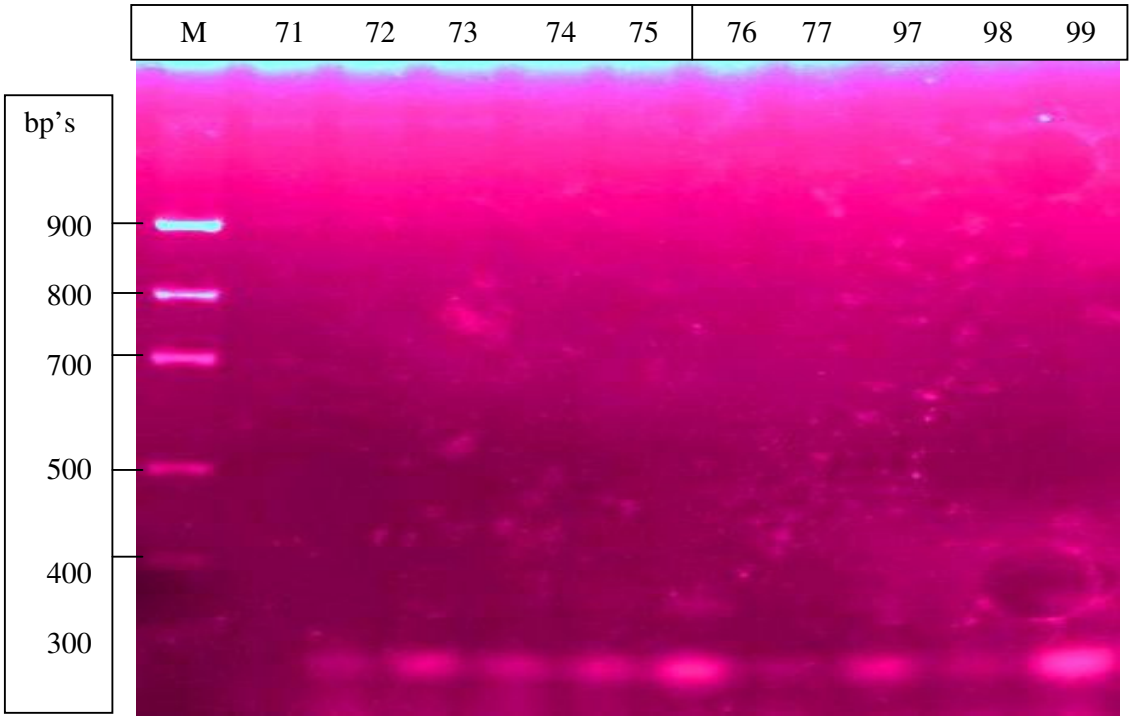

Plate 4.11. Agarose gel electrophoresis pattern of PCR amplification products of *ParC* genes from *S.enterica*.

KEY: Lanes 71-75 show isolates from UNTH Enugu with lane numbers 72, 73, 74, 75 having amplicon size (260 bp's) positive for *ParC* (Double mutation) genes in the DNA of *S.enterica* serovar.typhi. Lane numbers 76, 77, 97, 98 and 99 are isolates from FMC Abakaliki also having amplicons positive for *ParC*. Lane M shows DNA molecular marker (100- bp ladder).

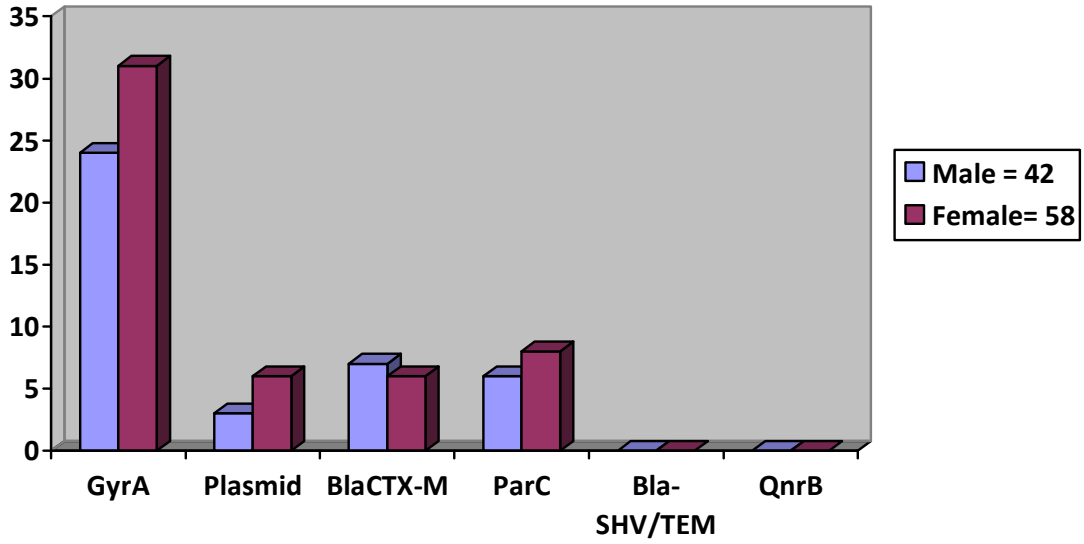

Fig. 4.1. The incidence rate of *qnrB*, *gyrA*, *parC*, *bla*SHV, *bla*TEM, bla_{CTX-M} and plasmids in *S. enterica* isolates from patients in Southeast region of Nigeria**.**

KEY: *GyrA* = gyrase A, *ParC*= Topoisomerase iv, Bla_{CTX-M} = B-lactamase cefotaxime –M type enzyme, N.D = Not detected

4.7. Antibiotics susceptibility of *S. enterica* in relation to their resistant determinants in the four (a-d) hospitals.

Table 4.7 a shows the antibiotic susceptibility of *S. enterica* isolates in relation to their resistance genes, antigenic properties and ESBL's production in the various hospitals**.** Isolates with code serial numbers SO1-SO25 were from the hospital in Owerri, the same applies to the hospitals in Umuahia (SU26- SU50) (Table 4.7 b), Enugu (SE51-75) Table 4.7 c and Abakaliki (SA76- SA100) Table 4.7 d. From Table 4.6 a for Federal Medical Centre (FMC) Owerri 13 (52%) of the *S.enterica* were ESBL's positive while 14 (56%) were positive for *GyrA*, 4 (16%) for Bla_{CTX-M}, 2 (8%) for plasmid and 13 (12%) for *ParC* (topoisomerase IV enzyme) while none was accounted for in *bla*-SHV, *bla*-TEM gene and the quinolone resistant protein of the isolates. The same applies to FMC Umuahia, UNTH Enugu and FMC Abakaliki respectively as shown below on Table 4.7 b-d.

Table 4.7 a. Antibiotic susceptibility of *S. enterica* in relation to their resistant determinants in FMC, Owerri.

Organism Code S/NO	Presence of *GyrA* genes	Presence of *ParC* genes	Presence of Bla_{CTX-M} genes	Presence of ESBL'S	Presence of Plasmids	Antibiotic resistant	Presence of *S.typhi* antigens
O1.	-	-	-	-	-	5	H &O
O2.	-	-	-	-	+	2	H &O
O3.	+	+	+	+	-	2	H &O
O4.	-	-	-	-	-	3	H &O
O5.	-	-	-	+	-	7	H &O
O6.	-	-	-	-	-	4	H &O
O7.	-	-	-	-	-	3	H &O
O8.	-	-	-	-	-	2	H &O
O9.	+	-	-	+	+	8	H &O
O10.	-	-	-	+	-	7	H &O
O11.	+	-	-	+	-	9	H &O
O12.	-	-	-	+	-	9	H &O
O13.	+	-	-	-	-	7	H &O
O14.	+	-	+	+	-	10	H &O
O15.	+	-	+	+	-	6	H &O
O16.	+	-	-	+	-	7	H &O
O17.	-	-	-	+	-	8	H &O
O18.	+	+	-	-l	-	10	H &O
O19.	+	+	-	-	-	10	H &O
O20.	-	-	-	+	-	8	H &O
O21.	+	-	-	-	-	5	H &O
O22.	+	-	-	-	-	3	H &O
O23.	+	-	-	+	-	4	H &O
O24.	+	-	+	+	-	6	H &O
O25	+	-	-	-	-	4	H &O
Percentage Prevalence	**14 (56%)**	**3 (12%)**	**4 (16%)**	**13 (52%)**	**12 (8%)**		

KEY: ***GyrA*** = gyrase A, ***ParC***= Topoisomerase iv, $\boldsymbol{Bla_{CTX-M}}$ = ß-lactamase cefotaxime –M type enzyme, **ESBL's** = Extended Spectrum ß-lacatamase enzyme, **H** = Flagellated antigen, **O** = Somatic antigen, **O1- 25**= Isolates code from Owerri, **Nil** = Negative , **+ve** = Positive, *bla*-SHV and *bla*-TEM = Not detected (not shown on the table).

Table 4.7 b. Antibiotic susceptibility of *S. enterica* in relation to their resistant determinants in FMC, Umuahia (continue)

Organism Code S/NO	Presence of *GyrA* genes	Presence of *ParC* genes	Presence of Bla_{CTX-M} genes	Presence of ESBL'S	Presence of Plasmids	Antibiotic resistant	Presence of *S.typhi* antigens
U26.	+	-	-	+	-	5	H &O
U27.	-	-	+	-	-	6	H &O
U28.	+	-	+	-	+	7	H &O
U29.	-	-	-	-	-	3	O
U30.	+	-	-	-	-	6	O
U31.	+	-	-	-	-	1	O
U32.	+	-	-	-	-	6	O
U33.	+	-	-	+	-	7	H &O
U34.	-	-	-	-	-	4	H &O
U35.	+	-	-	-	-	5	H &O
U36.	+	-	-	-	-	4	H &O
U37.	+	-	-	-	-	5	H &O
U38.	-	-	-	+	-	6	H &O
U39.	+	-	+	-	-	6	H &O
U40.	+	-	+	+	+	11	H &O
U41.	-	-	-	-	-	5	H &O
U42.	+	-	-	-	-	4	H &O
U43.	-	-	-	-	+	5	H &O
U44.	+	-	+	-	-	9	H &O
U45.	+	-	-	-	-	3	H &O
U46.	+	-	-	-	-	5	H &O
U47.	+	-	-	-	-	4	H &O
U48.	-	-	-	-	-	7	H &O
U49.	+	-	+	-	-	11	H &O
U50	+	-	+	-	-	7	H &O
Percentage Prevalence	**16 (72%)**	**0 (0%)**	**7 (28%)**	**4 (16%)**	**3 (12%)**		

KEY: ***GyrA*** = gyrase A, ***ParC*****=** Topoisomerase iv, $\boldsymbol{Bla_{CTX-M}}$ = ß-lactamase cefotaxime –M type enzyme, **ESBL's** = Extended Spectrum ß-lacatamase enzyme, **H** = Flagellated antigen**, O=** Somatic antigen, **U26- 50=** Isolates code from Umuahia, **Nil=** Negative , **+ve** = Positive, *bla*-SHV and *bla*-TEM = Not detected (not shown on the table).

Table 4.7 c: Antibiotic susceptibility of *S. enterica* in relation to their resistant determinants in UNTH, Enugu (continue)

S/NO	Presence of *GyrA* genes	Presence of *ParC* genes	Presence of Bla_{CTX-M} genes	Presence of ESBL'S	Presence of Plasmids	No of antibiotic resistant	Presence of *S.typhi* antigens
E51.	-	-	-	-l	-	7	H &O
E52.	-	-	-	-	-	9	H &O
E53.	+	-	+	+	-	8	H &O
E54.	+	-	+	-	-	5	H
E55.	-	-	-	-	-	7	H &O
E56.	-	-	-	-	-	6	H &O
E57.	-	-	-	-	-	6	H &O
E58.	+	+	-	-	-	5	H
E59.	+	-	-	-	-	4	H &O
E60.	+	+	-	-	-	7	H &O
E61.	+	-	-	-	-	9	H &O
E62.	+	-	-	-	-	3	H &O
E63.	-	-	-	-	-	6	H &O
E64.	-	-	-	-	-	8	H &O
E65.	-	-	-	+	-	8	H &O
E66.	-	-	-	+	-	13	H &O
E67.	-	-	-	+	-	11	H &O
E68.	-	-	-	+	-	10	H &O
E69.	-	-	-	+	-	12	H &O
E70.	-	-	-	-	-	7	H &O
E71.	-	-	-	-	-	9	H &O
E72.	-	+	-	+	-	11	H &O
E73.	-	+	-	-	-	8	H &O
E74.	+	+	-	-	-	6	H &O
E75	-	+	-	-	-	13	H &O
Percentage Prevalence	**8 (32%)**	**6 (24%)**	**2 (8%)**	**7 (28%)**	**0 (0%)**		

KEY: ***GyrA*** = gyrase A, ***ParC***= Topoisomerase iv, $\boldsymbol{Bla_{CTX-M}}$ = ß-lactamase cefotaxime –M type enzyme, **ESBL's** = Extended Spectrum ß-lacatamase enzyme, **H** = Flagellated antigen, **O** = Somatic antigen, **E51- 75**= Isolates code from Enugu, **Nil** = Negative , **+ve** = Positive, *bla*-SHV and *bla*-TEM = Not detected (not shown on the table).

Table 4.7d. Antibiotic susceptibility of *S. enterica* in relation to their resistant determinants in FMC, Abakaliki**.**

Organism Code S/NO	Presence of GyrA genes	Presence of ParC genes	Presence of Bla_{CTX-M} genes	Presence of ESBL'S	Presence of Plasmids	Antibiotic resistant	Presence of S.typhi antigens
A76.	+	+	-	+	+	12	H &O
A77.	+	+	-	+	-	14	H &O
A78.	+	-	-	-	-	13	H &O
A79.	+	-	-	-	-	10	H &O
A80.	-	-	-	-	-	13	H
A81.	-	-	-	+	+	10	H &O
A82.	+	-	-	+	-	10	H &O
A83.	-	-	-	+	+	12	H &O
A84.	-	-	-	-	-	13	H &O
A85.	-	-	-	+	+	10	H &O
A86.	+	-	-	-	-	12	H &O
A87.	+	-	-	-	-	9	H &O
A88.	+	-	-	-	-	11	H &O
A89.	-	-	-	-	-	6	H &O
A90.	-	-	-	-	-	9	O
A91.	+	-	-	-	-	8	H &O
A92.	+	-	-	-	-	1	H &O
A 93.	+	-	-	+	-	9	H &O
A 94.	+	-	-	+	-	11	H &O
A 95.	+	-	-	+	-	7	H &O
A 96.	+	-	-	-	-	6	H &O
A 97.	-	+	-	-	-	6	H &O
A 98.	+	+	-	+	-	8	H &O
A 99.	-	+	-	+	-	9	H &O
A100.	-	-	-	+	-	12	H &O
Percentage Prevalence	**15 (60%)**	**5 (20%)**	**0 (0%)**	**12 (48%)**	**4 (16%)**		

KEY: ***GyrA*** = gyrase A, ***ParC*****=** Topoisomerase iv, $\boldsymbol{Bla_{CTX-M}}$ = ß-lactamase cefotaxime –M type enzyme, **ESBL's** = Extended Spectrum ß-lacatamase enzyme, **H** = Flagellated antigen, **O** = Somatic antigen, **A76- 100=** Isolates code from Owerri, **Nil** = Negative , **+ve** = Positive., *bla*-SHV and *bla*-TEM = Not detected (not shown on the table).

