CHAPTER ONE

Introduction and Background of study

Salmonella is a foodborne pathogen of public health importance worldwide associated with varieties of illnesses (salmonellosis). *Salmonella* is a genus of the family Enterobacteriaceae and comprises a large and closely related population of medically important pathogens. Based on O (somatic) and H (flagellar) antigens, different antigenic types of salmonellae have been identified which are designated as 'serotypes' or 'serovars'. The terms 'serotypes' and 'serovars' are both recognized as the designations for the antigenic types of salmonellae by the Rules of the Bacteriological Code (1990 Revision) established by the Judicial Commission of the International Committee on the Systematics of Prokaryotes, but the term "serovar" is preferred to the term "serotype" (Su *et al.,* 2007).

There are three species in the genus *Salmonella* and these are S. *enterica, S. bongori and S. subterranean* (Shelobolina *et al.,* 2004; Judicial Commission, 2005; Su *et al.,* 2007). Currently, the genus *Salmonella* is divided into 2587 serovars (Grimont and Weill, 2007). Majority of the serovars are grouped into the species *S. enterica*, which contains all of the major serovars that cause infections in humans (Folley and Lynne, 2007). These serovars can be divided into typhoidal and non-typhoidal *Salmonella* based on the infections they cause. The typhoidal *Salmonella* are restricted to human hosts and these include *S. enterica* serotype Typhi (*S*. Typhi) and *S*. Paratyphi which cause enteric fever (Uzzau, 2000).

The non-typhoidal *Salmonella* are able to colonise the alimentary tracts of several animals including food-producing animals (such as poultry, cattle and pig). The infected animals may not show symptoms of infection thus acting as carriers of *Salmonella*. Such animals act as reservoirs of *Salmonella* from which these pathogens can be transmitted to humans (as zoonotic agents) through contacts and consumption of contaminated meat and other animal products (Mead *et al.,* 1999; Adak *et al.,* 2002; Hald *et al.,* 2007; Boyen *et al.,* 2008). Such animal carriers pose an important threat to both animal and human health because they have been associated with salmonellosis in human. Several outbreaks of human salmonellosis are associated with contaminated food products with food animals been the ultimate vehicles (Annonymous, 2009). Poultry are considered as one of the most common vehicles of human salmonellosis (Braden, 2006). Poultry is a potential source of salmonellosis in Nigeria because poultry farming is a thriving business mainly due to the reason that chicken-based dishes have become special delicacies especially in festive periods but in more recent times, with the increase in poultry farming and importation of chicken parts, it is becoming a staple diet especially in urban areas.

Salmonellosis is often associated with gastroenteritis, which is usually self-limiting. In some cases, particularly in children, elderly and immunocompromised patients, *Salmonella* infection can lead to invasive and focal infections which can be severe (Hohmann, 2001). The ability of *Salmonella* to cause invasive infection varies with serovar, age of patient and region. *S.* Typhi and *S.* Paratyphi A are highly invasive for both children and adults. Certain serovars of NTS such as *S.* Choleraesuis, *S.* Dublin, and *S.* Virchow have higher abilities than other serovars to cause invasive salmonellosis (Jones *et al.,* 2008; Morpeth *et al.,* 2009; Hendriksen *et al.,* 2009a). In Sub-Saharan Africa, non-typhoidal *Salmonella* (NTS) invasive infections are of public health concern in infants, young children and young adults suffering from malnutrition, malaria and

HIV infection (Gordon, 2008; Kingsley *et al.,* 2009; Vandenberg *et al.,* 2010). Most human infections are caused by a limited number of serovars; which may vary from country to country and over time. The predominant serovars in some African countries include *S.* Enteritidis, *S.* Typhimurium, *S.* Concord, and *S.* Isangi (Usha *et al.,* 2008; Sire *et al.,* 2008; Gordon *et al.,* 2008; Hendriksen *et al.,* 2009; Kingsley *et al.,* 2009;Vandenberg *et al.,* 2010).

Fluoroquinolones and third generation cephalosporins, are currently the drugs of first choice for treatment of invasive salmonellosis due to wide spread resistance to the traditional drugs such chloramphenicol, ampicillin and cotrimoxazole (Parry and Threlfall, 2008). Resistance of *Salmonella* to multiple antibiotics is increasingly reported and constitutes serious public health problems. For example, *S.* Typhimurium DT104 resistant to several antibiotics (ampicillin, chloramphenicol, streptomycin, sulfonamides and streptomycin) emerged in 1980s in U.K and spread across the globe with established endemicity in many countries (Helms *et al.,* 2005). The fluoroquinolones and third generation cephalosporins have not been spared in the development of resistance. Ciprofloxacin resistance increased from 0.02% in 1994-1995 to 2.5% in 2000 in the United States (Crump *et al.,* 2003). A recent study in Maiduiguri, Nigeria reported a high resistance rate to ciprofloxacin in chicken isolates of *Salmonella* (Raufu *et al.,* 2009). In 2004, *Salmonella* isolates resistant to third generation cephalosporins were identified in 43 countries spread over Europe, Americas, Africa and Asia (Arlet *et al.,* 2006). The reported resistance rates to third generation cephalosporins ranged between 0% to 3.4% (Dunne *et al.,* 2000; Winokur et al., 2001; Li *et al.,* 2005; Su *et al.,* 2005). Ciprofloxacin-resistant *S.* Kentucky has emerged and causing increasing human salmonellosis in Europe; majority of the infection have been linked with travels to many African countries where there is little data on *Salmonella* (Weill *et al.,* 2006; Collard *et al.,* 2007). Recently, there was a report of *Salmonella* strains resistant to

fluoroquinolones, third generation cephalosporins and cotrimoxazole which encompassed the three antibiotic classes recommended for the treatment of invasive salmonellosis (Collard *et al.,* 2007) thus limiting therapeutic options for the effective treatment of salmonellosis. Therefore, there is need for constant surveillance of antibiotic resistance in *Salmonella*.

Public health importance of antibiotic resistant *Salmonella* include increased hospitalization, blood stream infection and vulnerability to salmonellosis coupled with higher cost of diagnosis and treatment (Helms *et al.,* 2002; Su *et al.,* 2003; Crump *et al.,* 2003; Su *et al.,* 2004; Martin *et al.,* 2004; Varma *et al.,* 2005a,b). The high rates of antibiotic resistance in *Salmonella* is a serious public health issue that warrants an adequate understanding of the molecular basis for effective control strategies to be put in place. The development of resistance in *Salmonella* toward antibiotics is attributable to one of multiple mechanisms, including production of enzymes that inactivate antibiotics through degradation or structural modification, reduction of bacterial cell permeability to antibiotics, activation of antibiotic efflux pumps and modification of the cellular target for drug (Lacroix *et al.,* 1996; Poole, 2001; Soto *et al.,* 2003). Resistance to the β-lactam antibiotic (such as ampicillins and third-generation cephalosporins) by *Salmonella* is mainly due to the production of β-lactamase enzymes which are able to degrade the β-lactam antibiotics. The β-lactamases include the extended-spectrum β-lactamases (ESBLs) which have the ability to degrade penicillins, first, second and third generation cephalosporins (Arlet *et al.,* 2006). The most prevalent ESBLs are the CTX-Ms which have been reported in several African countries (Arlet *et al.,* 2006; Bouchrif *et al.,* 2009; Abdelghani *et al.,* 2010).

The genes encoding resistance determinants are usually located on mobile genetic elements such as plasmids, transposons and integrons which facilitate the acquisition and dissemination of

resistance traits through horizontal transfer (Hawkey, 1998; Carattoli, 2003). Integrons plays significant role in the development and spread of multidrug resistance in *Salmonella* (Krauland *et al.,* 2009). Integrons are able to perform this role because they have the ability to capture, integrate and express more than one resistance gene (Carattoli, 2003). Four different types of integrons have been described which are classes 1, 2, 3 and 4.

Understanding clonal relationship of *Salmonella* isolates is useful to determine the source and dissemination of *Salmonella* isolates. This is central to limiting the spread of *Salmonella* infections. Several epidemiological typing methods have been developed to investigate clonal relationship of bacteria isolates. Pulse-field gel electrophoresis (a molecular method) is considered the gold-standard for determination of clonal relationship of *Salmonella* isolates (Foley *et al.,* 2007). Antibiotic susceptibility testing (a phenotypic method) has also been used (Ammari *et al.,* 2009)

STATEMENT OF PROBLEM

Salmonella is a pathogen of global importance with only few of the known 2547 serovars having significant public health importance (Grimont and Weill, 2007). The clinical burden of *Salmonella* infections in Nigeria is not fully understood; most importantly, information about serovars of public health importance is generally sparse. Furthermore, *Salmonella* can be transmitted from animals as zoonotic agents to humans resulting in infections (mainly through consumption of meat, poultry and milk). There is the need to determine animal sources of *Salmonella* infections in Nigeria for the initiation of possible targeted control measures. Additionally, antibiotic treatment is crucial for the effective management of invasive and severe salmonellosis. But in recent times, treatment of salmonellosis has become a difficult task due to

widespread increase of *Salmonella* strains resistant to available antibiotics; use of antibiotics in animals has been reported to promote antibiotic resistance in humans. There is inadequate data about relative antibiotic susceptibility profiles of *Salmonella* isolates from different sources in Nigeria thus militating against measures to limit spread. Diverse molecular determinants have also been reported to mediate antibiotic resistance in *Salmonella* including the β-lactamase enzymes encoded by β-lactamase genes such as extended-spectrum β-lactamase enzymes (ESBLs) which can destroy the first three generations of cephalosporin antibiotics; additionally integrons (mobile genetic elements) play important role in horizontal transfer of resistance. Information about molecular determinants of resistance in *Salmonella* is generally lacking compared to other bacteria in Nigeria. Furthermore, understanding clonal relationship of *Salmonella* isolates is useful to determine the source and dissemination of *Salmonella* isolates; this is crucial to limiting spread of *Salmonella* infections.

AIM OF THE STUDY

To determine phenotypic and genotypic characteristics of *Salmonella* isolates from Ibadan.

OBJECTIVES OF STUDY

These are to:

1. Determine the sources and clinical burden of *Salmonella enterica*
2. Evaluate serovars of the *Salmonella* isolates
3. Analyse antibiotic resistance profiles of the *Salmonella* isolates

4. Elucidate molecular determinants of resistance and clonal relationship of isolates

RESEARCH QUESTIONS

1. What is the clinical burden of *Salmonella* in humans?
2. What food animals act as sources (reservoirs) of *Salmonella*?
3. Which serovars of *Salmonella* are found in humans and animals? Are the serovars similar to those in other environments?
4. What are the antibiotic profiles of the *Salmonella* isolates?
5. What are the molecular determinants of antibiotic resistance and clonality of isolates?

SIGNIFICANCE OF STUDY

The study showed *Salmonella* as an important human pathogen and identified several food animals (chicken, pig, cattle, goat and sheep) to constitute important reservoirs of this bacterium. Diverse serovars were obtained from both humans and animals: the study revealed *S.* Dublin as an important human pathogen in our environment in contrast to several reports from other African countrie*s.* Invasive ability of some uncommon *Salmonella* serovars previously associated with reptiles was also revealed. Chicken was shown to be reservoirs of serovars of *Salmonella* that constitute potential public health importance in Nigeria. High rates of antibiotic resistance were observed particularly among the human and chicken isolate*s.* The human isolates were generally resistant to the traditional antibiotics use in the treatment of salmonellosis. While, the chicken isolates additionally showed alarming high resistance rate to fluoroquinolones and

aminoglycosides, which are drugs of last resort for treatment of invasive salmonellosis. Furthermore, β-lactamase enzymes encoded mainly by *TEM* gene, was found to mediate resistance to β-lactam antibiotics; integrons were also found to play a significant role in antibiotic resistance. Clonal dissemination of fluoroquinolone resistant *Salmonella* was observed among chicken isolates from different poultry farms in Ibadan. It is hoped that this study will help in the empirical treatment of salmonellosis and control of antibiotic resistance and will further necessitate the initiation of control measures in food animal productions to prevent transmission of zoonotic agents to humans in Nigeria to enhance food safety.

LIMITATION OF STUDY

All the *Salmonella* isolates could not be serotyped because the antisera are very expensive and not available locally. High cost of reagents for molecular studies was also a limitation.

DEFINITION OF TERMS

Phenotype: Observable characteristics of an organism

Genotype: Genetic bases underlying observable characteristics of an organism

Epidemiology typing: Determination of relationship between different isolates of bacteria of the same species

Salmonellosis: Infection caused by Salmonella

Invasive salmonellosis: *Salmonella* infections that result from infection of blood streams

Pyrexia of unknown origin: Increase in body temperature without known cause

Animal reservoirs: Animals harbouring viable microorganisms and can transmit the organisms to humans thus acting as sources of infections

Food animals: animals that are sources of food to man

Pathogens: microorganisms able to cause diseases

Asymptomatic: presence of a pathogen in an individual without showing signs of disease

Carrier: individual harbouring pathogens without showing symptoms

Endonuclease: an enzyme able to cut DNA into small pieces at specific points

Integrons: mobile genetic material able to capture, integrate and express resistance genes

Bacteremia: Infection of blood stream by bacteria

Prevalence: the number of cases in a defined population at a specific point in time

CHAPTER TWO

LITERATURE REVIEW

Salmonella is a genus of the family Enterobacteriaceae and comprises a large and closely related population of medically important pathogen*s*. It has long been associated with a wide spectrum of infectious diseases, including typhoid fever and nontyphoid salmonellosis, which cause public health problems worldwide. *Salmonella* is named after an American bacteriologist, D. E. Salmon, who first isolated *Salmonella choleraesuis* from porcine intestine in 1884(Smith, 1894). The organism was originally called "*Bacillus choleraesuis*," which was subsequently changed to "*Salmonella choleraesuis*" . Based on O (somatic) and H (flagellar) antigens, different antigenic types of salmonellae have been identified which are designated as 'serotypes' or 'serovars'. The terms 'serotypes' and 'serovars' are both recognized as the designations for the antigenic types of salmonellae by the Rules of the *Bacteriological Code* (1990 Revision) established by the Judicial Commission of the International Committee on the Systematics of Prokaryotes, but the term "serovar" is preferred to the term "serotype". The taxonomy and nomenclature of salmonellae is very complex and has undergone several changes since the first isolation of these bacteria (Su *et al.,* 2007).