4.8 Percentage antibiotic susceptibility pattern of *S. enterica* in relation to the resistant determinants

Table 4.8 shows the percentage of antibiotic susceptibility pattern of *S. enterica*. From the results obtained 87% of the isolates were amoxicillin/clavulanic acid resistant and 80% were chloramphenicol resistant followed by amoxicillin which has 80% resistant isolates, and co-trimoxazole, sparfloxacin, streptomycin and gentamycin which had 78, 78, 77 and 51% resistant isolates respectively. Levofloxacin and ceftriaxone had 78% of the *S. enterica* being susceptible to them, followed by cefotaxime (73%), ofloxacin (72%), sparfloxacine (71%), ciprofloxacine (71%) and ceftazidime 56% susceptibility. Out of the 100, the isolates tested for genes encoding *GyrA* coding for point mutation in their chromosomal DNA, 48% of the isolates harbouring *GyrA* in their gene were found to be resistant to amoxiclavulanic acid while 11% showed $Bla_{CTX\text{-}M}$ type gene and *ParC*, and 7% produced plasmids. Others were chloramphenicol resistant isolates having 45% habouring *GyrA* gene, 12% of the isolates having $Bla_{CTX\text{-}M}$ type, 14% of isolates having *ParC* gene and 7% of the isolates harbouring plasmids, while none of the isolates harboured *qnrB*, *bla*-SHV and *bla*TEM gene respectively in all the 14 antibiotics used against them in this study. Others were as shown in the Table 4.8.

Fig.4.2. shows the graphical representation of the genetic constituents of the isolates of *S. enterica* and their resistance factors as related to each of the 5 selected antibiotics screened. Out of the twenty-nine (29) isolates of *S. enterica* resistant to ciprofloxacin, only fifteen (15) produced mutation in *GyrA*, eight (8) produced double mutation in *ParC*, one (1) produced $Bla_{CTX\text{-}M}$ and three isolates produced plasmids. Also out of twenty-two (22) isolates resistant to levofloxacin nine (9) produced mutation in *GyrA* genes, while *ParC*, $Bla_{CTX\text{-}M}$, and plasmids were produced by only two (2) isolates, each. Other resistance gene produced by each *S. enterica* isolates resistant to other antibiotics such as ceftriaxone (CRO), cefotaxime (CTX-M), and amoxiclavulanic acid (AMC) were as shown graphically.

Table 4.8. Percentage antibiotic susceptibility pattern of *S. enterica* in relation to the resistant determinants

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PERCENTAGE RESISTANT AND SUSCEPTIBILITY OF ISOLATES				RESISTANT DETERMINANTS			
S/NO	ANTIBIOTICS	SENSITIVE (%)	RESISTANT (%)	*GyrA* (%)	*ParC* (%)	Bla_{CTX-M} (%)	Plasmid (%)
1.	Co-trimoxazole 30 µg	22	78	40	12	9	9
2.	Chloramphenicol 30 µg	20	80	45	14	12	7
3.	Sparfloxacin 10 µg,	22	78	43	12	10	7
4.	Ciprofloxacin 10 µg	71	29	15	8	1	3
5.	Amoxicillin 30 µg	20	80	46	12	9	7
6.	Gentamycin 10 µg	49	51	27	11	7	6
7.	Pefloxacin 30 µg	71	29	17	7	4	4
8.	Ofloxacin 10 µg	72	28	16	7	4	3
9.	Streptomycin 30 µg	23	77	41	11	12	7
10.	Levofloxacin 5 µg	78	22	9	2	2	2
11.	Ceftriaxone 30 µg	78	22	9	4	4	5
12.	Cefotaxime 30 µg	73	27	9	5	4	5
13.	Ceftazidime 30 µg	56	44	21	6	3	4
14.	Amoxicillin/Clavulanic acid 30 µg	13	87	48	11	11	7

KEY: *GyrA* = gyrase A, *Par C*= Topoisomerase iv, Bla_{CTX-M} = ß-lactamase cefotaxime –M type enzyme, % = Percentage .Note; *QnrB*, *bla*-SHV/ TEM were not detected on the isolates during PCR.

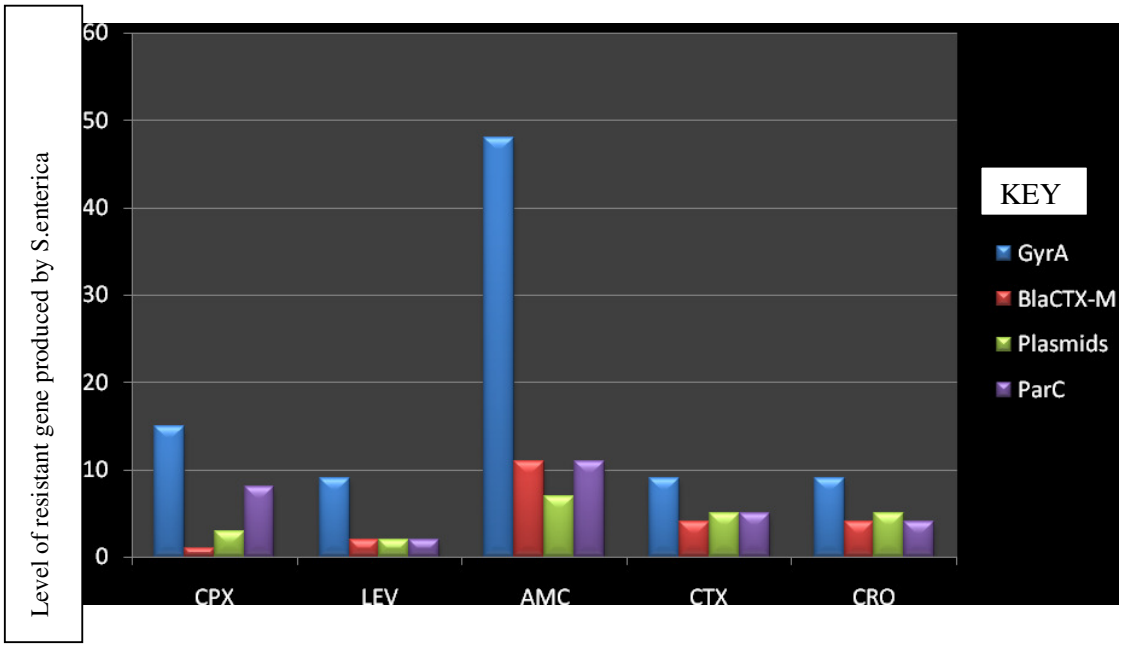

Fig.4.2. Shows graphical representation of the genetic constituents, the number of antibiotics and resistant pattern of isolates that produced Plasmids or a type of mutation in a gene detected using PCR amplification, out of the total isolates resistant to each antibiotics.

4.9 Phenotypic antibiotic resistance patterns of *S. enterica* from the four hospitals (a-d) in relation to Beta- lactamase and ESBLs production.

Table 4.9 shows the phenotypic pattern in isolates producing ß-lactamase enzyme and Extended Spectrum β-lactamases (ESBLs). As shown in Table 4.9 a- d, some isolates which showed Beta-lactamase negative were still resistant to at least 3-10 antibiotics, yet they were ESBL negative also. Also some ßeta-lactamase positive isolates that were also ESBL positive were resistanct to only two (2) antibiotics. These discripancies may be due to chromosomal constituents which might be constitutive or inductive (i.e some Beta-lactamase act only when there are presence of Beta-lactam drugs (inducive) while some Beta-lactamase enzymes are constituents of the chromosomal DNA and other are plasmids mediated). Table 4.9 a-d also show classes of ßeta-lactamase and ESBL, which might belong to several classes like TEM1, Bla_{CTX-M} , SHV, Metallo or Zinc- mediated Beta-lactamse etc. The phenotypic resistance was exhibited more on the isolates from the Federal Medical Centre (FMC) Abakaliki (SA76-SA100) Table 4.9 d.

Fig. 4.3 shows the graphical representation of the genetic constituents of *S. enterica* isolates received from different hospitals in the Southeast region of Nigeria. From the study, analysis revealed that from the hospital in Owerri (1-25), two (2) isolates produced plasmids, while 14, 4 and 3 *S. enterica* isolates produced *GyrA*, Bla_{CTX-M} and *ParC* respectively. From the hospital in Umuahia (26-50), 3, 18, and 7 *S.enterica* isolates produced plasmids, mutation in *GyrA*, Bla_{CTX-M} gene, while none of the isolates produced double mutation in *ParC* gene. Other isolates from the hospital in Enugu (51-75) and Abakaliki (76-100) were also reported graphically as shown in the Figure 4.3.

Table 4.9a. Phenotypic antibiotic resistance patterns of *S. enterica* from FMC Owerri in relation to Beta- lactamase and ESBL's production.

CODE S/NO	NUMBER OF ANTIBIOTICS	RESISTANCE PHENOTYPIC PATTERN	BETA LACTAMASE PRODUCTION	PRESENCE OF ESBL'S
O1.	**5**	SP,AM,STR,CAZ,AMC	**+**	-
O2.	**2**	CH,AMC	**+**	-
O3.	**2**	CH,AMC	**+**	+
O4.	**3**	SXT,CH,CAZ	**-**	-
O5.	**7**	SXT,CH,SP,AM,STR,CAZ,AMC	**+**	+
O6.	**4**	LEV,CTX,CAZ,AMC	**+**	-
O7.	**3**	SXT,CH,AMC	**+**	-
O8.	**2**	SXT,CAZ	**+**	-
O9.	**8**	SXT,CH,SP,CPX,AM,STR,CAZ,AMC	**+**	+
O10.	**7**	SXT,CH,SP,CPX,AM,CAZ,AMC	**+**	+
O11.	**9**	SXT,CH,SP,CPX,AM,GEN,STR,CAZ,AMC	**+**	+
O12.	**9**	SXT,CH,SP,CPX,AM,GEN,STR,CAZ,AMC	**+**	+
O13.	**7**	SXT,CH,SP,AM,GEN,STR,AMC	**+**	-
O14.	**10**	SXT,CH,SP,AM,STR,LEV,CRO,CTX,CAZ,AMC	**+**	+
O15.	**6**	SXT,CH,SP,AM,STR,AMC	**+**	+
O16.	**7**	SXT,CH,SP,AM,STR,CAZ,AMC	**+**	+
O17.	**8**	SXT,CH,SP,AM,GEN,STR,CAZ,AMC	**-**	+
O18.	**10**	SXT,CH,SP,CPX,AM,GEN,PEF,OFX,STR,AMC	**-**	-
O19.	**10**	SXT,CH,SP,CPX,AM,GEN,PEF,OFX,STR,AMC	**+**	-
O20.	**8**	SP,AM,STR,LEV,CRO,CTX,CAZ,AMC	**+**	+
O21.	**5**	SXT,SP,AM,STR,AMC	**+**	-
O22.	**3**	AM,STR,AMC	**-**	-
O23.	**4**	AM,STR,CAZ,AMC	**-**	+
O24.	**6**	AM,STR,CRO,CTX,CAZ,AMC	**+**	+
O25	**4**	CH,AM,CAZ,AMC	**+**	-

KEY: SXT= co-trimoxazole 30 µg, CH= chloramphenicol 30 µg, SP= sparfloxacin 10 µg, CPX= ciprofloxacin 10 µg, AM= amoxicillin 30 µg, GEN= gentamycin 10 µg, PEF= pefloxacin 30 µg, OFX= ofloxacin 10 µg, STR= streptomycin 30 µg, LEV= levofloxacin 5 µg, CRO= ceftriaxone 30 µg, CTX= cefotaxime 30 µg, CAZ= ceftazidime 30 µg, AMC= amoxicillin/clavulanic acid 30 µg, +ve = Positive, ESBL= Extended Spectrum ßeta Lactamase, - = Negative.

Table 4.9b. Phenotypic antibiotic resistance patterns of *S. enterica* from FMC Umuahia in relation to Beta- lactamase and ESBL's production (continue).

CODE S/NO	NUMBER OF ANTIBIOTICS	RESISTANCE PHENOTYPIC PATTERN	BETA-LACTAMASE PRODUCTION	PRESENCE OF ESBL'S
U26.	**5**	CH,AM,STR,CAZ,AMC	**-**	+
U27.	**6**	SXT.CH,SP,AM,STR,AMC	**+**	-
U28.	**7**	SXT,CH,AM,GEN,PEF,OFX,STR	**+**	-
U29.	**3**	AM,CAZ,AMC	**-**	-
U30.	**6**	SXT.CH.SP,AM,STR,AMC	**-**	-
U31.	**1**	AM	**+**	-
U32.	**6**	SXT,CH,SP,AM,STR,AMC	**+**	-
U33.	**7**	SXT,SP,AM,PEF,OFX,CAZ,AMC	**+**	+
U34.	**4**	CH,GEN,STR,AMC	**-**	-
U35.	**5**	SXT,CH,SP,AM,AMC	**+**	-
U36.	**4**	SXT,CH,SP,AMC	**+**	-
U37.	**5**	CH,SP,AM,STR,AMC	**-**	-
U38.	**6**	SXT,SP,AM,STR,CAZ,AMC	**-**	+
U39.	**6**	CH,SP,AM,GEN,PEF,STR	**+**	-
U40.	**11**	SXT,CH,SP,AM,GEN,PEF,OFX,STR,CRO,CTX,AMC	**+**	+
U41.	**5**	SXT,CH,SP,GEN,AMC,	**+**	-
U42.	**4**	CH,SP,GEN,AMC,	**-**	-
U43.	**5**	SXT,CH,SP,GEN,AMC	**+**	-
U44.	**9**	SXT,CH,SP,AM,GEN,PEF,OFX,STR,AMC	**+**	-
U45.	**3**	CH,SP,AMC	**+**	-
U46.	**5**	SXT,CH,SP,STR,AMC	**+**	-
U47.	**4**	SP,AM,STR,AMC	**+**	-
U48.	**7**	SXT,CH,SP,PEF,OFX,STR,AMC	**+**	-
U49.	**11**	CH,SP,GEN,PEF,OFX,STR,LEV,CRO,CTX,CAZ,AMC	**+**	-
U50	**7**	SXT,CH,SP,AM,GEN,STR,AMC	**+**	-

KEY: SXT= co-trimoxazole 30 µg, CH= chloramphenicol 30 µg, SP= sparfloxacin 10 µg, CPX= ciprofloxacin 10 µg, AM= amoxicillin 30 µg, GEN= gentamycin 10 µg, PEF= pefloxacin 30 µg, OFX= ofloxacin 10 µg, STR= streptomycin 30 µg, LEV= levofloxacin 5 µg, CRO= ceftriaxone 30 µg, CTX= cefotaxime 30 µg, CAZ= ceftazidime 30 µg, AMC= amoxicillin/clavulanic acid 30 µg, +ve = Positive, ESBL= Extended Spectrum ßeta Lactamase, - = Negative.

Table 4.9c. Phenotypic antibiotics resistance patterns of *S. enterica* from UNTH Enugu in relation to Beta- lactamase and ESBL's production (Continue).

CODE S/NO	NUMBER OF ANTIBIOTICS	RESISTANCE PHENOTYPIC PATTERN	BETA LACTAMASE PRODUCTION	PRESENCE OF ESBL'S
E51.	**7**	SXT,CH,SP,AM,GEN,STR,AMC	**+**	-
E52.	**9**	SXT,CH,SP,AM,GEN,PEF,OFX,STR,AMC	**+**	-
E53.	**8**	SXT,CH,SP,CPX,AM,GEN,STR,AMC	**+**	+
E54.	**5**	SXT,CH,SP,STR,AMC	**+**	**-**
E55.	**7**	SXT,CH,SP,AM,GEN,STR,AMC	**+**	**-**
E56.	**6**	SXT,CH,SP,GEN,STR,AMC	**+**	**-**
E57.	**6**	SXT,CH,SP,GEN,STR,AMC	**+**	**-**
E58.	**5**	SXT,CH,GEN,STR,AMC	**+**	**-**
E59.	**4**	SXT,AM,STR,AMC	**+**	**-**
E60.	**7**	CH,SP,AM,PEF,OFX,LEV,AMC	**-**	**-**
E61.	**9**	SXT,CH,SP,AM,GEN,PEF,OFX,STR,AMC	**-**	**-**
E62.	**3**	SXT,AM,STR	**-**	**-**
E63.	**6**	SXT,AM,OFX,STR,LEV,AMC	**+**	-
E64.	**8**	AM,PEF,OFX,STR,LEV,CRO,CTX,AMC	**+**	-
E65.	**8**	SXT,CH,SP,CPX,AM,STR,CTX,CAZ	**-**	**+**
E66.	**13**	SXT,CH,SP,CPX,AM,PEF,OFX,STR,LEV,CRO,CTX,CAZ,AMC	**-**	**+**
E67.	**11**	SXT,CH,SP,AM,PEF,OFX,STR,LEV,CRO,CTX,AMC	**+**	**+**
E68.	**10**	SXT,CH,SP,AM,PEF,STR,LEV,CRO,CTX,AMC	**+**	**+**
E69.	**12**	SXT,CP,SP,AM,PEF,OFX,STR,LEV,CRO,CTX,CAZ,AMC	**+**	**+**
E70.	**7**	SXT,CH,SP,AM,GEN,STR,AMC	**+**	-
E71.	**9**	SXT,CH,SP,CPX,AM,GEN,PEF,STR,AMC	**+**	-
E72.	**11**	SXT,CH,SP,CPX,AM,GEN,PEF,OFX,STR,CTX,AMC	**+**	+
E73.	**8**	SXT,CH,SP,AM,GEN,STR,CTX,CAZ	**+**	-
E74.	**6**	SXT,CH,SP,AM,STR,AMC	**+**	-
E75	**13**	SXT,CH,SP,CPX,AM,GEN,PEF,OFX,STR,CRO,CTX,CAZ,AMC	**+**	-

KEY: SXT= co-trimoxazole 30 µg, CH= chloramphenicol 30 µg, SP= sparfloxacin 10 µg, CPX= ciprofloxacin 10 µg, AM= amoxicillin 30 µg, GEN= gentamycin 10 µg, PEF= pefloxacin 30 µg, OFX= ofloxacin 10 µg, STR= streptomycin 30 µg, LEV= levofloxacin 5 µg, CRO= ceftriaxone 30 µg, CTX= cefotaxime 30 µg, CAZ= ceftazidime 30 µg, AMC= amoxicillin/clavulanic acid 30 µg, +ve = Positive, ESBL= Extended Spectrum ßeta Lactamase, - = Negative.

Table 4.9 d. Phenotypic antibiotic resistance patterns of *S. enterica* from FMC Abakaliki in relation to Beta- lactamase and ESBLs production (Continue).

CODE S/NO	NUMBER OF ANTIBIOTICS	RESISTANCE PHENOTYPIC PATTERN	BETA LACTAMASE PRODUCTION	PRESENCE OF ESBL'S
A76.	**12**	SXP,CH,SP,AM,GEN,PEF,OFX,STR,CRO,CTX,CAZ,AMC	**+**	+
A77.	**14**	SXT,CH,SP,CPX,AM,GEN,PEF,OFX,STR,LEV,CRO,CTX,CAZ,AMC	**+**	+
A78.	**13**	SXT,CH,SP,CPX,AM,GEN,PEF,STR,LEV,CRO,CTX,CAZ,AMC	**+**	-
A79.	**10**	SXT,CH,SP,CPX,AM,GEN,PEF,STR,CAZ,AMC	**+**	-
A80.	**13**	SXT,CH,SP,CPX,AM,GEN,PEF,STR,LEV,CRO,CTX,CAZ,AMC	**+**	-
A81.	**10**	SXT,CH,SP,CPX,AM,GEN,STR,CRO,CTX,CAZ	**+**	+
A82.	**10**	SXT,CH,SP,AM,GEN,OFX,STR,LEV,CAZ,AMC	**+**	+
A83.	**12**	SXT,SP,CPX,AM,GEN,PEF,OFX,STR,LEV,CRO,CTX,AMC	**+**	+
A84.	**13**	SXT,CH,SP,CPX,AM,GEN,OFX,STR,LEV,CRO,CTX,CAZ,AMC	**+**	-
A85.	**10**	SXT,SP,CPX,AM,STR,LEV,CRO,CTX,CAZ,AMC	**+**	+
A86.	**12**	SXT,CH,SP,CPX,AM,GEN,STR,LEV,CRO,CTX,CAZ,AMC	**+**	-
A87.	**9**	SXT,CH,SP,CPX,AM,STR,LEV,CAZ,AMC	**+**	-
A88.	**11**	SXT,CH,SP,CPX,AM,GEN,OFX,STR,LEV,CAZ,AMC	**+**	-
A89.	**6**	SXT,CH,AM,GEN,STR,AMC	**+**	-
A90.	**9**	SXT,CH,SP,AM,GEN,STR,CTX,CAZ,AMC	**+**	-
A91.	**8**	SXT,CH,SP,CPX,AM,GEN,PEF,OFX	**-**	-
A92.	**1**	AMC	**+**	-
A 93.	**9**	SXT,CH,SP,CPX,AM,GEN,PEF,OFX,STR	**-**	+
A 94.	**11**	SXT,CH,SP,CPX,AM,GEN,PEF,OFX,STR,CAZ,AMC	**+**	+
A 95.	**7**	SXT,CH,SP,AM,GEN,CAZ,AMC	**+**	+
A 96.	**6**	SXT,CH,SP,AM,GEN,AMC	**+**	-
A 97.	**6**	SXT,CH,SP,CPX,AM,GEN	**+**	-
A 98.	**8**	SXT,CH,SP,CPX,AM,GEN,STR,CAZ	**+**	+
A 99.	**9**	SXT,CH,SP,CPX,AM,GEN,STR,CAZ,AMC	**+**	+
A100.	**12**	SXT,CH,SP,AM,GEN,OFX,STR,LEV,CRO,CTX,CAZ,AMC	**-**	+

KEY: SXT= co-trimoxazole 30 µg, CH= chloramphenicol 30 µg, SP= sparfloxacin 10 µg, CPX= ciprofloxacin 10 µg, AM= amoxicillin 30 µg, GEN= gentamycin 10 µg, PEF= pefloxacin 30 µg, OFX= ofloxacin 10 µg, STR= streptomycin 30 µg, LEV= levofloxacin 5 µg, CRO= ceftriaxone 30 µg, CTX= cefotaxime 30 µg, CAZ= ceftazidime 30 µg, AMC= amoxicillin/clavulanic acid 30 µg, +ve = Positive, ESBL= Extended Spectrum ßeta Lactamase, - = Negative.

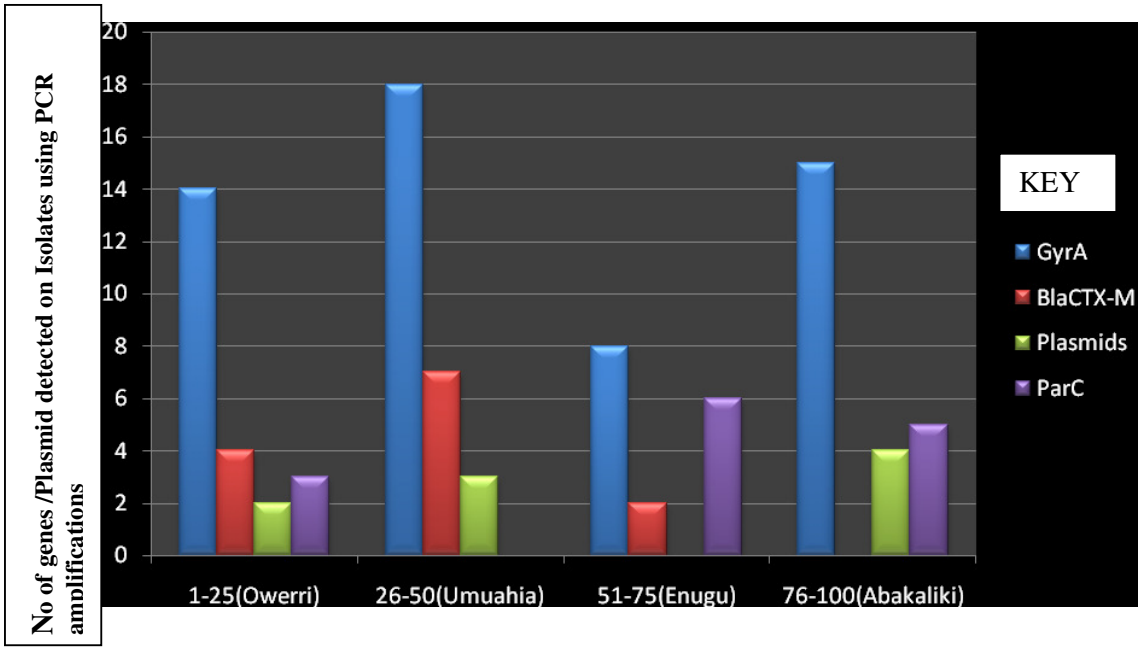

Fig. 4.3 shows graphical representation of the genetic constituents on the isolates from different hospitals in the Southeast Region and the relative distribution.

4.10 Phenotypic antibiotic resistance pattern of *S. enterica* against five selected antibiotics in relation to resistance genes.

Tables 4.10 a- d shows the phenotypic pattern of resistance to five selected antibiotics in relation to the plasmid, *GyrA, ParC*, and Bla_{CTX-M} gene produced. Of the isolates SO1-25, some were resistant to not less than four of the antibiotics tested, but notably, the isolates did not produce plasmid, no mutation in *GyrA* and *ParC* gene, or Bla_{CTX-M} gene. As stated in the earlier Tables 4.9 c in this study, an isolate was resistant to 13 antibiotics as shown in the phenotypic pattern obtained but the isolates exhibited the presence of ESBL only.

With the isolates, SU26 – 50, Out of the 5 selected antibiotics tested, 4 (16%) of the isolates were resistant to at least one antibiotics screened without harbouring mutant gene in *GyrA* and *ParC*, Bla_{CTX-M} , and the plasmid DNA. Such isolates were SU29, SU38, SU41 and SU48 (Table 4.10 b). The isolates SE51- 75 had the same trend of results except in some cases where up to 4-5 of the antibiotics selected were completely resisted by the isolates (Table 4.10 c). This may be implying that different types of enzyme apart from the ones detected were playing a major role in the phenotypic resistance pattern. Similar observations were made in SA76 - SA100 as shown in Table 4.10 d.

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Table 4.10 a. Phenotypic antibiotics resistance pattern of *S. enterica* against five selected antibiotics in relation to resistant genes from Owerri

CODE S/NO	NUMBER OF ANTIBIOTICS	RESISTANCE PHENOTYPIC PATTERN	PRESENCE OF *GryA*	PRESENCE OF *ParC*	PRESENCE OF Bla_{CTX-M}	PRESENCE OF PLASMID
O1.	**1**	AMC	-	-	-	-
O2.	**1**	AMC	-	-	-	+
O3.	**1**	AMC	+	+	+	-
O4.	**0**	NIL	-	-	-	-
O5.	**1**	AMC	-	-	-	-
O6.	**3**	LEV,CTX,AMC	-	-	-	-
O7.	**1**	AMC	-	-	-	-
O8.	**0**	NIL	-	-	-	-
O9.	**2**	CPX,AMC	+	-	-	+
O10.	**2**	CPX,AMC	-	-	-	-
O11.	**2**	CPX,AMC	+	-	-	-
O12.	**2**	CPX,AMC	-	-	-	-
O13.	**1**	AMC	+	-	-	-
O14.	**4**	LEV,CRO,CTX,AMC	+	-	+	-
O15.	**1**	AMC	+	-	+	-
O16.	**1**	AMC	+	-	-	-
O17.	**1**	AMC	-	-	-	-
O18.	**2**	CPX,AMC	+	+	-	-
O19.	**2**	CPX,AMC	+	+	-	-
O20.	**4**	LEV,CRO,CTX,AMC	-	-	-	-
O21.	**1**	AMC	+	-	-	-
O22.	**1**	AMC	+	-	-	-
O23.	**1**	AMC	+	-	-	-
O24.	**3**	CRO,CTX,,AMC	+	-	+	-
O25	**1**	AMC	+	-	-	-

KEY: **CPX=** ciprofloxacin 10 µg, **LEV=** levofloxacin 5 µg, **CRO** = ceftriaxone 30 µg, **CTX=** cefotaxime 30 µg, AMC= amoxicillin/clavulanic acid 30 µg**, +ve** = Positive, **ESBL=** Extended Spectrum Beta Lactamase, Nil = negative, ***GyrA*** = gyrase A, ***ParC*** = Topoisomerase IV, ***Bla*** $_{CTX-M}$ = Beta-lactamase cefotaxime -M type enzyme,

Table 4.10b. Phenotypic antibiotics resistance pattern of *S. enterica* against five selected antibiotics in rellation to resistant genes from Umuahia (Continue).