HISTORY OF TAXONOMY AND NOMENCLATURE OF *SALMONELLA*

From 1966, the different serovars were designated as different species of salmonellae based on the acceptance of a proposition for them to be so designated (Kauffmann, 1966) which led to having several species in the genus *Salmonella*, for example, *S. typhi* and *S. typhimurium*.

Because of the complexity of having several species in the genus *Salmonella*, several propositions were made for few species or even one.

There was a proposition that the genus *Salmonella* be sub-divided into three species, *S. choleraesuis* (the type species), *S. typhosa* (*S. typhi*), and *S. kauffmannii*, with the last containing all the other serovars (Borman *et al.,* 1944). Another proposition for three species but with different names was made in 1966 (Ewing, 1972). In 1970, a proposal for five species was put forward to represent the 'sub-genera' under *S. kauffmannii* (Ewing, 1972). A proposal for only one species (*S. enterica*) to encompass all salmonellae was also made (Kauffmann and Edwards, 1952).

Eventually in 1973, DNA-DNA hybridization experiments showed that all *Salmonella* strains belong to a single species (Crosa *et al.,* 1973). In 1982, on the basis of numerical taxonomy and DNA relatedness "*Salmonella choleraesuis*" was proposed as the single *Salmonella* species and six sub-species were defined (Le Minor *et al.,* 1982). Additionally, the name of serovars were proposed to be used without italicization or underlining (e.g., *Salmonella choleraeusis* subsp. *choleraesuis* ser. typhimurium). In 1989, the subspecies *Salmonella choleraesuis* subsp. *Bongori* was removed and designated as a unique *Salmonella* species due to differences shown by DNA relatedness studies (Reeves *et al.,* 1989).

To avoid the confusion of using "choleraesuis" as a name for both a species and a serovar, in 1986 "*Salmonella enterica*" was proposed again as the type species of *Salmonella* to replace "*Salmonella choleraesuis*" by the Sub-committee of Enterobacteriaceae of the International Committee on Systematic Bacteriology at the XIV International Congress of Microbiology (Penner, 1988) and presented to the Judicial Commission of the International Committee of

Systematic Bacteriology in 1987 (Le Minor and Poppoff, 1987). The epithet "*enterica*" was recommended because it has not been used previously for a serovar. Furthermore, seven sub-genera of *Salmonella* were proposed as subspecies (subspecies I, II, IIIa, IIIb, IV, V, and VI). Subgenus III was divided into IIIa and IIIb by DNA similarity and phenotypic characteristics. The suggestion was accepted by the Centers for Diseases Control and Prevention (CDC) and other experts and laboratories (Ewing, 1986; Old, 1992) but denied by the Judicial Commission due to concerns that the status of *Salmonella* serovar Typhi might be overlooked. This proposal was represented in 1999, with an amendment by Euzéby, proposing "*Salmonella enterica*" as the type species of *Salmonella* and reserved the name "*Salmonella typhi*" to reflect its clinical importance (Euzéby, 1999).

Consequent upon the amended proposal, The Judicial Commission gave an approval (the Judicial Opinion 80) that from January 2005, "*Salmonella enterica*" would replace "*Salmonella choleraesuis*" to become the type species of the genus *Salmonella*. According to the ruling of the Judicial Commission, the genus *Salmonella* consists of two species, "*Salmonella bongori*" and "*Salmonella enterica*". The latter includes six subspecies, "*arizonae*", "*diarizonae*", "*enterica*", "*houtenae*", "*indica*", and "*salamae*". In 2005, a new species, "*Salmonella subterranea*" was recognized and approved by the Judicial Commission (Shelobolina *et al.,* 2004).

All antigenic formulae of recognized *Salmonella* serotypes are listed in a document called the Kauffmann-White scheme (Popoff and Le Minor, 2001). The WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France is responsible for updating the scheme. Currently, there are 2,587 serotypes of *Salmonella*. The nomenclature of

genus *Salmonella* is currently according to the CDC nomenclature system which is based on recommendations from the WHO Collaborating Centre

According to the CDC system the genus *Salmonella* has three species: *S. enterica* (type species), *S. bongori* and *Salmonella subterranean. S. enterica* consists of six subspecies (Popoff and Le Minor, 2001; Brenner *et al.,* 1998): I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S. enterica* subsp. *indica*. In subspecies I, names of serovars are indicative of associated diseases, geographic origins, or usual habitat*s.* In the remaining subspecies, as well as those of *S. bongori*, antigenic formulae determined according to the Kauffmann-White scheme (Popoff and Le Minor, 2001) are used for unnamed serovar*s.* The serovars of other subspecies and species which had been named before 1966 retain such names and are cited as those of subspecies I.

Moreover, CDC nomenclature system avoids confusion between serovars and species names by not italicizing serovar name and starting with a capital letter. When writing a report, the genus name is written in full the first time it is mentioned followed by the word "serotype" (or the abbreviation "ser.") and then the serotype name, e.g., *Salmonella* serotype or ser. Choleraesui*s.* Afterward the name may be shortened with the genus name followed directly by the serotype name, e.g., *Salmonella* Choleraesuis or *S.* Choleraesuis (Popoff *et al.,* 2004). Because the type species name, *enterica*, was not approved before 2005, serotype names are used directly after the genus name without mention of the specie*s.* For those without attached serovar names but designated only by their antigenic formulae, the subspecies name is written in Roman letters (not

italicized) followed by their antigenic formulae. A colon is used between each antigen, e.g., *Salmonella* serotype II 39:z10:z6.

In the publications of American Society of Microbiology (ASM), the CDC nomenclature is used (Publications Board, 1999). But from 2006, instructions to ASM authors stipulate inclusion of species name in the nomenclature of salmonellae. These instructions stipulate that, for the species, "*Salmonella enterica*" is used at first mention and "*S. enterica* thereafter; for the subspecies, "*Salmonella enterica* subsp. *arizonae*" is used first, and "*S. enterica* subsp. *arizonae*" thereafter. Serovar names should be in Roman type with the first letter capitalized, e.g., *Salmonella enterica* serovar Typhimurium. After the first use, the serovar may be used without a species name, e.g., *Salmonella* Typhimurium.

EPIDEMIOLOGY OF *SALMONELLA* SEROVARS

Salmonella serovars can be broadly divided into typhoidal and non-typhoidal based on host-adaptability and infection. The typhiodal *Salmonella* serovars are host-restricted human pathogens and include *Salmonella enterica* serotype Typhi and *S.* Paratyphi (Uzzau *et al.,* 2000). Non-typhiodal *Salmonella* (NTS) are zoonotic agents and a wide variety of animals has been identified as their reservoirs (Mead *et al.,* 1999; Adak *et al.,* 2002; Hald *et al.,* 2007). The animal reservoirs include food animals and therefore can infect man through the food chain causing zoonotic infections. Poultry are often considered as one of the common vehicles of human salmonellosis (Braden 2006).

SALMONELLA INFECTIONS

Salmonellosis is often associated with gastroenteritis, which is usually self-limiting. In some cases, particularly in children, elderly and immunocompromised patients, *Salmonella* infection can lead to invasive and focal infections which can be severe (Hohmann, 2001). The ability of *Salmonella* to cause invasive infection varies with serovar, age of patient and region. *S.* Typhi and *S.* Paratyphi A are highly invasive for both children and adults; they cause enteric fever. On the other hand, certain serovars of NTS such as *S.* Choleraesuis, *S.* Dublin, and *S.* Virchow have higher abilities than other serovars to cause invasive salmonellosis (Jones *et al.,* 2008; Morpeth *et al.,* 2009). In Sub-Saharan Africa, NTS invasive infections are of public health concern in infants, young children and young adults suffering from malnutrition, malaria and HIV infection (Gordon 2008; Kingsley *et al.,* 2009; Vandenberg *et al.,* 2010). Most human infections are caused by limited serovars; such important serovars vary from country to country and there can be shift from time to time within a region. The predominant serovars in some African countries include *S.* Enteritidis, *S.* Typhimurium, *S.* Concord, and *S.* Isangi (Sire *et al.,* 2008; Gordon *et al.,* 2008; Usha *et al.,* 2008; Kingsley *et al.,* 2009; Hendriksen *et al.,* 2009a; Vandenberg *et al.,* 2010)

In Nigeria, there is limited data on *Salmonella* serovars causing human salmonellosis and the importance of transmitting foodborne pathogens from the food chain to humans is not well understood. Decades ago, a study showed that *S.* Typhi and NTS played significant role in septicemia in humans in Ibadan, Nigeria. However, the specific NTS serotypes (Alausa *et al.,* 1977) were not revealed probably due to the lack of resources and high quality antisera which often are lacking from many African countries (Hendriksen *et al.,* 2009b). Chicken is an

important food source to man; chicken meat dishes are special delicacies particularly during festive periods in Nigeria but the poultry industry is largely unregulated. The link between human salmonellosis and consumption of chicken is not known nor the serovars in Nigeria. A study found the prevalence of *S.* Enteritidis in chicken meat in Maiduguri, Nigeria to be 27% (Ameh *et al.,* 2001). A recent study in Maiduguri, in northern part of Nigeria, reported *S.* Hiduddify to be the predominant serovar in free chicken (Raufu *et al.,* 2009). Moreover, *S.* Dublin has also been reported from different animals including cattle and zoo animals in Nigeria (Collard and Sen, 1956, 1962; Falade and Durojaye, 1976; Okoh and Onazi, 1980). The public health importance of these serovars is not known.

SALMONELLA INFECTIONS: NON-TYPHOIDAL SALMONELLOSIS

Nontyphoidal salmonellae (NTS) are important causes of reportable foodborne infection. They are problematic, even in modestly compromised hosts, as a result of bacteremic spread, focal infection, and persistence in deep or endovascular sites (Mead *et al.,* 1999; Hohmann, 2001). NTS have a wide range of hosts and are strongly associated with agricultural products (Mead *et al.,* 1999). The increasing centralization and industrialization of food supply have enhanced the distribution of these hardy organism*s*. The incidence of the NTS has been on the rise since the 1970*s*.

HOST SUSCEPTIBILITY TO NON-TYPHOIDAL SALMONELLOSIS

Gastrointestinal salmonellosis caused by non-typhoidal *Salmonella* and its serious sequelae are linked to a wide variety of illnesses and therapies that affect the body's multiple defences against enteric and intracellular pathogen*s*. Gastric hypopacidity in infants, in pernicious anemia, or

caused by antacids and H-2 blockers may predispose individuals to salmonellosis. Risk factors for salmonellosis include extremes of age, alteration of the endogenous bowel flora of the intestine (e.g. as a result of antimicrobial therapy or surgery), diabetes, malignancy, rheumatological disorders, reticuloendothelial blockade (e.g., as a result of malaria, sickle-cell disease, or bartonellosis), HIV infection, and therapeutic immunosuppression of all types. Anatomical distruptions, including kidney stones and urinary tract abnormalities, gallstones, atherosclerotic endovascular lesions, schistomiasis, and prosthetic devices, may all serve as foci for persistent *Salmonella* infection. The occurrence of bacteremia without associated recent gastrointestinal symptoms is ominous and should prompt clinicians to consider whether an underlying immunosuppressive illness or anatomical risk factor is present (Hohman, 2001).

Non-typhoidal Bacteremia and Endovascular Infection: Serious Complications

Approximately 5% of individuals with gastrointestinal illness caused by nontyphoidal *Salmonella* will develop bacteremia, a serious and potentially fatal problem. Bacteremia is more likely to develop focal infection. In adults, non-typhoidal *Salmonella* bacteremia could carry higher fatality rate compared to children (Galofres *et al.,* 1994; Zaidi *et al.,* 1999). This reflects both the tenacity and comorbidities of adults who develop bacteremia; these bacteremic patients represent a small percentage of the large number of people with both overt and subclinical intestinal infection. A feared complication of *Salmonella* bacteremia in adults is the development of infectious endarteritis, especially that which involves the abdominal aorta (the arteritis formally known as mycotic) and was in the part almost uniformly fatal (Hohman, 2001), but combined surgical and medical therapy, and extra-anatomical bypass with construction of an axillobifemoral graft have significantly increased the survival rate (Oskoui *et al.,* 1993).

Blood invasion of *Salmonella* is a function of both the serotype and patient age (Weinberger and Keller, 2005). Each serotype can be characterized by its blood invasiveness ratio (BIR): the number of blood isolates per 100 blood plus stool isolates. Among NTS serotypes, *S. choleraesuis* and *S. dublin* have similarly high invasiveness with BIR ranges of 50 – 60 and 40 – 70, representatively (Blasser and Feldman, 1981; Weinberger *et al.,* 2004). Age-specific BIR could be high at the extremes of life and could vary between the various NTS serotypes. Thus, the overall invasiveness of NTS in a certain location depends on the relative frequency of the individual serotypes.

Non-typhoidal Focal Infections: Protean and Problematic

Virtually any anatomical site may be seeded hematogenously by non-typhoidal *Salmonella* and may evolve into local infection, even if the bacteremia is successfully treated (Hohman, 2001). Focal infections should be drained or debrided whenever possible. A minimum of 2 weeks of antimicrobial therapy is suggested for the treatment of a surgically eradicated soft-tissue focus in a normal host. Therapy for 4 – 6 weeks most often is advisable, given the known persistence of *Salmonella* species at compromised sites.

Other serious complications include osteomyelitis and joint infections, which are common in patients with sickle-cells anemia and are difficult to treat. Failure of fluoroquinolones and emergence of quinolone resistance have been demonstrated in patients with osteomyelitis (Workman *et al.,* 1996). Furthermore, severe and prolonged polyarticular reactive joint disease can occur after intestinal salmonellosis and is not altered by long-term antibiotic therapy (Sieper *et al.,* 1999). Splenectomy may be required for splenic abscesses. Central nervous system infection may also occur and this is fatal in approximately 50% of cases. Urinary isolates of

Salmonella are common (they accounted for 50 or 129 extraintestinal isolates found in patients at a general hospital in the U.*S.* in the 1990s) (Hohmann, 2001). Positive urine culture results may erroneously be attributed to fecal contamination or "urinary tract colonization," but they frequently reflect bacteremic seeding and secondary infection of the urinary tract.

SALMONELLA INFECTIONS: TYPHOIDAL SALMONELLOSIS

The typhoidal *Salmonella* are host-restricted to humans causing enteric fever (typhoid and paratyphoid). Typhoid fever is a systemic infection caused by *Salmonella* Typhi (*S.* Typhi). It was an important cause of illness and death in the overcrowded and unsanitary urban conditions of the United States and Europe in the 19th century (Osler, 1912) but the provision of clean of water and good sewage systems led to a dramatic decrease in the incidence of typhoid in these region*s*. Today most of the burden of the disease occurs in the developing world, where sanitary conditions remain poor (Parry *et al.,* 2002). According to the best global estimates, there are at least 16 million new cases of typhoid fever each year, with 600,000 deaths annually (Ivanof, 1995).

The bacterium is serologically positive for lipopolysaccharide antigens O9 and O12, protein flagellar antigen Hd, and polysaccharide capsular antigen Vi. The capsular antigen Vi is largely restricted to *S. enterica* serotype typhi, although it is shared by some strains of *S. enterica* serotypes hirschfeldii (paratyphi C) and Dublin, and *Citrobacter freundii*. A unique flagella type, Hj, is present in some *S. enterica* serotype typhi isolates from Indonesia (Grossman *et al.,* 1995). Phage typing, pulse-field gel electrophoresis, and ribotyping have shown that areas of endemic disease usually have many strains in circulation but that outbreaks are usually due to a restricted number of strains (Thong *et al.,* 2000; Connerton *et al.,* 2000).

Clinical Features of Typhoid Fever

The clinical manifestations and severity of typhoid fever vary with the patient population studied. Most patients who present to hospitals with typhoid fever are children or young adults from 5 to 25 years of age (Osler, 1912; Parry *et al.,* 2002). However, community-based studies in areas of endemic disease indicate that many patients with typhoid, particularly children under five years of age, may have a nonspecific illness that is not recognized clinically as typhoid (Sinha *et al.,* 1999). Between 60 and 90% of people with typhoid do not receive medical attention or are treated as outpatients (Sinha *et al.,* 1999).

After a person ingests *S.* Typhi, an asymptomatic period follows that usually lasts 7 to 14 days (range, 3 to 60). The onset of bacteremia is marked by fever (the fever rises progressively to 39°C to 40°C) and malaise. A relative bradycardia is considered common in typhoid, although in many geographic areas this has not been a consistent feature. Adults often have constipation, but in young children and in adults with HIV infection, diarrhoea is more common (Gotuzzo *et al.,* 1991). It is unusual for a patient hospitalized with typhoid to have no abdominal symptoms and normal bowel movements. A few rose spots, blanching erythematous maculopapular lesions approximately 2 to 4 mm in diameter, are reported in 5 to 30% of cases. They usually occur on the abdomen and chest and more rarely on the back, arms, and legs. These lesions are easily missed in dark-skinned patients.

Complications occur in 10 to 15% of patients and are particularly likely in patients who have been ill for more than two weeks. Many complications have been described (Table 1), of which gastrointestinal bleeding (the most common), intestinal perforation (the most serious), and typhoid encephalopathy are the most important (Bitar *et al.,* 1985). Intestinal (usually ileal)

perforation is the most serious complication, occurring in 1 to 3 percent of hospitalized patients (Bitar *et al.,* 1985). A reduced level of consciousness or encephalopathy, often accompanied by shock, is associated with high mortality (Rogerson *et al.,* 1991). The incidence of these neuropsychiatric presentations varies among countries. It ranges from 10 to 40 percent among hospitalized patients with typhoid in Indonesia and Papua New Guinea but is less than 2 percent in Pakistan and Vietnam (Parry *et al.,* 2002). This geographic variation is unexplained.

Relapse occurs in 5 to 10 percent of patients, usually two to three weeks after the resolution of fever. Relapse may follow recrudescence or reinfection (Wain *et al.,* 1999). Up to 10 percent of convalescing patients with untreated typhoid excrete *S.* Typhi in the feces for up to three months; 1 to 4% become long-term carriers, excreting the organism for more than one year. Up to 25% of long-term carriers have no history of typhoid. Chronic carriage is more common among women and the elderly and in patients with cholelithiasis (Levine *et al.,* 1982). Most carriers are asymptomatic. Patients with an abnormal urinary tract, such as those who have schistosomiasis, may excrete the organism in the urine for long periods.

The average case fatality rate is less than 1%, but the rate varies considerably among different regions of the world. Among hospitalized patients, the case fatality rate varies from less than 2 percent in Pakistan and Vietnam to 30 to 50% in some areas of Papua New Guinea and Indonesia (Parry *et al.,* 2002). The case fatality rates are highest among children under one year of age and among the elderly (Butler *et al.,* 1991). However, the most important contributor to a poor outcome is probably a delay in institution of effective antibiotic treatment.

Table 1: Important Complications of Typhoid Fever

Complications
Abdominal
Gastrointestinal perforation *
Gastrointestinal hemorrhage*
Hepatitis
Cholecystitis (usually subclinical)
Cardiovascular
Asymptomatic electrocardiographic changes
Myocarditis
Shock
Neuropsychiatric
Encephalopathy*
Delirium
Psychotic states
Meningitis
Impairment of coordination
Respiratory
Bronchitis
Pneumonia (*Salmonella enterica* serotype typhi, *Streptococcus pneumoniae)*
Hematologic
Anemia
Disseminated intravascular coagulation (usually subclinical)
Other
Focal abscess
Pharyngitis
Miscarriage
Relapse
Chronic carriage

Source: Parry *et al.* (2002)

SALMONELLA INFECTION IN HIV-INFECTED PATIENTS

The HIV/AIDS epidemic has been associated with many opportunistic infections, viral, bacterial, fungal and parasitic. Of the bacterial infections, non-typhoidal salmonellae (NTS) are the commonest bacterial isolates from adult blood culture series in African countries where HIV is

high (Gilks *et al.,* 1990; Archibald *et al.,* 1998) and were isolated from 37% of adult blood culture samples in Blantyre, Malawi (Gordon *et al.,* 2001), where HIV sero-prevalence is approximately 30% among urban women attending antenatal clinics (Saha *et al.,* 2001).

In a Kenyan study, a total of 138 NTS isolations were isolated from 1,220 episodes of fever in adults admitted to the Kenyatta Hospital, Nairobi (Kariuki *et al.,* 1996) and represented 11.3% of all blood cultures and well over 50% of positive blood cultures. Of these, 224 (19.7%) of the 1220 patients with febrile episodes were HIV sero-positive, though 95(68.8%) of the 138 with NTS bacteremia were HIV infected, demonstrating a strong association ($P < 0.001$) between being HIV sero-positive and having NTS bacteremia. Similar observations have also been made in some other parts of the world. For example, studies in the USA have estimated that NTS bacteremia is 100-fold more common in HIV-infected adults than in the general population (Levin *et al.,* 1991; Gruenevald *et al.,* 1994). Also in the U.K., NTS is common in patients infected with HIV; and NTS bacteremia without diarrhoea is a marker of immunosuppression (Brown and Eykyn, 2000).

In immunocompetent adults, NTS infection causes a self-limiting diarrhoea illness. Transient bacteremia occurs in 1 – 4% of cases, and overall mortality is 0.5% (Gordon *et al.,* 2002). By contrast, the available data suggest a devastating inpatient mortality of up to 80% among HIV-infected adults with NTS bacteremia (Gordon *et al.,* 2002). There are case reports of focal NTS infections in HIV, including endocarditis, intracrannial infections and pyomyostitis (Sperber and Schluepner, 1987). Pulmonary involvement in HIV-infected patients with NTS bacteremia is well recognized, and may represent either isolated NTS lung disease or co-infection with a second respiratory pathogen (Casado *et al.,* 1999). Despite these isolated reports, most case-

study series found that focal metastatic NTS infections in HIV are rare (Sperber and Schlueper, 1987; Casado *et al.,* 1999; Gordon *et al.,* 2002). Presentation may mimic enteric fever (Gilks *et al.,* 1992), and a lack of diarrhoea or other gastrointestinal symptoms is commonly reported in HIV-positive patients (Sperber and Gordon, 1987; Casado *et al.,* 1999).

Recurrent NTS bacteremia was recognized as a feature of AIDS in 1985 (Smith *et al.,* 1985; Nadelman *et al.,* 1985; Gordon *et al.,* 2002). In Africa, a study showed 43% of patients had a first recurrence of NTS bacteremia at 23 – 186 days; while 26% of the patients that showed first recurrence further developed multiple recurrences up to 245 days (Gordon *et al.,* 2002). In other parts of the world, between 22% (Casado *et al.,* 1999) and 44% (Hart *et al.,* 2000) of patients have recurrent bacteremia reported, in a median time of 87 days (Casado *et al.,* 1999). Recurrence may be as a result of re-infection with NTS or recrudescence of NTS bacteremia. In Africa, recurrence is commonly due to recrudescence (Gordon *et al.,* 2002). Recrudescence from intracellular tissue sequestration, rather foci of infections within the patients, has been suggested to be more common (Gordon *et al.,* 2002). Elsewhere recurrence is commonly associated with re-infections unlike African situation. Increased susceptibility among HIV-infected adults (Levine *et al.,* 1991) might be responsible for this. The role of antibiotics in preventing relapses is poorly understood.

Studies in African children have shown repeatedly that NTS are the commonest cause of bacteremia, being responsible for 40 – 60% of cases (Lepage *et al.,* 1987) and predisposing factors include malaria, severe malarial anaemia, sickle cell disease, malnutrition and schistosomiasis (Green and Checsbrongh, 1993; Mabey *et al.,* 1987; Gendrel *et al.,* 1994; Graham *et al.,* 2000). In contrast to the situation in adults, HIV infection does not appear to play

a major role in susceptibility to NTS bacteremia in African children (Lepage *et al.,* 1987; Green and Cheesbrough, 1993). However, there is a suggestion that relapse of NTS bacteremia after completion of appropriate antimicrobial chemotherapy is more common in HIV-infected children than non-HIV infected children (Graham *et al.,* 2000).

Of interest, despite the prominence of bacteremia infection with non-typhoidal *Salmonella* species in HIV-infected patients, there are few series that describe such infection with *S. typhi* or *S. paratyphi*, which are endemic in some areas (Hohmann, 2001). This could be related to (1) the fact that there are many different serotypes of nontyphoidal *Salmonella* species, and immunity is at least partially serotype specific; and (2) the fact that nontyphoidal *Salmonella* more vigorously and durably colonize the intestinal tract than do typhoidal strains, thereby providing more prolonged opportunity for dissemination. Dissemination might also be enhanced by intestinal inflammation resulting from chronic diarrheal disease, parasitic infection, or sub-optimal nutrition.

TREATMENT OF SALMONELLOSIS

Salmonella infections may manifest as self-limiting gastroenteritis in which case antibiotic treatment is not recommended. This is because antibiotic treatment does not reduce the duration or severity of the gastroenteritis and instead may result in prolonged fecal excretion and emergence of resistant strains (Aserkoff and Bennet, 1969). On the other hand, antibiotic treatment is important for invasive salmonellosis including bacteremia, enteric fever and metastatic infection (Guerrant, 2001).

Ampicillin, chloramphenicol and cotrimoxazole used to be the traditional antibiotics for treatment. But over the years, *Salmonella* has evolved multi-resistance traits to these antibiotics thus grossly eroding their efficacy. The high rates of multi-resistance have led to the use fluoroquinolones, particularly ciprofloxacin, in adults, as the drugs of first choice for treatment of invasive salmonellosis. The extended-cephalosporins are used in children because fluoroquinolones are contra-indicated because of toxicity (Parry and Threlfall, 2008).

The carbapenems have also been used as alternatives in the treatment of salmonellosis (Koc *et al.,* 1995; Ko *et al.,* 2005). However, the clinical data on the use of carbapenems against invasive *Salmonella* infection is limited. Imipenems therapy has cured an infant with *salmonella* meningitis, who had relapse after 1month of cefotaxime therapy (Koc *et al.,* 1995); a patient who had spinal osteomyelitis that relapsed after 7weeks of cefepime therapy (Ko *et al.,* 2005) and a patient infected with *Salmonella choleraesuis* strain resistant to all antibiotics commonly used in the treatment of salmonellosis (including ciprofloxacin and ceftriaxone) (Chiu *et al.,* 2004).

ANTIBIOTIC RESISTANCE IN *SALMONELLA*

Antibiotic treatment of salmonellosis is now a global public health problem due to wide spread resistance especially in developing countries, where the lack of economic resources does not allow a wide antibacterial armamentarium. Moreover, in some of these areas, both the social situation and the presence of other basal illness, such as malaria, favour the acquisition of systemic *Salmonella* infections (Graham *et al.,* 2000).

RESISTANCE TO FLOUROQUINOLONES IN NONTYPHOIDAL *SALMONELLA*

Because of the increased resistance to conventional antibiotics, extended-spectrum cephalosporins and fluoroquinolones have become the drugs of choice for the treatment of infections caused by multidrug-resistant *Salmonella* serotypes. Resistance to quinolones (e.g., nalidixic acid) and their derivatives, such such as fluoroquinolones (e.g., ciprofloxacin) is increasingly reported. However, the reports of *Salmonella* strains showing high-level resistance to fluoroquinolones are few compared to *E. coli* and several other enterobacteriaceae (Heisig *et al.,* 1995; Herikstad *et al.,* 1997; Piddock *et al.,* 2002; Nakaya *et al.,* 2003; Casin *et al.,* 2003).

In the United States, a national survey conducted in 1994-1995 found that, of the 4008 *Salmonella* isolates tested, 21(0.5%) were resistant to nalidixic acid and 1(0.02%) was resistant to ciprofloxacin (MIC>4ug/mL); by 2000, the rate of nalidixic acid resistance had increased to 5-fold, to 2.5% for ciprofloxacin (Crump *et al.,* 2003). Ciprofloxacin resistance has been exceptionally high in *S. enterica* serotype choleraesuis strains in Taiwan since 2001 (>60%) which is mainly due to clonal spread of resistant strains (Chiu *et al.,* 2002; 2004; Chiu *et al.,* 2004). A prevalence rate of 84.4% is recently reported (Yan *et al.,* 2005).