CODE S/NO	NUMBER OF ANTIBIOTICS	RESISTANCE PHENOTYPIC PATTERN	PRESENCE OF *GryA*	PRESENCE OF *ParC*	PRESENCE OF Bla_{CTX-M}	PRESENCE OF PLASMID
U26.	**1**	AMC	+	-	-	-
U27.	**1**	AMC	-	-	+	-
U28.	**0**	NIL	+	-	+	+
U29.	**1**	AMC	-	-	-	-
U30.	**1**	AMC	+	-	-	-
U31.	**0**	NIL	+	-	-	-
U32.	**1**	AMC	+	-	-	-
U33.	**2**	AMC	+	-	-	-
U34.	**1**	AMC	-	-	-	-
U35.	**1**	AMC	+	-	-	-
U36.	**1**	AMC	+	-	-	-
U37.	**1**	AMC	+	-	-	-
U38.	**1**	AMC	-	-	-	-
U39.	**0**	NIL	+	-	+	-
U40.	**3**	CRO,CTX,AMC	+	-	+	+
U41.	**1**	AMC,	-	-	-	-
U42.	**1**	AMC,	+	-	-	-
U43.	**1**	AMC	-	-	-	+
U44.	**1**	AMC	+	-	+	-
U45.	**1**	AMC	+	-	-	-
U46.	**1**	AMC	+	-	-	-
U47.	**1**	AMC	+	-	-	-
U48.	**1**	AMC	-	-	-	-
U49.	**4**	LEV,CRO,CTX,AMC	+	-	+	-
U50	**1**	AMC	+	-	+	-

KEY: **CPX=** ciprofloxacin 10 µg, **LEV** = levofloxacin 5 µg, **CRO** = ceftriaxone 30 µg, **CTX=** cefotaxime 30 µg, AMC= amoxicillin/clavulanic acid 30 µg**, +ve** = Positive, **ESBL=** Extended Spectrum Beta Lactamase, Nil = negative, ***GyrA*** = gyrase A, ***ParC*=** Topoisomerase IV, ***Bla*** $_{CTX-M}$ = Beta-lactamase cefotaxime -M type enzyme,

Table 4.10 c. Phenotypic antibiotics resistance pattern of *S. enterica* against five selected antibiotics in relation to resistant genes from Enugu (Continue).

CODE S/NO	NUMBER OF ANTIBIOTICS	ANTIBIOTIC RESISTANCE PHENOTYPIC PATTERN	PRESENCE OF *GRY A*	PRESENCE OF *ParC*	PRESENCE OF Bla_{CTX-M}	PRESENCE OF PLASMID
E51.	**1**	AMC	-	-	-	-
E52.	**1**	AMC	-	-	-	-
E53.	**2**	CPX,AMC	+	-	+	-
E54.	**1**	AMC	+	-	+	-
E55.	**1**	AMC	-	-	-	-
E56.	**1**	AMC	-	-	-	-
E57.	**1**	AMC	-	-	-	-
E58.	**1**	AMC	+	+	-	-
E59.	**1**	AMC	+	-	-	-
E60.	**2**	LEV,AMC	+	+	-	-
E61.	**1**	AMC	+	-	-	-
E62.	**1**	NIL	+	-	-	-
E63.	**2**	LEV,AMC	-	-	-	-
E64.	**4**	LEV,CRO,CTX,AMC	-	-	-	-
E65.	**2**	CPX,CTX	-	-	-	-
E66.	**5**	CPX,LEV,CRO,CTX,AMC	-	-	-	-
E67.	**4**	LEV,CRO,CTX,AMC	-	-	-	-
E68.	**4**	LEV,CRO,CTX,AMC	-	-	-	-
E69.	**4**	LEV,CRO,CTX,AMC	-	-	-	-
E70.	**1**	AMC	-	-	-	-
E71.	**2**	CPX,AMC	-	-	-	-
E72.	**3**	CPX,CTX,AMC	-	+	-	-
E73.	**1**	CTX	-	+	-	-
E74.	**1**	AMC	+	+	-	-
E75	**4**	CPX,CRO,CTX,AMC	-	+	-	-

KEY: **CPX=** ciprofloxacin 10 µg, **LEV** = levofloxacin 5 µg, **CRO** = ceftriaxone 30 µg, **CTX=** cefotaxime 30 µg, AMC= amoxicillin/clavulanic acid 30 µg**, +ve** = Positive, **ESBL=** Extended Spectrum Beta Lactamase, Nil = negative, ***GyrA*** = gyrase A, ***ParC*=** Topoisomerase IV, ***Bla*** $_{CTX-M}$ = Beta-lactamase cefotaxime -M type enzyme,

Table 4.10 d. Phenotypic antibiotics resistance pattern of *S. enterica* against five selected antibiotics in relation to resistant genes from Abakaliki.

CODE S/NO	NUMBER OF ANTIBIOTICS	ANTIBIOTIC RESISTANCE PHENOTYPIC PATTERN	PRESENCE OF *GryA*	PRESENCE OF *ParC*	PRESENCE OF Bla_{CTX-M}	PRESENCE OF PLASMID
A76.	**3**	CRO,CTX,AMC	+	+	-	+
A77.	**5**	CPX,LEV,CRO,CTX,,AMC	+	+	-	-
A78.	**5**	CPX,LEV,CRO,CTX,,AMC	+	-	-	-
A79.	**2**	CPX,AMC	+	-	-	-
A80.	**5**	CPX,LEV,CRO,CTX,AMC	-	-	-	-
A81.	**3**	CPX,CRO,CTX,	-	-	-	+
A82.	**2**	LEV,AMC	+	-	-	-
A83.	**5**	CPX,LEV,CRO,CTX,AMC	-	-	-	+
A84.	**5**	CPX,LEV,CRO,CTX,AMC	-	-	-	-
A85.	**5**	CPX,LEV,CRO,CTX,AMC	-	-	-	+
A86.	**5**	CPX,LEV,CRO,CTX,AMC	+	-	-	-
A87.	**3**	CPX,LEV,AMC	+	-	-	-
A88.	**3**	CPX,LEV,AMC	+	-	-	-
A89.	**1**	AMC	-	-	-	-
A90.	**2**	CTX,AMC	-	-	-	-
A91.	**1**	CPX	+	-	-	-
A92.	**1**	AMC	+	-	-	-
A 93.	**1**	CPX,	+	-	-	-
A 94.	**1**	CPX,,AMC	+	-	-	-
A 95.	**1**	AMC	+	-	-	-
A 96.	**1**	AMC	+	-	-	-
A 97.	**1**	CPX	-	+	-	-
A 98.	**1**	CPX	+	+	-	-
A 99.	**2**	CPX,AMC	-	+	-	-
A100.	**4**	LEV,CRO,CTX,AMC	-	-	-	-

KEY: **CPX=** ciprofloxacin 10 µg, **LEV** = levofloxacin 5 µg, **CRO** = ceftriaxone 30 µg, **CTX=** cefotaxime 30 µg, AMC= amoxicillin/clavulanic acid 30 µg**, +ve** = Positive, **ESBL=** Extended Spectrum Beta Lactamase, Nil = negative, ***GyrA*** = gyrase A, ***ParC*=** Topoisomerase IV, ***Bla*** $_{CTX-M}$ = Beta-lactamase cefotaxime -M type enzyme,

4.11 Prevalence of Beta-lactamase linked phenotypic resistance for the five selected antibiotics in relation to enzymatic production by *S. enterica*

Table 4.11 shows the prevalence of ßeta-lactamase linked resistance against the 5 selected antibiotics and their enzymatic production of ESBL's, mutation in *GyrA* and *ParC*, and Bla_{CTX-M} gene from *S. enterica*. The result showed phenotypic resistance pattern of the isolates to only one type of the antibiotics tested and the number of isolates involved. The isolates reduced in number in their resistance to only two antibiotics as compared to the one resistance to only one antibiotic. From the Table 4.11, a total of 87, 27, 29, 22, and 22% of the isolates were resistant to only one of either of these antibiotics; AMC, CTX, CPX, LEV, and CRO respectively, as compared to 23, 23, 20, and 13 % of the isolates to two different types of antibiotics namely CPX and AMC, CTX and AMC, LEV and AMC, and CPX and CTX respectively. The Table 4.11 also shows that only Nine (9) of the isolates were resistant to the whole five selected antibiotics tested, out of which 8 (88.8%) produced ßeta-lactamase, 4 (44.4%) had mutation in *GyrA* genes, 2 (22.2%) were ESBLs positive, 1 (11.11%) had double mutation in *ParC* gene while no isolate had Bla_{CTX-M} gene.

Note: The *qnrB* gene, *bla*-SHV and *bla*-TEM were not detected in this study.

Table 4.11. Prevalence of Beta-lactamase linked phenotypic resistance for the five selected antibiotics in relation to enzymatic production by *S. enterica*

RESISTANCE GROUP	RESISTANCE PATTERN	NUMBER OF RESISTANT ISOLATES	BETA LACTAMASE (%)	*GryA* (%)	ESBL (%)	*ParC* (%)	Bla_{CTX-M} (%)
SINGLE	AMC	87	70 (80.4)	48 (55.2)	31 (35.6)	3 (3.45)	6 (6.89)
	CTX	27	24 (88.8)	9 (33.3)	14 (51.8)	1 (3.70)	0 (0.0)
	CPX	29	24 (82.7)	15 (51.7)	13 (44.8)	2 (6.89)	0 (0.0)
	LEV	22	19 (86.4)	9 (40.9)	10 (45.5)	0 (0.0)	0 (0.0)
	CRO	22	20 (90.9)	9 (40.9)	12 (54.5)	0 (0.0)	0 (0.0)
	CPX, AMC	23	21 (91.3)	12 (52.2)	9 (39.1)	3 (13.0)	1 (4.35)
DOUBLE	CTX, AMC	23	22 (95.6)	9 (39.1)	12 (52.2)	0 (0.0)	0 (0.0)
	LEV, AMC	20	19 (95)	9 (45)	10 (50)	1 (5.0)	0 (0.0)
	CPX, CTX	13	11 (84.6)	4 (30.8)	5 (38.5)	0 (0.0)	0 (0.0)
	LEV, CTX, AMC	18	16 (88.8)	6 (33.3)	9 (50)	0 (0.0)	0 (0.0)
TRIPLE	LEV, CTX, CRO	17	15 (88.2)	6 (35.2)	9 (52.9)	0 (0.0)	0 (0.0)
	CRO, CTX, AMC	21	19 (90.4)	9 (42.8)	11 (52.4)	1 (4.76)	2 (9.52)
	CPX, CTX, AMC	11	10 (90.9)	9 (81.8)	3 (27.3)	1 (9.09)	0 (0.0)
	CPX, CRO, CTX	11	10 (90.9)	4 (36.4)	3 (27.3)	0 (0.0)	0 (0.0)
	CPX, LEV, AMC	10	9 (90)	5 (50)	2 (20)	0 (0.0)	0 (0.0)
QUADRUPLE	LEV, CRO, CTX, AMC	17	15 (88.2)	6 (35.3)	9 (52.9)	0 (0.0)	2 (11.76)
	CPX, CRO, CTX, AMC	10	9 (90)	4 (40)	2 (20)	1 (10.0)	0 (0.0)
PENTRUPLE	CPX, LEV, CRO, CTX, AMC	9	8 (88.8)	4 (44.4)	2 (22.2)	1 (11.1)	0 (0.0)

KEY: CPX = ciprofloxacin 10 µg, LEV= levofloxacin 5 µg, CRO = ceftriaxone 30 µg, CTX= cefotaxime 30 µg, AMC= amoxicillin/clavulanic acid 30 µg, ESBL= Extended Spectrum Beta Lactamase, *bla*-SHV, qnrB and *bla*-TEM= not detected.

4.12 Curing of antibiotic resistance in *S. enterica* isolates with ethidium bromide and acridine orange.

The Nine strains of *S.enterica* (SO2, SO9, SU28, SU40, SU43, SA76, SA81, SA83, and SA85) habouring plasmids and either of *GyrA* gene or Bla_{CTX-M} gene were subjected to various concentrations of ethidium bromide and acridine orange and thereafter were tested against the MIC's of the five selected antibiotics (amoxi/clavulanic acid ceftriaxone, cefotaxime, levofloxacin and ciprofloxacin), results showed that all the strains produce susceptibility as determined according to CLSI standard. As shown on the Appendix III.

Plate 4.12 shows the plasmid profile of *S. enterica* seravar. typhi after treatment with ethidium bromide at concentration of 1.25 µg/ml. Strains SO2, SO9, SU28 SU40, SU43, SA76, SA81, SA83 and SA85 harbouring low molecular weight plasmid of 1.37 kbs were completely cured, similarly as the strains in lane number 2 and 85 carrying 1.39 kbs plasmid DNA were also cured.

Plate 4.13 shows the plasmid profile of the Nine *S. enterica* serovar. typhi exposed to acridine orange at of 2.50 µg/ml. The plate indicates the absence of plasmid as evident in the susceptibility of the previously resistant strain, of *S. enterica* seravar. typhi to the MICs of the five antibiotic tested.