There is also public health concern for strains of *Salmonella*, though not resistant (MIC>4.0ug/mL), but showing reduced susceptibility (>0.125ug/mL) to fluoroquinolones, according to NCCLS standard. The clinical importance of such strains is that treatment with fluoroquinolones results in treatment failures (Brown *et al.,* 1996; Ouabedesselman *et al.,* 1996; Launay *et al.,* 1997); such strains could also mutate to high-level resistant strains. The reduced susceptibility to flouroquinolones in *Salmonella* is usually linked with nalidixic acid resistance, a nonfluoroninated narrow-spectrum quinolone (Piddock *et al.,* 1998). Therefore, resistance to

nalidixic acid has been proposed for screening for reduced susceptibility to fluoroquinolones in *Salmonella* (Launay *et al.,* 1997; Hakanen *et al.,* 1999).

Table 1: Global status of antimicrobial resistance in nontyphoid *Salmonella* isolates recovered from humans in the past decade

	Year(s) of study	*Salmonella enterica* serotype	No. of isolates	Resistance %					
				Any antibiotics	AM	C	SXT	CIP	CRO/CTX
Country									
United States	1989-1990	Unspecified	758	31	13	4	1	0	0
Greece	1993	*S. Enteritidis*	58	66	34	3	7	0	0
Thailand	1995	Unspecified	349	NA	25	24	40	0.3	NA
Turkey	1994-1996	Unspecified	259	20	19	17	8	0	5
United Kingdom	1996	*S. Typhimurium*	5849	90	80	75	86	12	NA
		S. Enteritidis	18,968	8	5	<1	1	<1	NA
		S. Hadar	633	94	59	<1	10	60	NA
United States	1997	*S. Typhimurium*	249	NA	66	47	2	NA	NA
France	1997	*S. Typhimurium*	992	NA	73	56	9	NA	NA
		S. Enteritidis	800	NA	7	4	3	NA	NA
		S. Hadar	141	NA	72	0	8	NA	NA
Italy	1990-1996	*S. Enteritidis*	1624	2.3	0.8	0.2	0.8	NA	0.4
Spain	2001	Unspecified	1051	73	45	26	14	0.6	0.2
		S. Typhimurium	284	NA	80	73	19	1	1
		S. Enteritidis	385	NA	23	0	1	0	0
		S. Hadar	101	NA	70	1	5	1	1
Taiwan	2003	Unspecified	675	69	44	49	31	8	1.5
		S. Choleraesuis	67	98	91	91	88	69	1.5

Source: Su *et al.,* (2004)

It is suggested that laboratories should screen for these strains (Hakanen *et al.,* 2001), using nalidixic acid screening test (Hakanen *et al.,* 1999) or the E-test. Change in breakpoints is been argued to accommodate these clinical important strains (Aarestrup *et al.,* 2003).

Increasing isolation of *Salmonella* strains showing reduced susceptibility to fluoroquinolones is now been reported in some parts of the world. For example, in England and Wales, the number of isolates of common serovars (Enteritidis, Typhimurium, Virchow and Hadar) showing reduced susceptibility to ciprofloxacin (MIC>1ug/ML) has increased (PHLS,2002). In 2000, 52% of the *S. enterica* serovar Virchow isolates tested exhibited decreased susceptibility to ciprofloxacin. From 1995 to 1999, the annual proportion of reduced ciprofloxacin susceptibility (MIC 0.125ug/mL) among all Finnish traveler's isolates increased from 3.9% to 23.5% with outstanding increase among the isolates from the Finnish returning from South east Asia; isolates from Thailand alone increased from 5.6% to 50.0% (Hakanen *et al.,* 2001). Reduced susceptibility has also been reported in *Salmonella* isolates from the African Continent (Hakanen *et al.,* 2001; Kariuki *et al.,* 2005).

In the United Kingdom, reduced ciprofloxacin susceptibility (0.25ug/mL) in *Salmonella* isolates increased from 0.3% in 1991 to 2.1% in 1994 and to 5% in 1996 (Threlfall *et al.,* 1997).

Reports suggested that high level fluoroquinolone resistance was re-emerging in *S.* Typhimurium, *S.* Cholereasuis and *S.* Schwarzengrums in different parts of the world (Olsen *et al.,* 2001; Casin *et al.,* 2003; Nakaya *et al.,* 2003). The reports of fuoroquinolone resistance in several serotypes of *Salmonella* suggests polyclonal dissemination; this is further confirmed by the presence of diverse mutations in the target genes: two different double mutations in gyrA (Olsen *et al.,* 2001), one single mutation in gyrB (Casin *et al.,* 2003), three different single

mutations in ParC (Nakaya *et al.,* 2003) and for the first time one single mutation in ParE (Ling *et al.,* 2003).

ANTIBIOTIC RESISTANCE IN *S.* TYPHIMURIUM

While resistance has been reported in almost all *Salmonella* serotypes, it is of special interest in *Salmonella enterica* serotype Typhimurium (Helms *et al.,* 2005) because of its leading role as a human and animal pathogen. *S.* Typhimurium is of interest as a vector of antibiotic resistance genes and, as such, is a likely indicator of therapeutic and nontherapeutic antibiotic use in the environment in which it grows (Levy *et al.,* 2004).

These aspects combined justify systematic, large scale, and detailed antibiotic resistance surveys of this serovar to monitor trends in the spread of established resistance traits and the appearance of new traits. Of particular concern, is the spread of multiresistant epidemic strains of *S.* Typhimurium of definitive phage type (DT) 104, with chromosomal integration of the genes coding for resistance to ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines (Threlfall *et al.,* 1994).

S. Typhimurium DT104 was first isolated in the early 1980s in the United Kingdom and later became endemic in bovine animals, from where it spread to the whole animal production in the country (Threlfall *et al.,* 1993; Threlfall *et al.,* 2000). Throughout the 1990s, it spread to other parts of the world, and it is now a common *Salmonella* type in many countries, including the United States, the United Kingdom, Germany, and France (Ward *et al.,* 1990; White, 1998; Glynn *et al.,* 1998; Tauxe, 1999; Rabsch *et al.,* 2001). Muti-drug resistance DT 104 now remains

the most common multidrug resistance *Salmonella* in the United Kingdom and many other European countries (Threlfall *et al.,* 2003).

Globally, the incidence of MDR *S.* Typhimurium and DT 104 strains is on continuous increase, particularly in Europe and North America. The increase appears to have peaked in the United Kingdom but not in other countries (Helms *et al.,* 2005). In England and Wales, a multiresistant *S.* Typhimurium DT104 was responsible for a major epidemic of human salmonellosis (Wall *et al.,* 1994) and was found in a wide variety of food animals (Threlfall *et al.,* 1993). There is some evidence now that this epidemic strain has spread efficiently through the food chain in the United States (Besser *et al.,* 1997), Canada (Khakhria *et al.,* 1997) and Europe (Kuhn and Tschape, 1996). There are estimates that up to 300,000 cases of infection with DT104 may occur annually in the USA (Glynn *et al.,* 1998).

DT 104 is also increasingly acquiring additional resistance traits to other clinical important antibiotics like nalidixic acid, fluoroquinolone and trimethoprim (Threlfall, 2000; Helms *et al.,* 2005) and has been associated with increasing outbreaks. This has eroded the effectiveness of antibiotics use in the treatment of salmonellosis.

ANTIBIOTIC RESISTANCE IN *S.* TYPHI

Resistance has over the years developed in *S.* Typhi. Chloramphenicol was the first-line drug from 1948 to the mid-1970s (Anderson and Smith, 1972). By mid-1970s, extensive outbreaks of typhoid fever in Mexico and India caused by epidemic strains resistant to chloramphenicol limited its use (Anderson and Smith, 1972) thus fostering the use of alternative antibiotics. By late 1980s and 1990s, there were outbreaks in the Indian subcontinent, the Arabian Gulf, the

Philippines, South Africa and South east Asia caused by strains resistant to the alternative drugs (ampicillin, trimethoprim) and chloramphenicol (Mirza *et al.,* 1996; Rowe *et al.,* 1997). By 1990, chloramphenicol resistance in UK isolates had increased to 20% (Rowe *et al.,* 1990) from 0.47% recorded from 1978 to 1985 (Rowe *et al.,* 1987), with most of the strains showing resistance to ampicillin and trimethoprim. The incidence then increased to 26% in 1999 (Threlfall *et al.,* 2001). During the period, multidrug resistant *S.* Typhi was not reported in Africa except in South Africa (Coovadia *et al.,* 1992), and Kenya (Kariuki *et al.,* 2001). Pansusceptible strains were reported from Papa New Guinea in addition to Chile and Spain (Thong *et al.,* 1996a,b; Rodriguez *et al.,* 1998). Currently, multiresitant strains are problematic in Nigeria and some other parts of Africa (Akinyemi *et al.,* 2005).

Because of the wide spread resistance to the old drugs, fluoroquinolone and third generation cephalosporin, have become the drug of choice. But *S.* Typhi is becoming increasingly resistant to fluoroquinolones in Uk (Threlfall and Ward, 2001), Vietnam (Parry *et al.,* 1998), Tajikistan (Murdoch *et al.,* 1998), India (Rodrigues *et al.,* 1998; Chaudhry *et al.,* 1998) and Africa (kariuki *et al.,* 2001). Such strains are usually multiply resistant to other antibiotics (Threlfall and Ward, 2001; Chandel and Chandhry, 2001) and are associated with treatment failures (Threlfall and Ward, 2001), making treatment very difficult.

CLINICAL IMPORTANCE OF ANTIBIOTIC RESISTANCE IN *SALMONELLA*

There is accumulating evidence that infection with antimicrobial-resistant salmonellae may be associated with some serious health consequence*s.* Increased hospitalization has been reported in persons infected with resistant salmonellae compared to susceptible strains in the United States (Varma *et al.,* 2005a). Also in the United States, resistant *Salmonella*e have been associated with

more bloodstream infections compared to the pansusceptible strains (Varma *et al.,* 2005b). Furthermore, studies from Canada and Denmark have indicated higher death rates with resistant *Salmonella* infection (Helms et al., 2002; Martin *et al.,* 2004). However, in England and Wales, no association is found between resistant salmonellae and bloodstream infection (Threlfall *et al.,* 1998).But the England and Wales study was criticized for some serious limitations including the failure to use pansuscepible salmonellae as a reference group and the failure to adjust for confounders, such as age (Varma *et al.,* 2005a).

Antibiotic résistance in salmonellae has further complicated and increased the burden of laboratory diagnosis. There is the need to detect ESBL-producing and fluoroquinolones resistant strains (Su *et al.,* 2003, 2004; Crump *et al.,* 2003). This has led to increased cost on the part of hospitals and patients; and delay in administration of appropriate antibiotics which could lead to further increase in morbidity and mortality (Helms *et al.,* 2002). This could compromise empirical treatment with greater danger for the developing countries where economic factors militate against extensive laboratory diagnosis.

There is also increased vulnerability to *Salmonella* infections in individuals receiving antibiotic treatment for unrelated conditions due to "selective effect" of antimicrobial resistance (Barza and Travers, 2002). It was estimated that, in the United States, antimicrobial resistance in non-typhoidal *Salmonella* serotypes has resulted in extra 29,379 infections annually, causing an additional 342 hospitalisations (or 8,677 days of hospitalization) and 12 deaths (Barza and Travers, 2002).

In view of the increasing rate of antimicrobial resistance among common pathogens, including salmonellae, broad-spectrum agents are now more likely to be used for empirical therapy. For

example, imipenem or cefepime might be used more frequently in a clinical setting where isolates that produced extended-spectrum cephalosporinases were frequently encountered (Su *et al.,* 2004). These antibiotics are usually more expensive and toxic, and more harmful to the commensal microfloral; which further lead to socioeconomic loses (Howard *et al.,* 2003). Even the effectiveness of these other drugs is being gradually eroded with the reports of *Salmonella* strains resistant to imipenem (Miriagou *et al.,* 2003).

RESISTANCE OF *SALMONELLA* TO β-LACTAMS

Ampicillin (a β-lactam) used to be the drug of first choice for treatment of salmonellosis but high rate of resistance to ampicillin coupled with multidrug resistance to the other traditional old drugs (chloramphenicol and cotrimoxazole) has led to the use of extended-spectrum cephalosporins (newer β-lactams) as the drugs of first choice for treatment (Parry and Threlfall, 2008). Multidrug resistance of greater than 90% has been reported for ampicillin, chloramphenicol and cotrimoxazole in Malawi (Gordon *et al.,* 2008). Multidrug resistance rate of 34.2% has been reported from Kenya (Kariuki *et al.,* 2006. In Senegal, resistance rate could be 10% or less (Sire *et al*., 2008). A high resistance rate (87%) was reported from Lagos, Nigeria (Akinyemi *et al.,* 2007). The multidrug resistance range from 7 to 65% in Asia (Chau *et al.,* 2007; Ochai *et al.,* 2008).

Enternet, the international surveillance network for human gastrointestinal infections, reported resistance rate of 20% to ampicillin during the period 2000 to 2004 (Meakins *et al.,* 2008); multidrug resistance rate was 15%. Various resistance rates have been reported from other regions in the world. In the Gulf states of Kuwait and the United Arab Emirates, various

resistance rates have been reported which included 17.1% to 26.5% (ampicillin), 8.9% to 26.1% (cotrimoxazole), 5.6 to 5.7% (chloramphenicol) (Rotimi *et al.,* 2008).

Salmonella is also becoming increasingly resistant to the extended-spectrum cephalosporins (ESCs). *Salmonella* resistant to the ESCs emerged worldwide since 1988. During the period 1999-2004, the number of publications reporting *Salmonella* resistant to the ESCs increased dramatically. Between 1999 and 2001 the number of new cases increased threefold and the number of countries involved has increased steadily (Arlet et al., 2006). In 2004*, Salmonella* resistant to ESCs were identified in 43 countries including Europe, Americas, Africa and Asia.

The prevalence of *Salmonella* resistant to ESCs ranged from 0 to 3.4% between continents (Winokur *et al*., 2001) and increased progressively from year to year from 0.1% in 1996 to 1.9% in 1999 in the USA (Dunne *et al*., 2000) and from 0.4% in 1999 to 1.5% in 2003 in Taiwan (Li *et al.,* 2005; Su *et al.,* 2005). Resistance to ESCs has been reported in diverse serovars of *Salmonella* worldwide (Arlet *et al.,* 2006).

Molecular basis of antibiotic resistance in *Salmonella*

Generally, antibiotic resistance in bacteria can result from decreased antibiotic accumulation, physical modification or destruction of the antibiotics, alteration of the enzyme target of antibiotic action and active efflux of antibiotics by multidrug efflux pumps (Lacroix *et al.,* 1996; Poole, 2001; Soto *et al.,* 2003). The underlying molecular basis include mutation (influencing the target or efflux of antimicrobial agent) or the acquisition of resistance genes via horizontal gene transfer (encoding antimicrobial or targ*et al*teration, or alternate pathways (Davies, 1994; Chadwick and Goode, 1997). Horizontal transfer of resistance genes can occur through plasmids, transposons, integrons, bacteriophage and transformation (Hawkey, 1998; Grubb, 1998).

Resistance can also result from mutation events in motifs for gene expression, such as promoters (Chen and Clowes, 1987), in regulatory modules, such as 2-component regulatory systems (Baptista *et al.,* 1997), or positioning upstream from a gene of a mobile (Goussard *et al.,* 1991) or stable (Magnet *et al.,* 1999) promoter. Enhanced expression of genetic information can also be caused by alterations in translation attenuation (Leclercg and Couvalin, 2002).

HISTORY OF QUINOLONES/FLUOROQUINOLONES AND RESISTANCE MECHANISMS

Quinolones were introduced into clinical use in 1962 in the form of nalidixic acid (Ball, 2000), a fully synthetic agent with bactericidal effects on most Enterobacteriaceae at clinical concentrations (Robicsek *et al.,* 2006). Addition of a fluorine at the C-6 position and piperazinyl or related ring at position C-7 of the quinolone molecule yielded the fluoroquinolones, first available clinically in the 1980s (Paton *et al.,* 1988). The fluoroquinolones have the advantages of achieving higher serum levels than the nalidixic acid and higher potency against Enterobacteriaceae; drug concentrations of 1000-fold higher than those required to inhibit growth are routinely achieved (Robicsek *et al.,* 2006).

In the over 20 years, that the fluoroquinolones have been in the clinical use, resistance to these agents by Enterobacteriaceae has become common and widespread, and, interestingly, is generally not clonal. Resistance mechanisms against quinolones and fluoroquinolones include: accumulation of mutations in the drug targets (DNA gyrase and DNA topoisomerase IV), decreasing intracellular drug accumulation by upregulation of native efflux pumps (Poole, 2005) either alone or together with decreased expression of outer membrane porins (Ruiz, 2003) and plasmid mediated determinants (Strahilevitz *et al.,* 2009).

Mutation in the drug targets: This is the most important resistance mechanisms in Enterobacteriaceae. The drug targets include DNA gyrase and topoisomerase IV. When bound to DNA, these enzymes transiently break the closed circular DNA molecule, pass another strand through the break and then reseal the DNA. This process effects changes in DNA topology that are essential in DNA replication, transcription, recombination, and repair. Quinolones bind to these enzymes and stabilize a drug-enzyme-cleaved DNA complex, allowing lethal double-stranded DNA breaks to accumulate unrepaired (Froelich-Ammon and Osheroff, 1995). Each of the target enzymes has a quinolone-resistance determining region (QRDR), a portion of the DNA-binding surface of the enzyme (Morais *et al.,* 1997) at which amino acid substitutions can diminish quinolone binding. Generally, multiple of such mutations are required to achieve clinically important resistance in Enterobacteriaceae; when such organisms are quinolone resistant they nearly always found to have one or more QRDR mutations.

Plasmid mediated quinolone resistance: This is relatively new resistance mechanism which includes qnr proteins, aac (6')-Ib-cr acetylase, and qepA which mediate low level resistance to the fluoroquinolones (Robicsek *et al.,* 2006). The qnr proteins belong to the pentapeptide family and are produced by plasmid-encoded *qnr* genes (*qnrA, qnrB, qnrD and qnrS*). The encoded proteins protect DNA gyrases and topoisomerase iv from quinolone attack by binding to these enzymes thus minimizing formation of lethal gyrase-DNA complex (Robicsek *et al.,* 2006). The *qnr* genes usually coexist on the same plasmid with genes encoding resistance to the latest cephalosporins. The plasmid encoding the *qnr* genes vary widely in size and associated resistances but almost all carry multiple resistant determinants.

The *qnrA* and sometimes *qnrB* are found as part of complex Sul1-type integrons containing presumed recombinase, orf513. On the other hand, qnrS has not been associated with integron, but in one plasmid it was found bracketed by inserted repeats with insertion sequence-like structure that could have been responsible for its mobilization (Gay *et al.,* 2006). Variants of *qnrA* (*qnrA3*), *qnrB* (*qnrB5*) and *qnrS* (*qnrS1 and qnrS2*) have been found in *Salmonella* (Cheung *et al.,* 2005; Gay *et al.,* 2006; Robicsek *et al.,* 2006).

For most fluoroquinolones, the presence of a qnr plasmid increased their MIC by between about 16-fold and 125-fold. Nalidixic acid is relatively stable against the qnr (two-fold to eight-fold MIC increase in MIC) (Wang *et al.,* 2004). Increasing the MIC of fluoroquinolones by qnr plasmids, also lead to increase in mutant prevention concentration (MPC) thus enhancing the number of resistant mutants that can be selected from a population. Qnr genes have also been shown to be able to complement other resistance mechanisms in bacterial cell additively (Rebicsek *et al.,* 2006).

Plasmid mediated resistance determinant *aac(6')-Ib-cr* gene was discovered in 2004 (Robicsek *et al.,* 2006). This gene is a variant of *aac(6')-Ib* which differed from the earlier variants by two codon changes which confer ciprofloxacin resistance. The encoded protein N-acetylates ciprofloxacin and norfloxacin leading to some loss of antimicrobial activity of the fluoroquinolne*s.* The gene also confers resistance to tobramycin, amikacin and kanamycin. It has been associated with modest increase (three-fold to four-fold) in MIC of ciprofloxacin and norfloxacin but causing marked increase in MP.

Molecular basis of resistance to β-lactams

The β-lactam antibiotics include penicillins, cephalosporins, carbapenems and monbactam and are characterized by a core β-lactam ring. Enzymatic hydrolysis of the β-lactam ring leads to lose of potency. This group of antibiotics represents 60% of all antimicrobial use by weight in medicine. Heavy use of these antibiotics have selected for resistance. Beta-lactamases are the major resistance determinants of Gram negative bacteria against this strategic group of antibiotics. Since β-lactams were introduced into clinical practice, β-lactamases have coevolved with them (Medeiros, 1997). The spread of β-lactamases has actually driven β-lactam development for roughly 60years.

The first analoque, benzylpenicillin, penetrated Gram negative bacteria poorly and was destroyed by penicillinases, which spread rapidly in *Staphylococcus aureus.* These problems were overcome in the early 1960s with the development of semi-synthetic penicillins (e.g. ampicillin and carbenicillins) that could penetrate gram negative bacteria, and those that were stable to staphylococcal penicillinases (methicillin and oxacillins). The anti-gram-negative analogues were compromised, in turn, by the spread of plasmid-mediated penicillinases (notably TEM-1) among Enterobacteriaceae. From the 1970s, this drove the development of (a) second - , third- and fourth- generation oxyimino-cephalosporins (e.g. cefuroxime, cefotaxime, ceftriaxone, ceftazidime and cefepime); (b) of β-lactamase inhibitors such as clavulanic acid (Livermore and Woodford, 2006).

In the following years, oxyimino-cephalosporins became workhorse antibiotics worldwide. Once again, clinical use selected for resistance and this cephalosporin resistance, along with

dramatically rising enterobacterial resistance to flouroquinolone (Livermore, 2005), is now propelling the use of carbepenems.

Classification of β-lactamases

Hundreds of β-lactamases have been described with intimidating variety of names. However, these enzymes can be classified on the basis of their primary structure into four molecular classes (A through D) (Bush *et al.,* 1995), or on the basis of their substrate spectrum and responses to inhibitors into a larger number of functional groups (Ambler, 1980). Class A and class C β-lactamases are the most common and have a serine residue at the active site, as do class D β-lactamases. Class B comprises the metallo-β-lactamases. The class A enzymes include the TEMs and SHVs; while the class D include the OXAs. Table 3 shows some examples of these enzymes.

Table 3: Selected β-lactamases of Gram-negative bacteria

Table 1. Selected β-Lactamases of Gram-Negative Bacteria.

β-Lactamase	Examples	Substrates	Inhibition by Clavulanic Acid*	Molecular Class
Broad-spectrum	TEM-1, TEM-2, SHV-1	Benzylpenicillin (penicillin G), aminopenicillins (amoxicillin and ampicillin), carboxypenicillins (carbenicillin and ticarcillin), ureidopenicillin (piperacillin), narrow-spectrum cephalosporins (cefazolin, cephalothin, cefamandole, cefuroxime, and others)	+++	A
	OXA family	Substrates of the broad-spectrum group plus cloxacillin, methicillin, and oxacillin	+	D
Expanded-spectrum	TEM family and SHV family	Substrates of the broad-spectrum group plus oxyimino-cephalosporins (cefotaxime, cefpodoxime, ceftazidime, and ceftriaxone) and monobactam (aztreonam)	++++	A
	Others (BES-1, GES/IBC family, PER-1, PER-2, SFO-1, TLA-1, VEB-1, and VEB-2)	Same as for TEM family and SHV family	++++	A
	CTX-M family	Substrates of the expanded-spectrum group plus, for some enzymes, cefepime	++++	A
	OXA family	Same as for CTX-M family	+	D
AmpC	ACC-1, ACT-1, CFE-1, CMY family, DHA-1, DHA-2, FOX family, LAT family, MIR-1, MOX-1, and MOX-2	Substrates of expanded-spectrum group plus cephamycins (cefotetan, cefoxitin, and others)	0	C
Carbapenemase	IMP family, VIM family, GIM-1, and SPM-1	Substrates of the expanded-spectrum group plus cephamycins and carbapenems (ertapenem, imipenem, and meropenem)	0	B
	KPC-1, KPC-2, and KPC-3	Same as for IMP family, VIM family, GIM-1, and SPM-1	+++	A
	OXA-23, OXA-24, OXA-25, OXA-26, OXA-27, OXA-40, and OXA-48	Same as for IMP family, VIM family, GIM-1, and SPM-1	+	D

* Plus signs denote relative sensitivity to inhibition.

Source: Jacoby and Munoz-Price, 2005

Extended-spectrum β-lactamases (ESBLs)

The ESBLs have the ability to hydrolyse the penicillins and the oxyimino-cephalosporins. These include the following:

1. TEM-type ESBLs: Amino acid substitutions at many sites in TEM-1 β-lactamases can be created in the laboratory without loss of activity (Joris *et al.,* 1988).Those

responsible for the ESBL phenotype change the configuration of the active site of the enzyme, making it accessible to oxyimino-β-lactams (Nukaga et at., 2003). More than 130 TEM enzymes are currently known (Jacoby and Munoz-Price, 2005).

2. SHV-type ESBLs: Just like TEM, SHV-type ESBLs have one or more amino acid substitutions around the active site. More than 50 varieties of SHV are currently known based on unique combinations of amino acid replacements (Jacob and Bush, 2005).

3. CTX-M-type ESBLs: This is the most common group of ESBL*s*. More than 40 CTX-M enzymes are currently known (Bonnet, 2004).

4. Other Class A ESBLs: These include PER, VEB, GES, IBC which are very uncommon.

5. OXA-type ESBLs (Class D): At least 12 esbls derived from OXA-10, OXA-1, or OXA-2 by amino acid substitutions are currently known (Jacoby and Bush 2005).

Plasmid-mediated AmpC enzymes

AmpC β-lactamases, usually inducible by β-lactams, are encoded by chromosomal genes in many gram-negative bacilli e.g. *Enterobacter.* The AmpC enzyme in E. coli is poorly expressed and AmpC gene is missing from the chromosome of klebsiella and *Salmonella* spp., but can acquire plasmid mediated AmpC gene*s.* There are at least 20 different plasmid-mediated AmpC (Philippon *et al.,* 2002).

Carbapenemases

Carbapenemases are a diverse group of enzymes. Though currently uncommon, they are great source of worries because they inactivate carbepenems in addition to the the oxyimino-cephalosporins and cephamycins (Nordmann and Poirel, 2002).They include IMP, VIM, and KPC.

MOLECULAR BASIS OF RESISTANCE TO B-LACTAMS IN *SALMONELLA*

Resistance to ampicillin is mainly due to the production of broad-spectrum β-lactamases predominantly the TEMs (Olsen *et al.,* 2002). On the other hand, resistance to extended-spectrum cephalosporins (ESCs) (such as cefotaxime, ceftriaxone, and ceftazidime) is mainly due to the production of extended-specrum cephalosporinases (Su *et al.,* 2004; Arlet *et al.,* 2006). These include the extended-spectrum β-lactamases (ESBLs) and plasmid-mediated AmpC.

Among the Ambler class A ESBLs, the TEM or SHV-types, along with the CTX-M types, are the most widespread enzymes (Miriagou *et al.,* 2004). These enzymes typically confer resistance to penincillins, ESCs, and aztreonam but not to cefoxitin and the carbapenems. Of the five groups of CTX-M enzymes, only the CTX-M-1, CTX-M-2 and CTX-M-9 have been widely reported in *Salmonella* (Table 3). Among the plasmid mediated Ambler class C enzymes, the CMY-type enzymes are the most prevalent but DHA- and ACC-types have also been reported (Philippon *et al.,* 2002).

Ambler class A carbapenemase (KPC-type) has also been reported in *Salmonella* (Miriagou *et al.,* 2003). The KPC-type enzyme confers resistance to all β-lactams. The CMY-2 has been

reported in many countries widely distributed in four continents including Europe, Americas, Africa and Asia. The TEM- and SHV-types as well as the CTX-M-1 group are also widely distributed (Arlet *et al.,* 2006). In contrast, some enzymes seem limited to specific continents, such as CTX-M-14 in Asia (Li *et al.,* 2005; Cheung *et al.,* 2005) or TEM-52 in Europe (Weill *et al.,* 2004; Politi *et al.,* 2005), and certain enzymes seem specific to individual countries such as CTX-M-2 and PER-2 to Argentina (Rossi *et al.,* 1995), PER-1 to Turkey (vahaboglu *et al.,* 1997), or TEM-63 and TEM-131 to South Africa (krugger *et al.,* 2004).