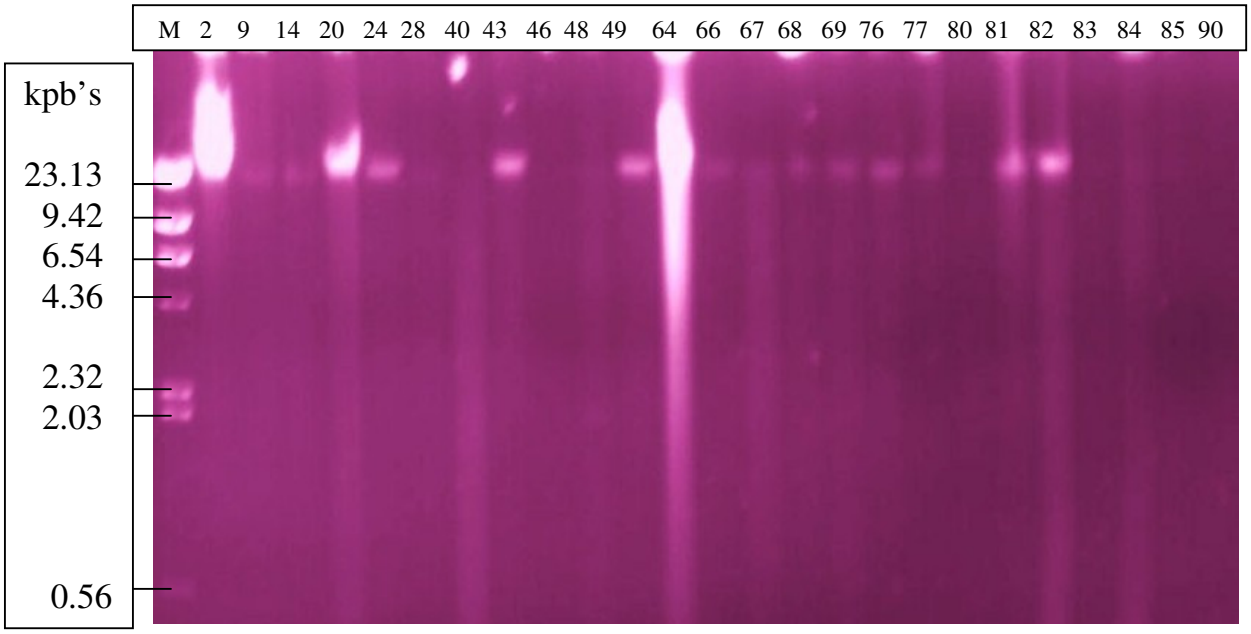


Plate 4.12. Agarose gel electrophoresis pattern of *S. enterica* serovar. typhi after exposure to Ethidium bromide (mutagen).

KEY: Lane 2, 9, 14, 20 and 24 from FMC Owerri. Lane 28, 40, 43, 46, 48 and 49 from FMC Umuahia. Lanes 64, 66, 67, 68 and 69 are from UNTH Enugu. Lane 76, 77, 80, 81, 82, 83, 84, 85, and 90 are from FMC, Abakaliki. Lane M is the lambda Hind III marker (0.12-23.1 kpb).

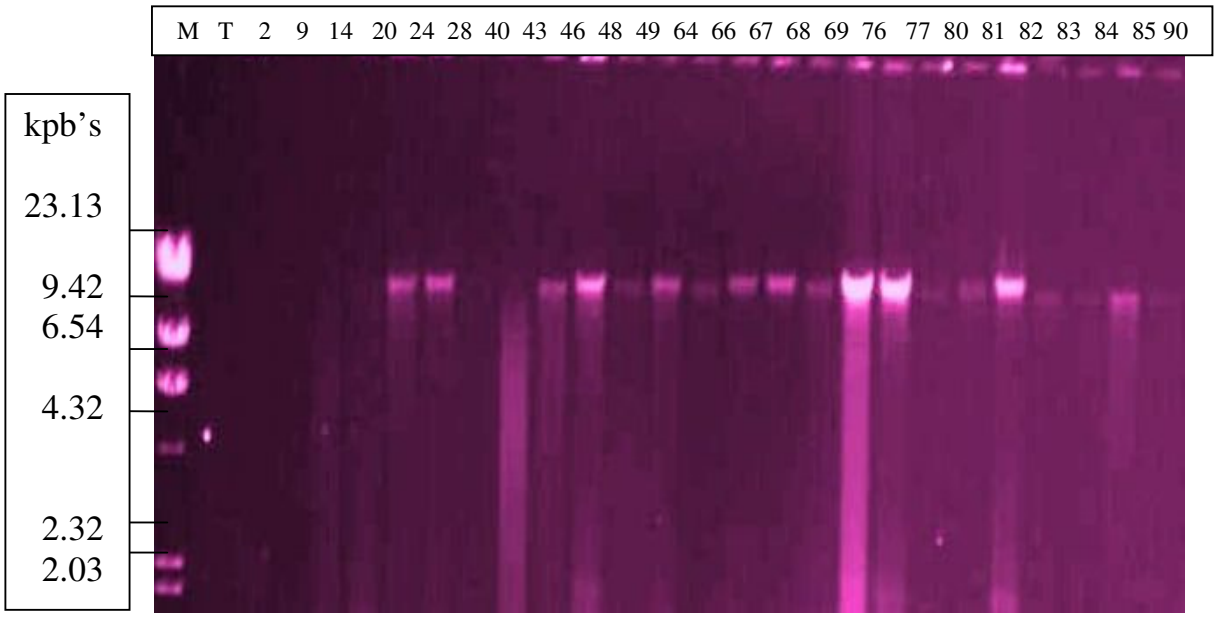


Plate 4. 13. Agarose gel electrophoresis pattern of *S. enterica* serovar. typhi after exposure to Acridine orange (mutagen).

KEY: Lanes 2, 9, 14, 20 and 24 from FMC Owerri. Lanes 28, 40, 43, 46, 48 and 49 from FMC Umuahia. Lane 64,66,67,68 and 69 are from UNTH Enugu. Lane 76, 77, 80, 81, 82, 83, 84, 85, and 90 are from FMC Abakaliki. Lane T (positive control) is the *S.typhi* typed culture ATCC14028. Lane M is the lambda Hind III marker (0.12-23.1 kpb).

4.13. Analysis of variance (ANOVA) to determine the treatment effect of the antibiotics on the human isolates of *S. enterica*.

Table 4.12 shows the ANOVA calculated using two- way grouping according to Martin *et al*. (2000), From the Table 4.12 it can be deduced that the block (drugs) degree of freedom of 3 and 12 is 3.49. While the treatment degree of freedom of 4 and 12 is 3.26 at $p>0.05$ level of significance, thus showing that the calculated F-value of block (7.21) and treatment (20.07) is higher than the tabulated F-value of block (3.49) and treatment (3.26). This is an indication that the null hypothesis of no treatment effect should be rejected. Thus there is enough evidence to suggest real significant differences in the individual performance of the five selected antibiotics drugs on the isolates of *S.enterica* (see appendix III, for the analytical calculation).

4.14: BLAST analysis of the gene sequencing and alignment of *S. enterica* from Southeast Nigeria

Table 4.13 a-b shows the result of the sequence alignment of the gene resulting from the PCR amplifications of the DNA complete genome of the isolate from the various hospitals (SO3, SO14, SA96 and SU33, SA98 as shown in Table 4.13a and Table 4.13b respectively. From the Table 4.13a, isolate SO3 had 95% and 94% maximum identity with the strains found in the gene bank (*Salmonella enterica* subsp. enterica serovar. Typhimurium str. UK-1 chromosome, complete genome and *Salmonella enterica* subsp. enterica serovar. Typhi str. CT18, complete genome) with the accession number NC016863 and NC_003198 after 100% blast hits (query coverage) on the BLAST soft ware respectively. Also isolate SA98 (Table 4.13b) had 99% and 94% identity with the strain found in the genebank (*Salmonella typhimurium* strain 580 *GyrA* gene, partial cds and *Salmonella enterica* subsp. enterica serovar.Typhimurium strain ATCC 307) with the acession number EF059893.1 and CP009102.1, after 97% blast hits (query coverage) on the BLAST soft ware. Another gene (isolate SO14) sequenced was found to be 89% identical to a strain, *Salmonella enterica* subsp. enterica serovar Typhi strain B/SF/13/03/195, complete genome. Others were as shown on Table 4.13a-b.

Fig.4.4 and 4.5 shows the Fast Minimum Evolution and Neighboring joining Taxonomic tree of some strains sequenced including their relatedness was as stated in Fig.4.4 and 4.5 and Appendix IV for isolates SO3, SO14, SU33, SA96, and SA98.

Table 4.12: Analysis of variance (ANOVA) to determine the treatment effect of antibiotics on the clinical isolates of *S. enterica*

VARIATION SOURCE	D. F	S. SQUARE	MEAN SQUARE	F-VALUE
BLOCK	3	209.75	69.9	7.21**
TREATMENT	4	778.3	194.57	20.07**
ERROR	12	116.3	9.69	
TOTAL	19	1104.55		

KEY: ** = Highly Significant Treatment Effect, S.S = Sum of Square, D.F = Degree of freedom.

Table 4.13a: BLAST Analysis of the Gene sequencing and Alignment of *S. enterica* from Southeast Nigeria.

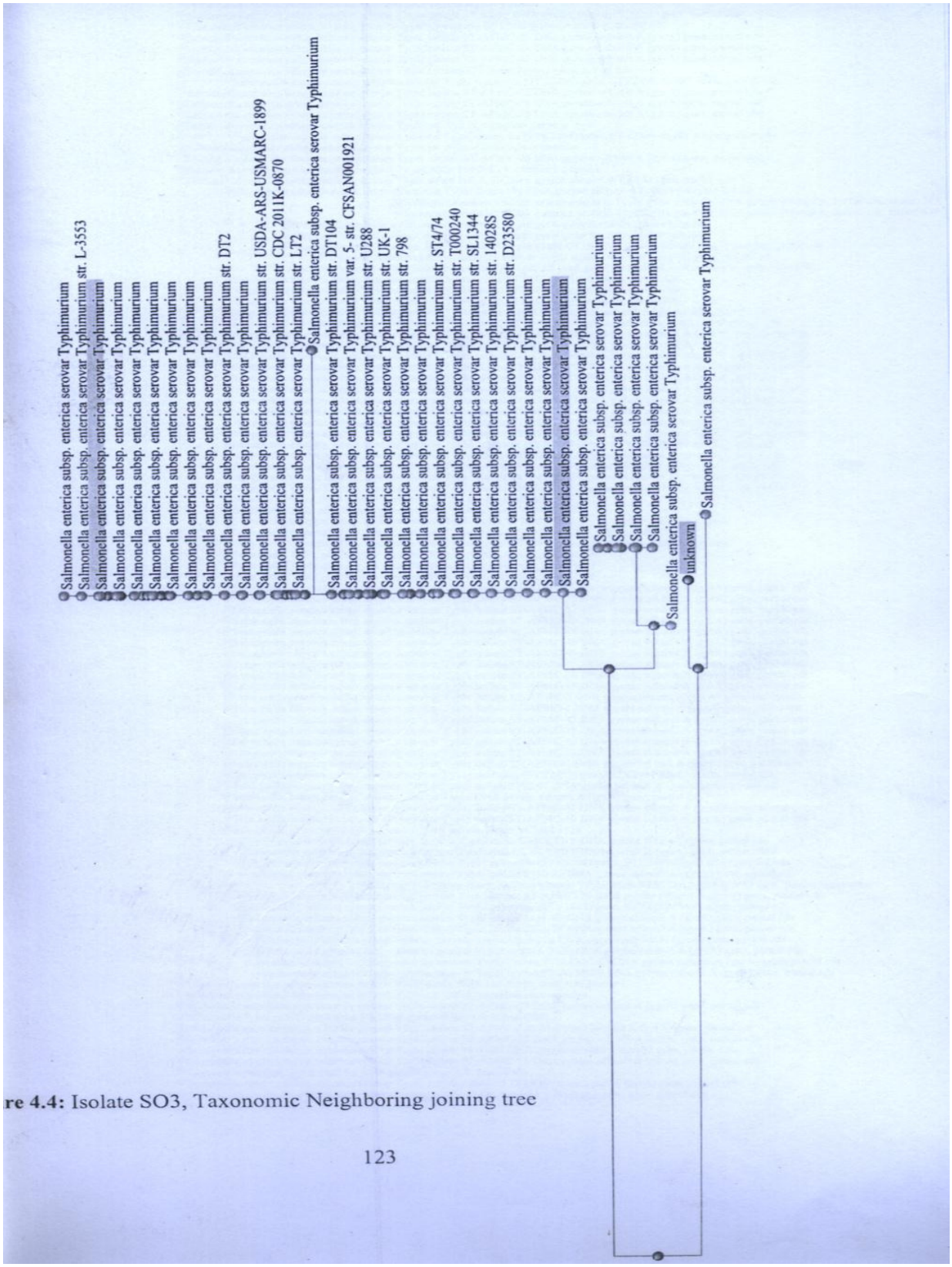
Sequenced gene	Description of significant alignment	Acession No. of aligned gene	Max. score	Total score	Query coverage	E-value	Max. Identity
SO3	1.*Salmonella enterica* subsp. enterica serovar Typhimurium str. UK-1 chromosome, complete genome	NC016863.1	174	174	100%	3e-43	95%
	2.*Salmonella enterica* subsp. enterica serovar Typhi str. CT18, complete chromosome	NC_003198.1	169	169	100%	2e-41	94%
	3.*Salmonella enterica* subsp. enterica serovar Typhi str. Ty2 chromosome, complete genome	NC_004631.1	169	169	100%	2e-41	94%
SO14	1.*Salmonella enterica* subsp. enterica serovar Typhi strain B/SF/13/03/195, complete genome	CP012151.1	147	147	100%	2e-35	89%
	2.*Salmonella enterica* subsp. enterica serovar Typhi strain PM016/13, complete genome	CP012091.1	147	147	100%	2e-35	89%
SA96	1.*Salmonella enterica* subsp. enterica serovar Typhi isolate 9618-2K DNA gyrase subunit A (gyrA) gene, partial cds	AY302588.1	274	274	100%	1e-73	92%
	2.*Salmonella enterica* subsp. enterica serovar Typhi strain CMCSTDY DNA gyrase II (gyrA) gene, partial cds	KTI62085.1	268	268	100%	6e-72	91%

Key: SO3,SO14= Isolates from owerri**, SA96=** Isolates from Abakiliki**, BLAST** = Basic Local Alignment Search Tool, **Query length of SO3** = 109nts, **Query I.D of SO3** = /c/2251., **Query length of SO14** = 118nts, **Query I.D of SO14** = /c/_47953. , **Query length of SA96** = 196nts**, Query I.D of S96** = /c/_82899.

Table 4.13b: BLAST Analysis of the Gene sequencing and Alignment of *S. enterica* from Southeast Nigeria.