The ESCs has been reported in at least from 53 serotypes but predominantly reported in *Salmonella* Typhimurium and *Salmonella* Enteritidis, which are the most prevalent serotypes in many countrie*s.* At least 18 and 11 different enzymes have been reported in *S.* Typhimurium and *S.* Enteritidis respectively. Some enzymes are widely distributed in several serotype*s.* For example, CMY-2 has been described in 32 serotypes, suggesting the widespread dissemination of plasmids or mobile genetic elements in the environment (Arlet *et al.,* 2006).

Several ESBLs and AmpCs have been reported in *Salmonella* from some African countries such as Algeria, Gambia, Libya, Mali, Morocco, Senegal, South Africa, Tanzania, Egypt and Tunisia (Arlet *et al.,* 2006; Usha *et al.,* 2008; Bouchrif *et al.,* 2009). The reported ESBLs in Africa include SHV-12, TEM-63, TEM-116, TEM-131, SHV-2, CTX-M-3, CTX-M-14, CTX-M-15 and CTX-37 (Arlet *et al.,* 2006; Bouchrif *et al.,* 2009). The AmpC reported from Africa include CMY-2, CMY-4 and ACC-1 (Arlet *et al.,* 2006; Bouchrif *et al.,* 2009; AbdelGhani *et al.,* 2010).

Epidemiological typing of *Salmonella*

The clonal relatedness of isolates is manifested by their display of a significantly higher level of similarity in their genotype and/or phenotype than can be expected for randomly occurring and epidemiologically unrelated isolates of the same species. The interest in clones has increased over the past decades, due to the emergence of multiresistant or highly virulent clones of pathogenic bacteria that have become widespread and seem to remain stable for prolonged periods (Selander *et al.,* 1987; Achtman and Pluschke, 1986; Monzo-Moreno *et al.,* 1991; Pitt *et al.,* 1989; Maynard Smith *et al.,* 1993; Dijkshoorn *et al.,* 1993; Van Embden *et al.,* 1993; Van Dessel *et al.,* 2002; Van Dessel *et al.,* 2004). Ørskov and Ørskov (1983) proposed the following formulation: 'The word clone will be used to denote bacterial cultures isolated independently from different sources, in different locations, and perhaps at different times, but still showing so many identical phenotypic and genotypic traits that the most likely explanation of this identity is a common origin.

Pathogenic bacteria are found in various ecological niches called reservoirs. Reservoirs include humans, animals and other ecological niches in the environment. Transmission of bacteria from any of these sources may lead to clusters of colonisation or infection among humans, which are mostly recognized as infectious disease outbreaks. When these outbreaks are not controlled, major epidemics (due to unrestricted further transmission) may arise. Bacterial epidemiological typing generates isolate-specific genotypic or phenotypic characters that can be used to elucidate the sources and routes of spread of bacteria (Miller, 1993; Jang *et al.,* 2001). Typing helps to monitor dissemination of infections from patients, animals or other sources to non-colonised and

uninfected individuals. Typing may also be used to identify emerging pathogenic strains or clones within a species.

Salmonella enterica is a foodborne pathogen of significant public health importance worldwide. This pathogen is a zoonotic agent associated with a variety of animals. Human salmonellosis is mostly linked to the consumption of contaminated foods such as poultry, beef, pork, eggs, milk, seafood, and fresh produce. Direct contact with infected animals has also been associated with salmonellosis (Tauxe, 1991; Benenson and Chin, 1995).Because most cases of salmonellosis are food related, knowledge of how *Salmonella* disseminates through the food chain is important in understanding how food animals and food processing procedures contribute to infection. The ability to characterize specific strains and determine the primary sources of *Salmonella* contamination provides valuable insights into the epidemiology and natural history of this pathogen and provides important tools to improve public health.

A variety of methods have been developed to generate isolate-specific fingerprints, for epidemiological typing. These methods facilitate the determination of the relatedness among isolates derived from outbreak situations or obvious and recent chains of transmission, in order to support or reject the hypothesis that the isolates come from a single source.

Typing data should always be considered within the time-frame and current epidemiological context that are being evaluated and from which bacterial isolates have been obtained. For example, more variability can be expected between related isolates when longer time periods are studied. Typing applies distinct labels to bacterial isolates. These labels facilitate identification of transmission routes and sources. However, they can also contribute to in-depth investigations of infectious disease pathogenesis, bacterial population structures and bacterial genetics.

Typing can be considered as either comparative or definitive (library) typing. In comparative typing, outbreak-related and unrelated isolates are compared, since comparison of outbreak related isolates with isolates from the past or the future is not relevant. This is sometimes considered sufficient for outbreak investigation (Pitt, 1994). However, in many outbreak settings, be they nosocomial or community-based, it is often useful to compare strains from a current outbreak with previous strains, in which case a definitive (library) typing method should be used. Therefore, it is important to set up and maintain collections of alert organisms in any typing laboratory. Library systems are those that can be used in different laboratories, by different investigators at various time intervals, with the aim of generating high-quality data to be aggregated in a single database for comparative assessment, in great detail at any time (Garaizar *et al.,* 2000). It is thus important that the typing methods are robust and sufficiently standardised to monitor the organisms of interest.

Typing can be undertaken at different levels, depending on the situation: locally, at a hospital or other primary laboratory, for small investigations; regionally or nationally, in a reference laboratory, to bear upon wider issues of public health and surveillance; or internationally through collaborative networks, to define or survey the worldwide dissemination of major bacterial clones. At each of these levels, different methods may be applied.

EPIDEMIOLOGICAL TYPING METHODS

Over the past two decades, a plethora of novel and often innovative typing methods has been developed. These range from methods that assess simple phenotypic traits to DNA sequencing. DNA molecules (or restriction fragments or amplified sections thereof) can be separated on the basis of their molecular size by gel electrophoresis. Such size comparisons assess differences in

the length of DNA fragments obtained from DNA from different bacterial strains. Whether the fragments of DNA are natural (e.g., plasmids) or generated at random, by restriction enzymes or after amplification of the DNA using enzymatic DNA replication (PCR), does not matter; size differences, provided that they are accurately determined, can be excellent markers of strain differences.

Phenotypic Typing methods

Phenotyping results in the grouping of organisms according to their similarity in characters resulting from the expression of their genotypes. These may include colony morphology, colour, odour and other macroscopic features. Others are ability of isolates to grow in the presence of specific substances (be they metabolites, drugs, bacterial toxins or bacteriophages) and their expression of specific molecules (be they surface antigens or allelic variants of housekeeping enzymes). All methods require strict standardization of experimental conditions, since phenotypes are generally quite susceptible to changes in environmental conditions.

Biotyping

Biotyping assesses biochemical characteristics that are known to vary within a given species. Typeability is usually excellent. The methods are usually technically easy and inexpensive, the data generated are simple to score and interpret, and all tests can be performed, even in the smallest of laboratories, on large numbers of isolates. If reproducibility is demonstrated, it can be used as a library typing method (Bouvet and Grimont, 1987; Kuhn *et al.,* 1991).

Antimicrobial susceptibility testing (antibiogram-based typing) can be performed either by drug diffusion in solid growth media or drug dilution in liquid media using a variety of

measurement systems. Antibiogram- based typing can, with appropriate selection of drugs, be applied to most species. Testing for resistance to heavy metals (resistotyping), as well as to disinfectants and antiseptics, can provide useful typing information. Susceptibility profiles expressed as diameters of inhibition zones combined with cluster analysis can provide useful typing data as an adjunct to data generated by other methods (Dijkshoorn *et al.,* 1996; Sloos *et al.,* 1998).

Serotyping is traditionally the most important phenotypic method that has been developed from the early days of microbiology. It has led to comprehensive systems for typing of, for example, *Salmonella* and *E. coli* isolates. Most typing sera react with surface antigens. High-throughput procedures using defined sets of polyclonal or monoclonal antibodies have been made available (Frasch *et al.*, 1994). Genetic instability per se, horizontal gene transfer and convergence due to natural or vaccine-driven herd immunity intrinsically limit the power of serotyping methods.

Other phenotypic methods include phage typing (Godovannyi *et al.,* 1974; Weller, 2000); SDS-PAGE of cellular and extracellular components (Aucken and Pitt *et al.,* 1993; Pantophlet *et al.,* 1998); Multilocus enzyme electrophoresis (MLEE) and mass spectrometry (Du *et al.,* 2002; Dieckmann *et al.,* 2005).

Genotypic typing methods for *Salmonella*

Pulsed-field gel electrophoresis (PFGE): DNA fragments are separated under conditions with alternating electric fields; DNA fragments up to 800 kb in size can be resolved (Schwartz and Cantor, 1984). When used in conjunction with rare cutting restriction enzymes, PFGE profiles provided a DNA ‘‘fingerprint’’ that reflect the DNA sequence of the entire bacterial genome

(Busch and Nitschko, 1999). PFGE is considered as the ‘‘gold standard’’ molecular typing method for bacteria (Olive and Bean, 1999). It is used by public health monitoring systems such as the CDC’s PulseNet program to track the spread of foodborne pathogens and assist in determining sources of *Salmonella* outbreaks (Swaminathan *et al.,* 2001).

A major advantage of PFGE typing is that the method is highly reproducible for the vast majority of strains allowing for data sharing among multiple laboratories. The reproducibility is an offshoot of less exposure of DNA to mechanical shearing effects of pipetting because the DNA is embedded in agarose (Birren and Lai, 1993). Furthermore, the fingerprint generated is reflective of the entire genome unlike PCR-based methods which amplify specified portion of the genome (Swaminathan and Barrett, 1995; Millemann *et al.,* 2000). Additional, PFGE results are comparable between laboratories if standardized protocol is followed ((Tenover *et al.,* 1995) such as the one proposed by by PulseNet (Swaminathan *et al.,* 2001). However, PFGE has a number of drawbacks.

PFGE is labour intensive, as results may not be available until several days. Additionally, genetic variation which does not affect electrophoretic mobility of a fragment is not captured in the generated fingerprint. The latter drawback may be overcome by use of more than one restriction enzyme (Joyner and Kincaid, 2006; Zhao *et al.,* 2006). On the whole, PFGE is an excellent typing method for *Salmonella* (Amavisit *et al.,* 2001; Folley *et al.,* 2007).

Other genotypic methods include RFLP-PCR (Dauga *et al.,* 1998; Kwon *et al.,* 2000); amplified fragment length polymorphisms (Lindstedt *et al.,* 2000; Scott *et al.,* 2001); infrequent-restriction-site PCR (IRS-PCR) IRS-PCR (Riffard *et al.,* 1998); random amplified polymorphic DNA PCR (RAPD-PCR) and arbitrarily primed PCR ((AP)-PCR) (Swaminathan and Barrett,

1995; Busch and Nitschko, 1999); repetitive element (Rep)-PCR (Versalovic *et al.,* 1991; Chadfield *et al.,* 2001); variable number tandem repeat analysis (Keim *et al.,* 2000; Lindstedt *et al.,* 2003, 2004, 2005); multilocus sequence typing (MLST) (Enright and Spratt, 1999; Kotetishvili *et al.,* 2002) and single nucleotide polymorphism analysis (Mortimer *et al.,* 2004; Cebula *et al.,* 2005).

CHAPTER THREE

Materials and Methods

Study site: This included seven different hospitals, a diagnostic laboratory and thirteen poultry farms in Ibadan. Two streams: one at Agbowo and the other at Ijokodo, within Ibadan metropolis were also included.

Study populations: These included humans (febrile patients and healthy persons) and food animals. The food animals included cattle, pigs, sheep, goat and chickens ready for slaughter for meat. The patients included febrile patients suspected of having bacteremia. The febrile patients were those clinically suspected of having bacteremia; these patients came from six different local government areas of Ibadan and were attending seven different hospitals denoted as A to G (hospital E is a tertiary hospital while the others are secondary). The clinical diagnosis of the patients together with their age and sex were extracted from the case notes. The healthy humans were those not having clinical signs of infections and they included pregnant women consulting for antenatal care. The pregnant women came from different local government areas of Ibadan.

Cattle, pigs, sheep and goat were sampled from Bodija abattoir. Chickens were sampled from thirteen different poultry farms in eight local government areas of Ibadan. The workers on the various poultry farms were interrogated for antibiotics used on the farms.

Justification for study populations: Although *Salmonella* can cause both bactetemia and gastroenteritis, bacteremia is an invasive disease that is usually very severe and requires the use of antibiotics for proper management. On the other hand, gastroenteritis is generally self-limiting and does not require the administration of antibiotics; moreover, patients with gastroenteritis

generally do not go to hospital. Therefore, only febrile patients suspected of having bacteremia were recruited into the study. Pregnant women were recruited as part of healthy humans because they are generally well and come from different parts of Ibadan coupled with the fact that they were easy to access.

Food animals are reservoirs of *Salmonella* and the infection can be transferred to humans through contact and consumption of contaminated meat and other animal products. Cattle, pigs, sheep, goat and chickens were chosen because they represent animals mostly eaten in our environment and thus represent potential sources of *Salmonella* infection to humans.

Sample Collection: The minimum study population was eighty-seven as estimated by the statistical formula;

$N=Z^2 P (1-P)/D^2$ - where: N is the required sample size

Z is the standard deviation for a confidence level of 95% (1.96)

P is 6%=0.06; the estimated prevalence of *Salmonella* in the study population

D is the desired level of precision or tolerable/allowed sampling error set at 5% (0.05) (Lemeshow *et al.,* 1990).

(A). **Febrile patients**

Venous blood (10-15mL from adults; and 2-5mL from children) was aseptically collected from the forearm of 984 febrile patients after swabbing with methylated spirit and cultured following standard procedure (Hoa *et al.,* 1998).

(B). **Healthy humans**

One fecal sample was collected into sterile universal bottle from each of the healthy humans including 960 pregnant women attending antenatal clinic.

(C). **Food animals**

Fecal samples were collected from chickens (n=1201), cattle (n=500), goat (n=500), sheep (n=500) and pig (n=500).

D. Water samples were collected from the streams using the Moore swab technique.

Ethical issues: The study was reviewed and approved by Institutional Review Boards of the Hospitals. The consent of the patients was obtained after the study was explained to them. Blood samples were collected by the clinicians (hospitals) and medical laboratory scientists (diagnostic laboratory).