Sequenced gene	Description of significant alignment	Acession No. of aligned gene	Max. score	Total score	Query coverage	E-value	Max. Identity
SA98	1.*Salmonella typhimurium* strain 580 GyrA gene, partial cds	EF059893.1	363	263	97%	1e-99	99%
	2.*Salmonella enterica* subsp. enterica serovar Typhimurium genome assembly NCTC13348, chromosome	LN829401.1	307	307	97	5e-83	94%
	3.*Salmonella enterica* subsp. enterica serovar Typhimurium strain ATCC 13311, complete genome	CP009102.1	307	307	97	5e-83	94%
SU33							
	1.*Salmonella enterica* subsp. enterica serovar Typhi strain 21g DNA gyrase subunit A (gyrA) gene, partial cds	KC773840.1	246	246	96%	2e- 65	92%
	2.*Salmonella enterica* subsp. enterica serovar Typhi strain ST33 GyrA (gyrA) gene, partial cds	HQ176354.1	246	246	100%	2e-65	91%

Key: SA98= Isolates from Abakiliki**, SU33=** Isolates from Umuahia**, BLAST** = Basic Local Alignment Search Tool, **Query length of SA98** = 208nts, **Query I.D of SA98** = /c/_221473, **Query length of SU33** = 184nts, **Query I.D of SU33** = /c/_65033.



re 4.4: Isolate SO3, Taxonomic Neighboring joining tree

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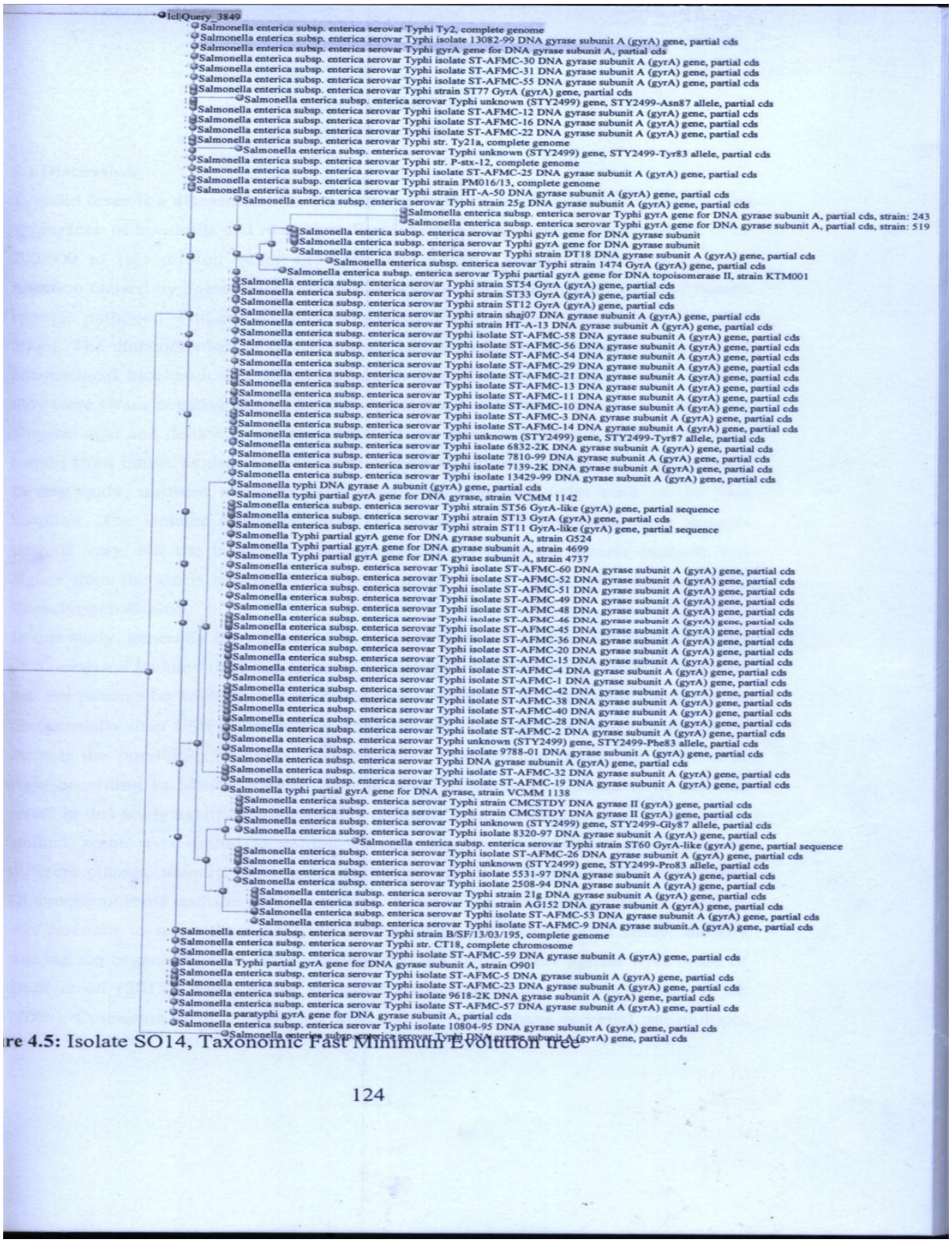
Figure 4.4: Isolate SO3, Taxonomic Neighboring joining tree



re 4.5: Isolate SO14, Taxonomic Fast Minimum Evolution tree

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Figure 4.5: Isolate SO14, Taxonomic Fast Minimum Evolution tree

CHAPTER FIVE
DISCUSSION

5.1 Discussion

Typhoid fever is a disease that has been associated with human since close to the first appearance of hominids and may have first infected human ancestors anywhere from 200,000 to two million years ago (Mustaq, 2006). Typhoid fever is a systemic infection caused by *Salmonella enterica* Serotype typhi. It is a highly adapted human specific pathogen with remarkable mechanism for persistence in its host (Mustaq, 2006). The authentication of the *S.enterica* isolates used in this study, applying the conventional biochemical test and Microbact identification techniques, showed that they were Gram negative tiny rods, showing deposit of black residues on *Salmonella Shigella* agar and deoxycholate agar, ferment mannose and glucose, and also utilised carbon from citrate as the sole source of carbon as reported by Cheesbrough (2006). In this study, uniform numbers of isolates were collected from each of the four hospitals. The isolates recovered from the wards and departmental units in each hospital vary, but the number of isolates recovered from the female patients was higher than the once recovered from the male. These agree with the report of Cheesbrough (2006).

In this study, generally the reports of In-patients units (IPU) recorded higher number of *S. enterica* isolates than the General out patient department (GOPD), suggesting that the patients on admission were more prone to getting infected with the organism nosocomially than GOPD patients. Among the IPU isolates of *S. enterica* therefore, there is the possibility of clonal dissemination of individual multidrug resistant *S. typhi* according to Mustaq (2006). Out of the 100 isolates of *Salmonella enterica* tested in this study against the antibiotics normally used for treatment in the hospitals studied, some were found to exhibit varying degrees of resistance cutting across different classes, namely; β-lactams, chloramphenicol, aminoglycosides, quinolones (fluoroquinolones) and the sulphonamides. It could be deduced that the *S. enterica* was resistant to more than three classes of antibiotics used in this study, thereby making the organism multi-drug resistant (MDR). This report agrees with those of Deak *et al*. (2015), Yah (2010), Brusch (2010) and Mustaq (2006). Thong *et al*. (2000), Connerton *et al*. (2000) and Mirza *et al*. (2000) have reported the multiple

outbreaks of infections with resistant strains of *Salmonella spp*. in India, Pakistan, Bangladesh, Middle East and Africa.

In this study, the highest number of *S. enterica* isolates were recovered from patients between 18 years and above, followed by the ages between 21- 30 years, also supporting the reports of Brusch (2010) and Mustaq (2006). Brush *et al*. (2010) stated that genes for antibiotic resistance in *S.typhi* were acquired from *Escherichia coli* and other Gram negative bacteria via plasmids. They further stated that the plasmid contains cassettes of resistance genes that are incorporated into a region of the *Salmonella* genome called integron. Some of these plasmids also carry multiple cassettes and immediately confer resistance to multiple classes of antibiotics. This may be the reason why the isolates of *S. enterica* were resistant to more than three different classes of antibiotics and could also explain the appearance of multi-drug resistant (MDR) strains of *S. typhi* (Brush *et al.,* 2010). According to Mandal *et al*. (2010) the original indication of chloramphenicol was in the treatment of typhoid. Currently, however there is a universal presence of multi-drug resistant *Salmonella typhi* as observed in this study such that chloramphenicol is seldom used for this indication except when the organism is known to be sensitive. Based on the CLSI standard used for interpreting the sensitivity screening results in this study, few of the isolates of *S.enterica* were sensitive to chloramphenicol. This has shown that the cause of resistance to these antibiotics was due to both the plasmid and chromosomal mediating enzymes. Enzymes such as β-lactamase and Extended spectrum β-lactamase were detected to account for the antibiotic resistance observed in this study, manifested in different forms of enzyme inhibition and resistant mutants, noticeable especially against cephalosporins and floroquinolones. These antibiotics have been recommended as replacements for chloramphenicol by most authorities for the treatment of typhoid fever. Some authors have reported the effectiveness of fluoroquinolones (Nathan *et al*., 2005; Noel *et al*., 2007; Brush *et al.,* 2010) against susceptible organisms with better cure rate than cephalosporins. These workers further reported the emergence of resistance to first generation fluoroqninolones in many parts of Asia (Nelson *et al*., 2007; Brush *et al.*, 2010). Brush *et al.* (2010) reported that third generation cephalosporins have been used in regions with high fluoroquinolone resistance rates, particularly in South Asia and Vietnam. However, in this study, both the third generation cephalosporin and the fluoroquinolone still maintained their choice as effective antibiotics for the treatment of chloramphenicol-

resistant and multi drug resistant strains of *S. enterica* serovar typhi in this part of Nigeria. The results obtained showed that among the antibiotics tested ceftriaxone (a third generation cephalosporin) and levofloxacine (a third generation fluoroquinolone) were each effective on 78 of the isolates out of the total 100 isolates, creened for sensitivity; cefotaxime was effective against 73 of the isolates, followed by ofloxacin, pefloxacin, ciprofloxacin and ceftaxidime with 72, 71, 71, and 56 isolates of *S. enterica* respectively.

Analysis revealed that not all the isolates were β-lactamase positive, as only 80% of the isolates produced β-lactamase, while 36% of the isolates were positive to extended spectrum β-lactamases. Also, it was found that only 28 isolates had both the conventional β-lactamase and the ESBLs co-existing. According to Yujuan and Ling (2006), Valverde *et al.* (2008), and Yah (2010) resistance to broad-spectrum β-lactams was highly mediated by ESBL enzyme, which has been increasing the world health problems in clinical settings, coupled with the hydrolytic activity of the plasmid mediated β-lactamases on β-lactams (Yah, 2010). In this study, plasmids were not found in most of the isolates of *S.enterica* yet; they were resistant to a wide range of antibiotics including the third generation cephalosporins and fluoroquinolones. Thus, the resistance found in them may not be due to plasmid. This study reported low molecular weight plasmids which were recovered from 9 (9%) of the resistant isolates of which were recovered mainly from adult patients, but only one patient, between the age range of 0-10years and the age range of 11-20 years in which the isolates were recovered from, had plasmids.

According to Soge *et al.* (2005) the first CTX-M-type *β*-lactamases were identified as plasmid-encoded enzymes in clinical isolates from the Enterobacteriaceae, and all the 30 isolates of *Klebsiella pneumoniae* in their study produced at least one β-lactamase and 17 (57%) produced CTX-M *β*-lactamase (Soge *et al*., 2005). Furthermore, reports have also shown that the resistance of gastroenteric *Salmonella* strains to antimicrobial agents was largely due to the production of extended-spectrum β-lactamases (ESBLs) encoded on plasmids, as well as on chromosomes (David and Frank, 2000; Yujuan *et al*., 2006 and Yah, 2010). In this study amoxicillin/ clavulanic acid was expected to be effective on the isolates because this combination of antibiotic is known to be a β- lactamase inhibitor, but the reverse was the case, as it was observed that out of the 100 *S. enterica* serovars isolate 87% of the organism were resistant to amoxicillin/clavulanic acid. It was also found that out of the 87

isolates of *S. enterica* resistant to amoxicillinl/clavulanic acid, 70 (80.4%) produced β-lactamase, while 31(35.6%) were positive for ESBL and were resistant to some of the cephalosporin tested in this study. This may be due to the fact that some isolates will not exhibit β-lactamase unless the enzyme has been induced, by exposing them to beta-lactam antimicrobial. This mechanism is achieved when the drug comes in contact with the organism; this initial contact will trigger the reaction or production of this enzyme by the organism as the organism tends to initiate resistance. It has been reported that beta-lactamases that hydrolyze extended spectrum cephalosporins have an oxyimino side chain which include cefotaxime, ceftriaxone and ceftaxidime (Philippon *et al.,* 2002). Also reports stated that such ESBLs confer resistance to these antibiotics and related oxyiminino β-lactams (Philippon *et al*., 2002). According to Woodford *et al.* (2006), CTX-M beta-lactamase (class A) enzymes were named for their greater activity against cefotaxime than other oxyimino β-lactam substrates e.g ceftazidime, ceftriaxone or cefepime which are examples of plasmid acquisition of β-lactamase genes normally found on the chromosome of kluyvera species, a group of rare pathogenic commensal organism (Woodford *et al.*, 2006).

Woodford *et al.* (2006) stated that these enzymes are not very closely related to TEM or SHV beta-lactamases in that they show only approximately 40% identity with these two commonly isolated β-lactamases relative to CTX-M genes (Woodford *et al*., 2006). Thus, in this study in order to detect the type of ESBL that confers resistance on some of the third generation cephalosporins, a universal Bla_{CTX-M} , SHV and TEM type primers were used for the Polymerase Chain Reaction (PCR) analysis on the DNA of the isolates of *S.enterica*. It was found that at least 13 (13%) of all the *S. enterica* isolates produced Bla_{CTX-M} type genes while only 9 (9%) of the *S. enterica* had plasmids. According to Woodford *et al*. (2006) more than 80 CTX-M enzymes are currently known and are mainly found in strains of *S. enterica* serovar. typhimurium and *E. coli*. They further stated that despite their name CTX-M enzymes, a few of the enzymes are more active on ceftazidime than on cefotaxime. Interestingly, the enzyme produced by bla_{CTX-M} gene detected in this study was more active on ceftazidime in view of the fact that out of the 44 isolates of *S. enterica* that were resistant to ceftazidime alone, 3 (6.8%) produced bla_{CTX-M} type enzyme, while out of the 27 isolates with resistance to cefotaxime alone, 4 (14.8%) had the gene producing the enzyme.