Criteria for inclusion in the study:

1. Healthy people who did not present with diarrhea within seven days of fecal sample collection were considered asymptomatic
2. Febrile patients were defined as patients with body temperatures greater than 37°C.
3. Isolation of bacteria from a blood sample is defined as bacteremia.
4. Streams were located in thickly populated area with human defecation at the bank.
5. Animals: must be apparently healthy.

Sample processing

(A). All the blood samples collected from the febrile patients were investigated for different bacteria including *Salmonella*. Briefly, each 2-5mL of blood sample was inoculated into 50mL brain-heart infusion broth (Lab M, Lancashire, UK) and incubated at 37°C aerobically for maximum of seven days. The broth was sub-cultured after 24hrs, 48hrs, and 96hrs and terminally at seventh day onto fresh 10% sheep blood agar and MaCconkey agar plates (Lab M, Lancashire, UK); all plates were incubated aerobically at 37°C for 18 to 48hr*s*. Presumptive *Salmonella* colonies were identified by standard methods (Cowan and Steel, 1974; Cruickshank *et al.*, 1975). Other bacterial isolates were also identified.

(B). All the fecal samples collected from the asymptomatic humans were investigated only for *Salmonella*. Briefly, formed fecal sample was first emulsified in normal saline, after which 1g of fecal sample was inoculated into 10ml of selenite F broth (Lab M, Lancashire, UK) and incubated at 37°C aerobically overnight. Thereafter, the broth was sub-cultured onto dried plates of Salmonella-Shigella agar plates (Lab M, Lancashire, UK) and incubated at 37°C aerobically overnight. Presumptive *Salmonella* colonies were identified by standard methods (Cowan and Steel, 1974; Cruickshank, 1975).

(C). All the fecal samples collected from the food animals were investigated only for *Salmonella*.

(i) Chickens: fecal samples were emulsified in normal saline and 1g was inoculated into 10ml of sterile selenite F broth (Lab M, Lancashire, UK), incubated at 37°C aerobically for 24-48hr*s*. Thereafter, the broth was sub-cultured onto dried plates of Salmonella-Shigella agar plates

(Lab M, Lancashire, UK) and incubated at 37°C aerobically overnight. Presumptive *Salmonella* colonies were identified by standard methods (Cowan and Steel, 1974; Cruickshank, 1975).

(ii) Cattle, Pigs, Goats and Sheep: fecal samples were emulsified in normal saline and 1g was inoculated into 10ml of sterile selenite F broth (Lab M, Lancashire, UK), incubated at 37°C aerobically for 24-48hrs. Thereafter, the broth was sub-cultured onto dried plates of salmonella-shigella agar plates (Lab M, Lancashire, UK) and incubated at 37°C aerobically overnight. Presumptive *Salmonella* colonies were identified by standard methods (Cowan and Steel, 1974; Cruickshank, 1975).

(D). The streams were investigated for *Salmonella* using the Moore swab method. Each stream was screened at least three separate times at intervals of 1 - 2 weeks. Briefly, Moore swabs were prepared by wrapping sterile cotton gauze, 15cm by 120cm long, around one end of a sterile stiff wire. The other end of the wire was attached to a nylon cord. Three swabs were suspended into each stream (for three days) at different locations with the nylon cord attached to heavy stones at the bank to prevent the swabs from been washed away by the water current. After three days, each swab was removed and put in a sterile nylon bag and then transported on ice to the laboratory. In the laboratory, each swab was removed aseptically and placed directly into a wide-mouth jar containing 500ml of sterile selenite-F broth (Lab M, Lancashire, UK), incubated aerobically at 37°C/24 hrs and subcultured onto Salmonella-Shigella agar plates (Lab M, Lancashire, UK) and incubated at 37°C/24 hrs. Presumptive *Salmonella* colonies were identified by standard methods (Cowan and Steel, 1974; Cruickshank, 1975). The *Salmonella* isolates were hereafter referred to as environmental isolates.

Laboratory identification of bacterial isolates

Salmonella isolates were identified by Gram stain, cultural morphology, biochemical and serological tests.

(i) **Cultural:** The cultural properties of the bacterial isolates were studied on salmonella-shigella agar (SSA). Bacterial colonies that gave rabbit eye appearance on SSA were presumptively identified as *Salmonella* spp.

(ii) **Morphological:** Morphological properties were studied by gram staining. Presumptive *Salmonella* isolates were Gram negative rods.

(iii) **Biochemical Identification:** All presumptive *Salmonella* isolates were further identified by biochemical tests: urease, sugar fermentation, indole, citrate utilization, malonate utilization, potassium cyanide, methyl-red, voges-proskauer, hydrogen sulphide, lysine decarboxylase and motility tests.

(a) **Urease Test:** Urea agar (Lab M) slants were used. All the suspected *Salmonella* isolates were heavily smeared onto the slants, incubated at aerobically at $37^{o}C/7$ days. The slants were observed at each day of incubation for a colour change; a pink colour change indicated positive test while no colour change indicated negative test. Urease positive isolates were taken as non-salmonellae. The urease negative isolates were regarded as suspected *Salmonella* and were subjected to other biochemical tests.

(b) **Sugar Fermentation Test:** The ability of the suspected *salmonella* isolates to ferment various sugars was investigated. The sugars tested were glucose, lactose, sucrose, dulcitol, salicin and manitol. Peptone water (Lab M) containing each sugar (1% w/v), and Andrade's

indicator was used; Durham's tubes were inverted in the glucose peptone water to collect gas. The Durham's tubes were filled up with the broth. Each suspected *Salmonella* isolate was inoculated into the peptone water, incubated aerobically at 37°C/48hrs. After incubation, a colour change to pink indicated acid production from the sugar and the displacement of broth in the Durham's tubes indicated gas production.

(c) **Indole Test:** All the suspected *Salmonella* isolates were inoculated into peptone water, incubated aerobically at 37°C/48hrs. After incubation 4 - 5 drops of Kovac's reagent were added drop wise and observed for the appearance of a pink ring on top of the broth; the pink ring indicated positive test.

(d) **Citrate Test:** Simmon citrate agar slants (Lab M) were prepared in bijou bottles. Each test bacterial isolate was inoculated onto the slant by smearing, incubated at 37°C/96hrs. The inoculated slants were observed for a colour change each day of incubation. A blue colour change indicated positive test.

(e) Methyl Red-Voges Prokauer Test: Glucose-phosphate broth (Lab M) was used. The broth was prepared in 10ml amounts in bottles. Each test bacterial isolate was inoculated into the broth, incubated at 37°C/3 days. After incubation, the broth was divided into two equal portions: Methyl-red test - to one portion was added 5 drops of methyl-red. A red colour was considered a positive result; a yellow colour negative; pink or pale red was equivocal. To the other portion was added a trace of creatine and 5ml of 40% potassium hydroxide for Voges-proskauer test; the mixture was then shaken very well. A pink colour was taken as positive while no colour change was taken as negative.

(f) Malonate Utilization Test: Malonate broth medium was used. The broth was inoculated with young agar slope culture (24 hr old) of each of the suspected *Salmonella* isolates, incubated at 37^oC/48 hrs. After incubation the broth was observed for malonate utilization (blue colour change).

(g) Potassium Cyanide Test: Potassium cyanide broth was used. The broth was dispensed into bijou bottles in 3ml amounts. The broth was inoculated with 24 hrs nutrient broth culture of each suspected *Salmonella* isolate and incubated aerobically at 37^oC/24 - 48 hrs with the caps of the bijou bottles tightly screwed down to prevent air exchange. After incubation, the broth was observed for turbidity (growth of the inoculated isolate) indicating positive test.

(h) Motility Test: Semi-solid nutrient agar was prepared inside bijou bottles. Each suspected *Salmonella* isolate was inoculated into the medium by stabbing into the medium, not reaching the bottom of the medium, with an inoculating needle. The medium was incubated aerobically at 37^oC/24hrs. After incubation, the growth of the test organism into the medium indicated positive test; while restriction of growth of the test organism to the line of inoculation indicated negative test.

(i) Hydrogen Sulphide Test: Hydrogen sulphide test was carried out on sodium thiosulphate containing medium (Salmonella-Shigella agar). Each of the suspected *Salmonella* isolates was streaked onto the medium, incubated at 37^oC/24 hrs and observed for black colonies development. Black colonies indicated hydrogen sulphide production.

(j) Serological Test: Slide agglutination test was carried out on presumptive *Salmonella* strains using *Salmonella* polyvalent O and H antisera separately. A suspension of each strain was

prepared from the overnight slope culture in 0.5ml of sterile normal saline in a test tube. A drop of the suspension was placed on a glass slide and mixed with a loopful of one of the antisera and rocked for 30 - 60 seconds while viewing under a good light against a dark background with naked eyes. Distinct clumping within 60s indicated positive result. All positive strains were identified as *Salmonella*. Positive isolates were subjected to Serotyping according to White-Kauffmain-Le Minor Scheme.

Serotyping: Serotyping using the slide agglutination method was performed to determine the type of O and H antigens present in the isolates using antisera obtained from Statens Institute, Copenhagen.

(i) Testing for O-antigens

Briefly, the test isolate was streaked onto nutrient agar and incubated aerobically at 37°C for 18hr. A loopful of the test isolate was emulsified in a loopful of saline on a glass slide to make a thick suspension. Another suspension of the bacterial growth is mixed with a second drop of saline on the same slide to act as negative control. One drop of poly O antisera was mixed with the first suspension for a minute. The two drops on the glass slide were then compared for agglutination. The presence of agglutination in the two suspensions was taken as auto-agglutination meaning that the organism was untypeable. A positive finding is reported when agglutination occurs only in the suspension of isolate but not in the saline suspension. Any isolate that is positive with the poly-O antisera is then tested against all the specific antisera mixed in the poly-antisera to determine the O antigens. The antigens corresponding to the positive specific antisera were taken as the O antigens of the isolates.

(ii) Testing for H-antigens.

Phases 1 and 2 of H-antigen types were determined.

(a). **Phase 1.** This was determined by sub-culturing the isolate on swarm agar (Oxoid) and incubating overnight at 37°C. Next a slide agglutination test is done using a loopful of the test isolate taken from edge of bacterial growth on the swarm agar. The negative control was a suspension of test organism mixed only with normal saline. Poly-H antisera containing a mixture of antisera were first used and then all isolates positive to poly antisera were tested with the specific antisera contained in the poly antisera to determine the phase 1 of H-antigen. The antigens corresponding to the positive specific antisera were taken as the phase 1 antigens.

(b). **Phase 2.** This was determined by adding 10µl of the positive specific antisera in phase 1 into petri dishes together with 5ml of warm (55-60°C) swarm agar and allowed the agar to solidify. The swarm agar was then inoculated at the centre with 24hr-old culture of the test isolate grew on nutrient agar and then incubated overnight at 37°C. Next slide agglutination was repeated as described under phase 1. The antigens corresponding to the positive specific antisera were taken as the phase 2 antigens.

(iii). Assignment of serotypes to isolates

The combination of O and H antigens detected in the isolates were used to assign serotype names to the isolates using the Kauffmann-White scheme. The isolates were assigned serotype names in the Kauffmann-White scheme having the same O and H antigens.

Antibiotic susceptibility testing

The minimum inhibitory concentrations (MIC) of the *Salmonella enterica* isolates were determined against antibiotics following the standard microdilution (two-fold dilution) method according to CLS guidelines (Clinical Laboratory Standard, 2007). Muller-Hinton broth (Lab M) was used as culture medium. The following antimicrobials and resistance cut-off values/break point were used in the study: ampicillin, AMP (R> 4 mg/mL); amoxicillinþclavulanic acid, AUG (R>4 mg/mL); apramycin, APR (R>4 mg/mL); ceftiofur, XNL (R>2 mg/ mL); chloramphenicol, CHL (R>16 mg/mL); ciprofloxacin, CIP (R>0.06 mg/mL); colistin COL (R>8 mg/mL); florfenicol, FFN (R>2 mg/mL); gentamicin, GEN (R>2 mg/mL); nalidixic acid, NAL (R>18 mg/mL); neomycin, NEO (R>8 mg/ mL); spectinomycin, SPE (R>64 mg/mL); streptomycin, STR (R>16 mg/mL); sulfamethoxazole, SMX (R>256 mg/mL); tetracycline, TET (R>8 mg/mL); and trimethoprim, TMP (R>2 mg/mL); kanaycin, Km (R>25mg/mL); amikacin, Ak (R>32mg/mL); ceftazidme, Cz (R>32mg/mL); ceftriazone, Ct (R>64mg/mL). All antibiotics were Oxoid product, UK.

The method was standardized using the recommended control strains: ATCC 25922 (*E. coli*) and ATTC 27853 (*Pseudomonas aeruginosa*)

MOLECULAR INVESTIGATION OF THE *SALMONELLA* ISOLATES

This entailed genotypic investigation of the molecular basis of antibiotic resistance in the *Salmonella* isolates. β-lactamase enzyme production, β-lactamase genes, integrons and clonal relationship of isolates were investigated by molecular techniques.

Determination of β-lactamases production by nitrocefin test

This was carried out to determine β-lactamase production by the *Salmonella enterica* isolate*s.* Crude enzyme preparation of a test isolate was first prepared by sonication. Briefly, the test isolate was inoculated into Trypticase soy broth and incubated overnight aerobically at 37°C. Thereafter, 50ml of the overnight culture was pipetted into a sterile tube and centrifuged at 5,000 rpm for 5min*s.* The supernatant was eliminated and the cells resuspended in sterile distilled water (weight/weight). The tube was kept in ice and the bacterial suspension was then disrupted by sonication, twice for 30s each at 4OHz using Vibracell 300 (Bioblock, IIkirch, France). Then one drop of the enzyme preparation was mixed with a drop of of nitrocefin solution (0.5mg/mL) on a clean glass slide. A change in colour from yellow to red was taken as positive (production of β-lacatamase enzyme by the test isoate).