According to the classification of Richmond and Sykes in 1987 as reported by Onyenwe *et al*. (2011) chromosomal cephalosporinase is inducible, and cannot be inhibited by clavulanic acid, thus having the ability to affect all cephalosporin drugs (including other generations) while the class 2 that is also chromosomally mediated is constitutive and is inhibited by clavulanic acid but affect only the penicillins. In this study, 87% of the isolates were resistant to amoxiclavulanic acid. Out of 13 isolates positive for bla_{CTX-M} type gene, only 2 harbored plasmids, while all the 9 isolates carrying R- plasmids produced β-lactamase but only 2 of them were positive for bla_{CTX-M} type gene. This implies that most of the bla_{CTX-M} type genes were found in the chromosomes, all produced β-lactamase emzymes and all of them were also resistant to clavulanic acid inhibition, based on Richmond and Sykes classification. It could be deduced that the chromosomal broad- spectrum β- lactamases in the class 1 chromosomal-mediated cephalosporinase and most of the resistance encountered in this study were mostly chromosomal as few plasmids were found in the isolates.

The results obtained in this study showed that some of the isolates that harbored plasmids and produced β-lactamase were resistant to high percentage of the antibiotics, such as isolate SO9 from a female adult patient in the General out – patient department (GOPD) unit that was resistant to 8 (57.1%) out of the 14 different antibiotics studied. The results also showed that isolate SA76 recovered from a male adult patient and isolate SA83 recovered from a female (58 years) adult patient both in the GOPD unit were resistant to 12 (85.7%) of the antibiotics. Analysis revealed that isolate SU40 recovered from a female adult patient in the In-patient units (IPU), was resistant to 11 (78.5%) antibiotics while isolate SA85 from female adult in IPU unit and isolate SA81 from 6 years old female patients in National Health Insurance Scheme unit (NHIS) were both resistant to 10 (71.4%) of the antibiotics each. Analysis showed that antibiotic resistant isolates of *S. enterica* harboring plasmid-mediated β-lactamase were still prevalent around the Southeast region of Nigeria as shown in this study, but chromosomal mediated β-lactamase was higher among the resistant isolates. This study showed that the *S. enterica* isolated from the male patients (42) irrespective of their age group produced more resistant genes of *GyrA* 24 (57.14%), *ParC* 6 (14.3%) and Bla_{CTX-M} genes 7 (16.7%), as against the isolates from female patients (58) infected with *S. enterica* that had *GyrA* 31 (53.4%), *ParC* 8 (13.7%) and bla_{CTX-M} genes 6 (10.3%).

The isolates of *S. enterica* harbouring plasmids were prevalent in the female patients, as the 10.3% of the isolates from them were found to be higher than the 7.14% of isolates harboring plasmid and producing β-lactamase in isolates from male patients. The *S. enterica* isolates in this study were resistant to the three first line drugs used for the treatment of typhoid; chloramphenicol (80%), co-trimoxazole (78%) and amoxicillin (80%). This report is in line with the reports of Brusch *et al.* (2010), where antibiotic resistant strains of *S. typhi* and *S. paratyphi* inactivated chloramphenicol via acetylation involving an enzyme that carries chloramphenicol acetyl transferase type I and multi drug resistant strains that may carry dihydrofolate reductase type VII, which confers resistance to trimethoprim. It has also been reported that amoxicillin and trimethoprim-suphamethaxazole were effective alternatives to chloramphenicol-resistant isolates till the end of 1990's (Mustaq, 2006).

According to Nathan *et al.* (2005) and Griggs (2007) chloramphenicol remains the drug of choice in the treatment of meningitis caused by *Neiseria meningitis* in patients with severe penicillin or cephalosporin allergy. Due to the increased resistance to the first line antibiotics, drugs such as cephalosporins and the fluoroquinlones have been recommended by most authorities for the treatment of typhoid fever. It has also been recommended (Nathan *et al*., 2005; Griggs, 2007) that in case where the origin of infection is unknown, a combination of a first- generation fluoroquinolone and a third-generation cephalosporin should be used. This study showed that 78% of the *S. enterica* serovar. typhi were susceptible to levofloxacin (a third-generation fluoroquinolone), 71% to ciprofloxacin (a second generation fluoroquinolone), 71% to pefloxacin (a 2^{nd} generation fluoroquinolone) and 72% to ofloxacin (a 2^{nd} generation fluoroquinolone). Interestingly, the third generation cephalosporins tested were active against *S. enterica* isolates in decreasing order of 78% > 73% > 56% for ceftriaxone, cefotaxime and ceftazidime respectively.

The MIC obtained for the five drugs selected for testing; levofloxacin, ciprofloxacin, amoxicillin/clavulanic acid, ceftriaxone and cefotaxime retained their efficacy as the current drugs of choice in the treatment of typhoid fever, except amoxicillin/clavulanic acid (Parry and Beeching, 2009; Brusch *et al.*, 2010). Screening for the gene constituents of the isolate, showed that out of 27 isolates of *S. enterica* that were resistant to only cefotaxime, 24 (88.8%) produced β-lactamase, 9 (33.3%) *GyrA* gene (topoisomerase II enzyme that codes for point or single mutation) and only one produced mutation in *parC* (topoisomerase IV enzymes) that codes for

double mutation. Also 87 isolates were resistant to amoxicillin/clavulanic acid among which 70 (80.4) isolates produced β-lactamase, 48 (55.2%) had single mutation in *GyrA* and 3 (3.45%) with double mutation in *parC*.

Moreover, out of the 22 isolates resistant to ceftriaxone 20 (90.9%) produced β-lactamase, 9 (40.9%) had single mutation in *GyrA* gene, but none had double mutation in *ParC* gene. Of the 29 isolates of *S. enterica* resistant to ciprofloxacin 24 (82.7%) produced β-lactamase, 15 (51.7%) had *GyrA* and 2 encoded *ParC*. Finally, out of 22 isolates resistant to only levofloxacin 19 (86.4%) produced β-lactamase enzyme, 9 (40.9%) had single mutation in *GyrA* and none in *ParC* genes. These trend of results showed that there have been some forms of resistant mutants among the *S. enterica* serovar family, because the presence of mutations in *GyrA* gene in the isolates showed that resistance to fluoroquinolone is evolving in an ominous direction according to Brusch *et al*. (2010). This implies that the patients from whom the isolates were obtained could have been on fluoroquinolone drug for treatment. It has been recognized that *S.typhi* most commonly develops fluoroquinolone resistance through specific mutations in *GyrA* and *ParC* which codes for the binding region of DNA gyrase and topoisomerase IV respectively. In this study the failure rates of ceftriaxone and cefotaxime were (22%) and (27%) respectively just as levofloxacin and ciprofloxacin had 22% and 29% respectively against the isolates tested, suggesting mutational resistance and clonal spread. The single and double mutation in *GyrA* and *ParC* respectively in this study correlates with the works of Randal *et al*. (2005) and Brusch *et al*. (2010). According to Brusch *et al*. (2010), a single point mutation in *GyrA* confers partial resistance while a mutation in *ParC* coupled with a single *GyrA* mutation confers full in vitro resistance to fluoroquinolone. Remarkably, Ackers *et al*. (2000) found no resistance against ciprofloxacin, ceftriaxone and gentamycin when 350 isolates of *Salmonella* were tested, even though 16% of the isolates were multidrug resistant *Salmonella typhi* (MDRST). Nadeem and Colleagues in 2002 reported from Pakistan that 69% of isolates were found to be MDRST (Nadeem *et al*., 2002). In another report from Bahawalpur Pakistan, 53.8% of isolates were found to be MDRST and all the strains were sensitive to fluoroquinolone and the third generation cephalosporins (Munir *et al*., 2001).

According to Munir *et al*. (2001) 28 isolates were exposed to all the three first-line anti-salmonella drugs, out of which 18 (64.3%) isolates turned out to be MDRST. In this study, 79% of the isolates were MDRST, showing a high resistance profile in the

Southeast region of Nigeria. Concerning ciprofloxacin and ceftriaxone, the results obtained were contrary to the works of Ackers *et al*. (2000) and Nadeem *et al*. (2002), but support the works of Mustaq (2006) who reported that out of 18 MDRST four of the isolates were resistant to ciprofloxacin and four to ceftriaxone while two were resistant to both drugs, and that the overall resistance to ciprofloxacin was 19.2%, ceftriaxone 17.9% and cefotaxime 21.1% out of 76 isolates. This agrees substantially with the findings in this study whereby resistance rates for ciprofloxacin, ceftriaxone and cefotaxime were 29%, 22% and 27% respectively. Threlfall and Ward (2001) reported decreased sensitivity to ciprofloxacin which supports the findings in this study and suggested possible alternatives as ceftriaxone and cefotaxime which correlates with the data obtained in this study, showing that ceftriaxone and cefotaxime had better activity against the isolates of *S.enterica*, than ciprofloxacin. Mustaq (2006) reported that resistance to trimethroprime/sulphamethoxazole was 94.2%, chloramphenicol 65.3%, and amoxiclavulanic acid 42.5% all of which were first line anti-Salmonella drugs. These reports clearly supported the results obtained in this study with 78%, 80% and 87% of the isolates were found to be resistant to co-trimoxazole, chloramphenicol and amoxicillin/ clavulanic acid respectively. As rightly stated by Mustaq (2006), the pattern of *S.typhi* resistance was changing rapidly and that Multi-Drug Resistant *Salmonella Typhi* resistant to ciprofloxacin and ceftriaxone was a major threat in the developing world, including the Southeast part of Nigeria as revealed in this study. It might be interesting to note that *S.enterica* had high susceptibility rate of 78% to levofloxacin, a racemic isomer of ofloxacin, even though some resistant mutant strains were detected in the present work. These resistant strains of *S.enterica* were found to harbor mutant genes in the gyrase A region (topoisomerase II enzyme) and *ParC* (topoisomerase IV enzyme) gene in their chromosomes and few produced plasmids which are of low molecular weight of about 1.37- 1.39kbs. This appears to be the first documented report of levofloxacin resistant *S.enterica* mutants in Southeast Nigeria. This high incidence of fluoroquinolone resistant *S. enterica* serovar. typhi could be attributed to some factors, namely:

i. the isolates were from the In-patients Units, which means that these patients have been hospitalized before the organism was isolated from their stool sample, therefore might have been infected with resistant strain of nosocomial origin as reported by Martin *et al*. (2004) and Varma *et al*. (2005).

ii. the isolates were from a University affiliated medical centre (Federal Medical Centre Owerri) and University Teaching Hospital (UNTH Enugu) that treat patients with severe infection, indicated in the fact that the isolates from GOPD were the second highest *S. enterica* isolates recovered from the patients. This could be indicative that these patients would have been using these antibiotics (fluoroquinolone) inappropriately.

Hakanem *et al*. (2001) stated that transferable resistance against quinolones was sometimes rare in bacteria invivo, but clonal resistance due to mutation in chromosomal gene remains the potential mechanisms, accounting for high level of reduced fluoroquinolone susceptibility in Southeast Asia. This report agrees with the findings in this study whereby chromosomal mediated resistance genes for mutation, clonal resistance and R-plasmids were detected. Statistical analysis of results obtained on the treatment effect of five selected antibiotics using Analysis of Variance (ANOVA) by two way grouping according to Martin *et al*. (2000) revealed significant differences at (p=0.05) level of significance (n=100) in the individual performance of the drugs on the isolates of *S. enterica.*

Curing analysis carried out in this study using two dyes, ethidium bromide and acridine, orange showed that the effect of ethidium bromide at 0.625 µg/ml and 1.25 µg/ml were more pronounced than the acridine orange used at different concentrations (5.0, 2.5, 1.25, 0. 625, 0.3125 µg/ml) revealing ethidium bromide as a more effective curing agent than the acridine orange even at the reduced concentration of the ethidium bromide. Acridine was effective on *Salmonella enterica* only when its concentration was increased to 2.5 µg/ml, thus supporting the reports of Adeleke *et al*. (2002) on the use of acridine dyes as mild agents of curing for recognizing resistant plasmids in resistant *S. aureus.*

The emergence of mutation- based resistance may be fostered by selection pressure caused by the use of antibiotics agents in either human medicine or agriculture, according to Parveen *et al*. (2007), corroborating the alarming increase in quinolone resistance observed during the past few years among food borne pathogens. In this study *Salmonella enterica* recognized as a food or water borne pathogen, showed increased quinolone chromosomally-mediated resistance and exhibited several mutations in their genes, an indicative of poor hygiene in the part of the patients, implicated in the study. This could therefore, have produced the increased level of mutation discovered among these *S. enterica* strains studied. It was reported by

Parveen *et al*. (2007) and Griggs (2007), that enrofloxacin (a fluoroquinolone used in agriculture) can select *Salmonella* mutants, resistant to nalixidic acid and fluoroquinolones. The results obtained in this study were in line with the above statement as up to 48% of the *Salmonella enterica* serovars were found to possess mutation in the gyrase A gene which codes for point mutation in the Quinolone Resistant Determining Region (QRDR), showing chromosomal- mediation and thus, created an increase in resistance to fluoroquinolone drugs.

The Minimun Inhibitory Concentrations (MIC's) of five selected antibiotics (ciprofloxacin, levofloxacin, amoxil/clavulanic acid, ceftriaxone and cefotaxime) obtained in this study increased from 1.56 µg/ml – 12.0 µg/ml in levofloxacin and ciprofloxacin, cefotaxime and ceftriaxone, except in few highly resistant mutants which had their MIC's at 25.0 µg/ml. The MICs of amoxillin/ clavulanic acid were observed to be very high ranging from 12 µg/ml – 25 µg/ml except in few cases where some isolates were susceptible with the MIC of 6.0 µg/ml-12.0 µg/ml. Thus in this study, the MIC for levofloxacin was within 1.5 µg/ml and 3.0 µg/ml against 41% and 25% of the isolates, ciprofloxacin was within 1.5 µg/ml and 3.0 µg/ml against 43% and 23% of the isolates, cefotaxime was within 1.5 µg/ml and 3.0 µg/ml against 8% and 25% of the isolates, and ceftriaxone was within the same range against 56% and 15% of the isolates tested respectively. These showed that each of the five drugs had its MIC at 1.5 µg/ ml against the isolates indicating some level of susceptibility to the range of drugs selected. These results fall within an indication of 4 µg/ml as the MIC break point by CSLI (2007) and CLSI (2011) on quinolones, especially ciprofloxacin. The cephalosporins used in this study showed equal match in the treatment of *S. enterica,* especially ceftriaxone. About 78% of the isolates were susceptible to the drug while 56% of the isolates had MIC 1.5µg/ml, indicating ceftriaxone as one of the best against *Salmonella* infection in adult and children, as they are also more tolerated than ciprofloxacin in the case of the children. These agree with the most recent professional guidelines for the treatment of typhoid fever in South Asia, issued by the India Association of Pediatrics (IAP) in October, 2006 according to Brusch *et al*. (2010). According to the guidelines, for empiric treatment of uncomplicated typhoid fever, the IAP recommends cefixime and as a second- line agents, azithromycin, but for complicated typhoid fever they recommended ceftriaxone.

This indication agrees with the findings in this study because a very good number of the *S. enterica* isolates screened were positive to ESBL production, with most of the isolates found having their resistant genes located on chromosomes rather than on plasmid. Based on the trend of results obtained, recommendation could be made to physicians to prescribe the combination of fluoroquinolone and third generation cephalosporins, especially the ceftriaxone, as supported and stated by many authors such as Montaz *et al*. (2002) that according to the data from their study in Egypt, neither fluoroquinolone nor third generation cephalosporins resistance has emerge.

Montaz *et al*. (2002) further reported that there could be a resurgence of quinolone resistance unless the use of this drug is restricted. Interestingly, the workers recorded a resurgence of chloramphenicol- susceptible *S.typhi* strains agreeing with the findings in this study that 20% of the isolates screened show susceptibility to chloramphenicol. This could be attributed to the previous long time restricted use of chloramphenicol to *Salmonella* treatment in Nigeria by health workers and possibly, the patients who indiscriminately use them. It was interesting to note that the level and degree of resistance vary globally and there are geographical variations in the epidemiology of *Salmonella* infections. In this study, Some isolates possessed no resistance gene or mutation in either *gyrA* or *ParC* in the chromosome, while some produced no β-lactamase and no plasmids but were only ESBL posistive strains, yet they were resistant to more than four antibiotics which included levofloxacin, ceftriaxone, cefotaxime, and amoxillin/clavulanic acid. Secondly, If these particular strains of *Salmonella enterica* serovars were resistant to cefotaxime as observed in this study, then it could be attributed to either the $Bla_{CTX\text{-}M}$ type gene detected (ESBL), but for the fact that it could not be inhibited by the action of amoxillin/ clavulanic acid which is a Beta-lactamase inhibitor, then this type of resistance may be chromosomal. Thirdly, no mutation in *gyrA* and *ParC* genes was detected on some isolates in the case of fluoroquinolone resistant strains after specific primers were used for the amplification of the determinant gene. Harbottle *et al*. (2006) suggested that there is a continuing need for increased surveillance of antimicrobial –resistance phenotypes in *Salmonella* isolates of animal and human origin on a global basis (Harbottle *et al*., 2006).

According to Haugum *et al*. (2006) and Deak *et al.,* (2015), important mechanisms for quinolone-resistance are mutations accumulating in the genes encoding the DNA gyrase and topoisomerase *gyrA, gyrB, ParC, Par E*. In this study only the mutant

genes encoding *gyrA* and *parC* were detected in the topoisomerase enzyme. Report from Eaves *et al*. (2004) stated that in *Salmonella* and *E.coli*, the majority of mutations in DNA gyrase are found between residues 67 and 106 in *gyrA*, in a region called the quinolone- resistant determining region (QRDR) while in *ParC* mutations in *Salmonella* have been found between residues 57 and 84 (Eaves *et al*., 2004). Hopkins *et al*. (2005) indicated that the mutations may only be required to achieve high level resistance.

In this study, at least three (3) *S. enterica* serovars harboring plasmids were found to be resistant to some groups of antibiotics (SA81, SA83, and SA85 isolate) including 2 members of the fluoroquinolone (ciprofloxacin and levofloxacin) without harboring any resistance gene or mutation in their *gyrA* and *parC* regions in the chromosomes, hence an indication of plasmid- mediated quinolone resistance (PMQR). According to Hopkins *et al*. (2005) and Haugum *et al*. (2006) such resistance was reported only in *Klebsiella* and *E.coli*. Earlier studies have observed that in Salmonellae, the relative frequency of different mutations in *gyrA* was dependent on the quinolone antibiotics used for selection (Levy and Manshall, 2004). According to Lindtedt *et al*. (2004) it was discovered that a geographically dependent distribution of *GyrA* mutation was at codons 83 and 87 in *S. hadar* while Haugum *et al*. (2006) stated that the position and type of amino acid substitution in *gyrA* varied with the serovars. It has also been reported that among *S.typhi* isolates obtained in the United States between 1999 and 2006, 43% were resistant to at least one antibiotic. In this study, 98 % of the *S.enterica* serovars isolates were resistant to at least one of the 14 antibiotics tested. According to Hirose *et al*. (2002) fluoroquinolones have become the first-line drugs for the treatment of typhoid, active against isolates of *Salmonella* species. However, several reports have declared treatment failures when these antimicrobials were used to treat *Salmonella* infections caused by strains with reduced fluoroquinolone susceptibility (Hakanem *et al*., 2001), in agreement with the present study. Several clinical treatment failures after the administration of ciprofloxacin and other fluoroquinolone to patients with typhoid fever due to strains with decreased susceptibility to the fluoroquinolone have also been reported by Threlfall and Ward (2001).

In this study, the mutations responsible for the fluoroquinolone resistance in the *gyrA* and *ParC* genes of the *Salmonella enterica* serovars were investigated. The sequences for the Quinolone Resistance Determining Region (QRDR) of the *gyrA* gene of the

isolates which showed reduced susceptibility to some fluoroquinolone were detected. There was single mutation at the Ser-83 – Tyr, Ser-87- Gly and Ser-83- Phe, while some were found in Asp-87- Gly or Asp-86- Gly in *ParC* gene. The sequence analysis also revealed that some of the positions of the amino acids in the *gyrA* mutation were identified as Asp-87- Asn or at Ser-83- Tyr. The gene (from isolate SA98) sequence alignment revealed that the *S. enterica* characterised in this study had 99% similaritiy or identity to the typed gene of *Salmonella typhimurium* strain 580 *GyrA* gene, partial cds (conservative domains) and 94% identical to the ATCC (American Typed Culture Centre) strain known as *Salmonella enterica* subsp. enterica serovar Typhimurium strain ATCC 307 found in the genebank respectively. Also in this study, some NCTC13348 (National Centre for Typed Culture) strains chromosome were found to be 94% identical to the quinolone resistant gene of the isolate sequenced in this study. Another gene (isolate SO3 having point and double mutation at position 83 and 87 codon) sequenced was also observed to be 94% identical to a strain, *Salmonella enterica* subsp.enterica serovar Typhimurium str. DT104, which is also similar to the gene of the clinical isolate reported by Hirose *et al.* (2002), which caused nosocomial infections in the United States with high level of fluoroquinolone resistance as seen in this study, according to the Fast Minimum Evolution and Neighboring joining tree, taxonomic analysis (Zheng, *et al.,* 2000; Aleksandr *et al.* 2008) .

These reports are in line with those reports of Hirose *et al*. (2002) where a single mutation at either the Ser-83 or the Asp-87 codon was found after sequencing the genes. According to Hirose *et al*. (2002), this indicated that *gyrA* mutations are of principal importance for the fluoroquinolone resistance of serovars typhi and para typhi A, among the salmonellae. Alterations at positions 83 and 87 of the *gyrA* amino acid sequence have also been described previously for *Salmonella* strains by Hirose *et al*. (2002). Double mutations at position 83 or 87 of the *gyrA* amino acid sequence were also reported in clinical isolates of serovars Schwarzengrund, which caused nosocomial infections in the United States and also exhibited ciprofloxcin resistance as earlier stated (Hirose *et al.*, 2002). Nevertheless, these workers stated that strains with high level fluoroquinolone resistance due to double mutations at codons in positions 83 and 87 in the *gyrA* amino acid sequence have not been found in clinical isolates of serovar Typhi and Paratyphi A.

In this study, however, double mutations were detected in *ParC* from *S. enterica* isolates SO3, 18, 19 strains and SE58 and 60 strains (Plate 4.10). Also double

mutation in *ParC* was detected from SE72, 73, 74, 75, and SA76, 77, 97, 98 and 99 strains (Plate 4.11), though in few cases of the clinical isolates. It could be deduced that the difference in fluoroquinolone resistance between two closely related species may be explained by differences in outer membrane permeability's for fluoroquinolones and differences in active efflux activities (Hirose *et al*., 2002). Fluoroquinolone resistance in *S.enterica* is usually mediated by at least one mutation in a DNA topoisomerase gene as observed in this study. According to Gaind *et al*. (2006) and Deak *et al.,* (2015) there was a single *gyrA* mutation in Ser-83-Phe or Ser-83- Tyr which was associated with reduced susceptibility to ciprofloxacin with MIC's of 0.125- 1.0 mg/L. In this study, there was reduced susceptibility to ciprofloxacin and levofloxacin with MIC ranging from 1.56- 12.5µg/ml in both drugs which also correlates with the mutation in *gyrA*, supporting Gaind *et al*. (2006) and Deak *et al.,* (2015). Futhermore, in this study it was observed that there was a slight increase in MIC of ciprofloxacin and levofloxacin which is ≥ 1.56µg/l, when an additional mutation occurred in *ParC* gene in the amino acid position of Asp-87- Gly or Asp- 86- Gly with MIC range between 1.56- 12.5µg/ml leading to reduced susceptibility of the two drugs, thus correlating with the report of Gaind *et al*. (2006), that an additional mutation in *ParC*, as seen in this study was observed in Ser-80- ile, Ser-80- Arg, Asp- 69- Glu or Gly-78-Asp and was accompanied by an increase in ciprofloxacin MIC which ≥0.5mg/L.

Gaind *et al*. (2006) further showed that three mutations conferred ciprofloxacin resistance, two in *gyrA* (Ser-83 –Phe and Asp-87- Asn or Asp -87 –Gly) and one in *ParC*. In this study, four was observed, three in *gyrA* (Ser-83 – Tyr, Ser-87- Gly and Ser-83- Phe) and one in *ParC* (Asp-87- Gly or Asp-86- Gly). Haugum *et al*. (2006) also indicated *gyrA* codon 83 and 87 as the main targets for mutation in *Salmonella enteritidis.* Based on the results obtained, the number of isolates with reduced susceptibility to fluoroquinolone such as ciprofloxacin and levofloxacin appeared to be increasing. Of a particular note was the emergence of quinolone resistance in some clones of the widespread *Salmonella enterica* serotype Typhimurium definite phage type 104 as earlier reported by Hakanem *et al*. (2001).

According to Gaind *et al*. (2006) the presence of plasmid-borne integron in ciprofloxacin-resistance (though not determined in this study) may lead to a situation of untreatable enteric fever. Nevertheless, the fluoroquinolones such as ciprofloxacin, and the cephalosporins such as ceftriaxone are best indicated for the treatment of

severe *S. enterica* serovars in adult while ceftriaxone and cefotaxime are best recommended for children based on the reports in this study.

CHAPTER SIX
CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The original indication of chloramphenicol was in the treatment of typhoid and chloramphenicol may have been effective against a wide variety of Gram negative bacterial (Mandal *et al*., 2010), including most anaerobic organisms (Falagas *et al*., 2008). However the almost universal occurence of multi-drug resistant *Salmonella typhi* meant that it is seldom used for this indication except when the organism is known to be sensitive (Mandal *et al*., 2010). There have been reports by Hakanem *et al*. (2001) that in this era of frequent international connections, microbes may be easily transmitted from one place to another. Correspondingly, factors furthering the emergence and spread of antimicrobial resistance in any country may soon have an impact on resistance of bacterial pathogens, or even of normal human flora, in faraway regions, even different continents. Thus, on this basis, the emergence of antimicrobial resistance in any part of the world may have a global bearing and thus deserves universal attention (Hakanem *et al*., 2001).

Chloramphenicol is no longer a first line agent for any indication in both developed and developing nations like Nigeria. In low income countries like Nigeria unfortunately, chloramphenicol is still widely used mainly because it is cheap and readily available. The most serious adverse effect associated with its treatment is bone marrow toxicity. This may occur in two distinct forms- bone marrow suppression, a direct toxic effect of the drug, usually reversible. Secondly, aplastic anaemia which is idiosyncratic (rare, unpredictable and unrelated to dose) are generally fatal according to Mustaq (2006). Arising from this study, fluoroquinolones such as levofloxacin should only be used in patients who have failed at least one prior therapy, and also reserved for use in seriously ill patients who may require immediate hospitalization as specified by Shin *et al*. (2003), Jonhson and Johnson (2004) and Janssen Pharmaceuticals (2008).

6.2. Recommendations

This study has highlighted the problem of bacterial resistance. Levofloxacin, ciprofloxacin (fluoroquinolone) and the cephalosporins (ceftriaxone and cefotaxime) were seen as possible drugs of choice in this study. Therefore the combination of ciprofloxacin and cefriaxone or levofloxacin and cefotaxime would be recommended for the treatment of *Salmonella* infections. It is also recommended that these drugs should be administered with utmost care and caution to avoid further resistance. According to Shin *et al*. (2003) and Fraundfelder and Fraundfelder (2009), the overuse of antibiotics has given rise to a breed of super- bacteria that are resistant to antibiotic entirely. This research correlates with and supports the report of Linder *et al*. (2005) that fluoroquinolone, including levofloxacin had become the most commonly prescribed class of antibiotics to adult.

Based on this study carried out in the Southeast part of Nigeria, fluoroquinolone and the third generation cephalosporins would be regarded as the drugs of choice for the treatment of chloramphenicol- resistant *Salmonella enterica* and other multi- drug *Salmonella* species. Alternatively, doctors and Pharmacist who prescribe and dispense these drugs indiscriminately in the hospitals and government healthcare centers, should be discouraged. Also, hand washing in the hospital or at home is also recommended as a useful, safe and aseptic technique to patients to prevent diseases such as Typhoid fever caused by *Salmonella enterica* species.

The control of indiscriminate intake of antibiotics such as fluoroquinolones and cephalosporins in animals and humans respectively can help in the control of MDRST; thus the low prevalence of *Salmonella* due to strict control programs result in a relatively low frequency of multi-drug resistance isolates. The degree of communication between veterinary organizations and health care providers in the various hospitals and tertiary health institutions is important for effective management of the menace of multidrug resistance. The government at all levels should be aware of the danger of the spread of antibiotic resistance and continue to enforce the existing laws guiding the sale and use of antibiotics. Also, the use of lactic acid bacillus (LAB) as probiotics in the treatment and management of the infections caused by this pathogen would assist in mitigating the high level of resistance from this organism.

Lastly, the establishment of bioscience and research institutes in Nigeria higher institution of learning, especially in Southeast Nigeria will aid the molecular studies of this type.

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