Test for production of extended-spectrum β-lactamases by Double-disk synergy test

The *Salmonella* isolates were screened for the production of extended-spectrum β-lactamase enzymes (ESBLs) by double-disk synergy test. Briefly, a suspension of the test organism was made to correspond to 0.5 McFarland turbidity and vortexed for 60s. Then a sterile cotton-swab was dipped into the suspension, pressed against the side of the tube to remove excess fluid and used to make a uniform lawn on the surface of a dry Muller-Hinton agar and allowed to dry. A Disk of amoxicillin+clavulanic acid was then placed in the center of the agar; then one disk of each of cefotaxime and ceftazidime was placed 20mm from the disk of amoxicillin+clavulanic acid. The plate was then incubated at 37°C for 18hrs aerobically. Enhanced zone of inhibition between the amoxicillin-clavulanic acid and cefotaxxime and/or ceftazidime disk was taken as positive (production of ESBL by the test isolate).

Isoelectric Focusing of β-lactamases

This was carried out to determine the iso-electric focusing points and types of the β-lactamase enzymes produced by isolates. This was done by performing analytical isoelectric focusing (IEF) with a Model 111 Mini IEF Cell (Bio-Rad). Briefly, a 5% polyacrylamide gel containing ampholytes (pH range, 3-10; Bio-Rad Laboratories, USA) was poured on a rectangular glass gel cast and allowed to solidify in a flat position. The IEF special comb was then placed under the glass at one end to serve as guide during loading. A filter paper (8mm by 2mm) was dipped into crude enzyme preparation of a test isolate (prepared as earlier described) using a forcep; excess fluid was removed from the filter paper by pressing it against the side of the crude enzyme tube. The filter paper was then placed on the gel using the comb wells as a guide to place the filter paper appropriately. The forcep was dipped into absolute alcohol and flamed before used for loading another enzyme preparation to prevent cross contamination. Specific β-lactamase enzymes with known focusing points were also loaded simultaneously to serve as markers; these include SHV-1 (pI 7.6), OXA-14 (pI 6.2) and TEM-1 (pI 5.4). One drop each of nitrocefin solution was placed at the opposite ends of the gel to monitor movement progress during electrophoresis. The gel was then put face downward inside the IEF electrophoresis chamber and then connected to a power source.The gel was run at 1watt and 300V. After running for three minutes, the electrophoresis was stopped and gel brought out to gently remove the filter papers with forcep.Then the gel was returned to the chamber to continue the electrophoresis. The electrophoresis was stopped when the two drops of nitrocefin met; the gel was was then brought out and put on a table facing upward. Then the gel was laid with nitrocefin solution (0.5mg/mL) to stain the electrophoretic bands. The β-lactamase enzyme in a test crude enzyme preparation

Preparation of DNA templates for polymerase chain reactions

β-lactamase gene and integrons were investigated in *Salmonella* isolates by polymerase chain reaction using whole DNA preparation from test isolates and control strains as templates.. Breifly, test isolate was inoculated into 5ml of LB broth (Oxoid) and incubated for 20hr at 37°C with shaking aerobically. Then 1.5ml of the broth culture was poured into an eppendorf tube and centrifuged at 13,000 rpm/5mins. The supernatant was decanted and the pellet resuspended in 500µl of sterile distilled water. The cells were then lysed by heating at 95°C for 10min and the suspension centrifuged at 13,000rpm for 5mins. The supernatant was used as the template for PCR.

Identification of β-lactamase genes by polymerase chain reaction

Polymerase chain reaction was carried out to investigate the β-lactamase genes mediating resistance to the β-lactam antibiotics in the test isolates. Separate (uniplex) polymerase chain reaction was performed to investigate each of TEM, SHV and OXA β-lactamase genes. Each polymerase chain reaction was performed in total volume of 50µl: Sterile distilled water (39.25µl), 10x buffer (5µl), dNTP (1.0µl), 50mM $MgCl_2$ (2µl), forward primer (100pmoles/µl) (0.25 µl), reverse primer (100pmoles/µl) (0.25 µl), Taq polymerase (5U/µl) (0.25µl) and DNA template (2µl). All the PCR materials were obtained from Invitrogen (USA). The primers used are specific for each gene and are as shown in the table 4.The used PCR conditions consisted of initial denaturation at 95°C/5mins (all uniplex PCR), 30 cycles of denaturation at 94°C/4mins (all uniplex PCR), 30 cycles of annealing at 55°C/30s for each of TEM and OXA uniplex PCR and 50°C/30s for SHV uniplex PCR. Other conditions are extention at 72°C/2min (for all uniplex PCR) and final extention at 72°C/7min (for all uniplex PCR). After completion of PCR, gel

electrophoresis was performed to separate the PCR products. Briefly, 2% (w/v) agarose was prepared in 1X TAE buffer and then casted using a gel cast and comb. The cast together with the gel was placed in a electrophoresis tank and covered with with 1X TAE buffer.Then 5µl of a PCR product was mixed with 1µl of loading buffer and loaded into a well in the gel. The tank was then covered and connected to a power source and the electrophoresis was run at 60V. After completion of electrophoresis, the gel was carefull taken out of the tank and placed briefly in an ethidium bromide solution for staining. The gel was then washed thoroughly in distilled water to remove excess stain and then placed in imaging device to visualize the bands and to take a picture of the gel.

Table 4: Primers used for PCR identification of β-lactamase genes

Genes	Primers	Primer sequences (5' to 3')+
TEM-like	TEMprime1F	AGA TCA GTT GGG TGC ACG AG
	TEMprimeEndR	CTT GGT CTG ACA GTT ACC
SHV-like	SHVprime2F	GGG AAA CGG ACC TGA ATG AG
	SHVprimeEndR	TTA GCG TTG CCA GTG CTC A
OXA-1	OXA1B14	CGACCCCAAGTTTCCTGTAAGTG
	OXA1F2	TGTGCAACGCAAATGGCAC

+Hanson *et al.,* 2002

Sequencing of β-lactamase genes

This was carried out to determine nucleotide sequence of a gene. A uniplex PCR was first performed as described above but HIFI Taq polymerase was used instead of Taq polymerase because it is less error prone; furthermore, extention was carried out at 68°C/2mins and final extention at 68°C/7mins because HIFI Taq polymerase works best at 68°C. After completion of PCR, gel electrophoresis was performed as described above but using 0.8% (w/v) agarose and loading by mixing 40µl of PCR mixture with 8µl of loading buffer. The gel was stained briefly in ethidium bromide solution and washed thoroughly in distilled water to remove excess stains.A nylon was placed on the surface of imaging device and the gel was placed on top of the nylon; the imaging device was then switched on to visualize the bands. The band corresponding to the specific PCR product was then excised from the gel and trimmed with sterile scapel to remove excess gel. The specific PCR product was purified from the excised band using Q1Aquick PCR purification kit (QIAgen, Hilden, Germany) following the manufacturer instructions. The purified amplicon was sequenced by PCR cycle-sequencing with dye-terminator chemistry according to Biosystems (Applied Biosystems, Foster city, CA, USA) using the same primers used in the uniplex PCR. Sequence analysis and alignment was performed using Vector NTI suite 9 (InforMax, Inc.). The obtained nucleotide sequence was compared with those in the GenBank using Blast programs (http://www.ncbi.nlm.nih.gov/Blastn).

Identification of Integrons by polymerase chain reaction

Integron identification was performed to investigate different classes of integrons in antibiotic resistant *Salmonella* isolates. Uniplex PCR was carried out to detect the presence of each of classes 1, 2 and 3 integrons in a test isolate. Each uniplex PCR was carried out in 50µl: Sterile

distilled water (39.25µl), 10x buffer (5µl), dNTP (1.0µl), 50mM $MgCl_2$ (2µl), forward primer (100pmoles/µl) (0.25 µl), reverse primer (100pmoles/µl) (0.25 µl), Taq polymerase (5U/µl) (0.25µl) and DNA template (2µl). All the PCR materials were obtained from Invitrogen (USA). The primers used are specific for each class of integrons and are as shown in the table 5.The used PCR conditions consisted of initial denaturation at 95^oC/5mins (all uniplex PCR), 30 cycles of denaturation at 94^oC/4mins (all uniplex PCR), 30 cycles of annealing at 50^oC/30s (all uniplex PCR). Other conditions are extention at 72^oC/2min (for all uniplex PCR) and final extention at 72^oC/7min (for all uniplex PCR). After completion of PCR, gel electrophoresis was performed to separate the PCR products. Briefly, 2% (w/v) agarose was prepared in 1X TAE buffer and then casted using a gel cast and comb. The cast together with the gel was placed in an electrophoresis tank and covered with with 1X TAE buffer.Then 5µl of a PCR product was mixed with 1µl of loading buffer and loaded into a well in the gel. The tank was then covered and connected to a power source and the electrophoresis was run at 60V. After completion of electrophoresis, the gel was carefull taken out of the tank and placed briefly in an ethidium bromide solution for staining. The gel was then washed thoroughly in distilled water to remove excess stain and then placed in imaging device to visualize the bands and to take a picture of the gel.

Table 5: Primers used for PCR identification of integrons

Genes	Primers	Primer sequences (5' to 3')	Source
IntI1	IntI1-F	GGT CAA GGA TCT GGA TTT CG	Rao *et al.,* 2006
	IntI1-R	ACA TGC GTG TAA ATC ATC GTC	Rao *et al.,* 2006
	5'CS	GGC ATC CAA GCA GCA AG	Rao *et al.,* 2006
	3' CS	AAG CAG ACT TGA CCT GA	Rao *et al.,* 2006
IntI2	IntI2-F	CAC GGA TAT GCG ACA AAA AGG T	Vinue *et al.,* 2008
	IntI2-R	GTA GCA AAC GAG TGA CGA AAT G	Vinue *et al.,* 2008
	att2-F	GTA GCA AAC GAG TGA CGA AAT G	Vinue *et al.,* 2008
IntI3	IntI3-F	AGT GGG TGG CGA ATG AGT G	Vinue *et al.,* 2008
	IntI3-R	TGT TCT TGT ATC GGC AGG TG	Vinue *et al.,* 2008

Sequencing of integron

This was carried out to determine nucleotide sequence of an integron to know integrated resistance genes. A uniplex PCR was first performed as described above but HIFI Taq polymerase was used instead of Taq polymerase because it is less error prone; furthermore, extention was carried out at 68°C/2mins and final extention at 68°C/7mins because HIFI Taq polymerase works best at 68°C. After completion of PCR, gel electrophoresis was performed as described above but using 0.8% (w/v) agarose and loading by mixing 40µl of PCR mixture with 8µl of loading buffer. The gel was stained briefly in ethidium bromide solution and washed thoroughly in distilled water to remove excess stains.A nylon was placed on the surface of imaging device and the gel was placed on top of the nylon; the imaging device was then switched on to visualize the bands. The band corresponding to the specific PCR product was then excised from the gel and trimmed with sterile scapel to remove excess gel. The specific PCR product was purified from the excised band using Q1Aquick PCR purification kit (QIAgen, Hilden, Germany) following the manufacturer instruction*s*. The purified amplicon was sequenced by PCR cycle-sequencing with dye-terminator chemistry according to Biosystems (Applied Biosystems, Foster city, CA, USA) using the same primers used in the uniplex PCR. Sequence analysis and alignment was performed using Vector NTI suite 9 (InforMax, Inc.). The obtained nucleotide sequence was compared with those in the GenBank using Blast programs (http://www.ncbi.nlm.nih.gov/Blast).

Digestion of integron amplicons by endonucleases

This was carried out to determine restriction length polymorphism of the integron amplicons using endonucleases *Hinf1, Pst1, Bcn* and *Hind III* following the method of Vinue *et al.* 2008.

Integron amplicons were restricted using the endonucleases one after the other. Each restriction consisted of: 1µl 10X buffer, 6.5µl sterile distilled water, 2µl of an integron amplicon 0.5µl enzyme added into a PCR tube in this order and incubated at 37°C/1hr in water bath. Then gel electrophoresis was run as earlier described for the uniplex PCR.

Investigation of transfer of integron by conjugation

Conjugation was used to determine if integron genes can be transferred from *Salmonella* isolates to other bacteria thus indicating the location of the gene on mobile genetic elements and possible disseminaton in the environment. Conjugation was carried out by mating test *Salmonella* isolate (donor) with recipient (*E. coli* J53-2, resistant to rifampicin). Briefly, the test *Salmonella* isolate (donor) was inoculated into 5ml of trypticase soy broth in a test tube and the recipient (*E. coli* J53-2) was inoculated into another test tube containing 5ml of trypticase soy broth. The two test tubes were then incubated at 37°C for 18hrs aerobically. The overnight broth culture of the donor and recipient were mixed on Muller-Hinton agar at a ratio of 1:1 (200 µl of donor and 200 µl of recipient cultures) using a sterile bent glass rod and incubated at 37°C for 18hr*s*. The mixed culture from the agar was harvested, suspended in 5 ml of trypticase soy broth and vortexed. Then 200 µl of the suspension was plated on Muller-Hinton agar containing 250µg/mL rifampin and sulfamethoxazole (256 mg/L) and incubated aerobically at 37°C for 18hrs. The isolated colonies (transconjugants) were then evaluated for integron by PCR (as described above).

Investigation of transfer of β-lactamase gene by conjugation

Conjugation was used to determine if β-lactamase genes can be transferred from *Salmonella* isolates to other bacteria thus indicating the location of the gene on mobile genetic elements and

possible disseminaton in the environment. Conjugation was carried out by mating test *Salmonella* isolate (donor) with recipient (*E. coli* J53-2, resistant to rifampicin). Briefly, the test *Salmonella* isolate (donor) was inoculated into 5ml of trypticase soy broth in a test tube and the recipient (*E. coli* J53-2) was inoculated into another test tube containing 5ml of trypticase soy broth. The two test tubes were then incubated at 37°C for 18hrs aerobically. The overnight broth culture of the donor and recipient were mixed on Muller-Hinton agar at a ratio of 1:1 (200 µl of donor and 200 µl of recipient cultures) using a sterile bent glass rod and incubated at 37°C for 18hr*s*. The mixed culture from the agar was harvested, suspended in 5 ml of trypticase soy broth and vortexed. Then 200µl of the suspension was plated on Muller-Hinton agar containing 250µg/mL rifampin and ampicillin 100mg/L and incubated at 37°C for 18hrs aerobically. The isolated colonies (transconjugants) were then evaluated for β-lactamase gene by PCR (as described above) and susceptibility to β-lactam antibiotic*s*.

Molecular subtyping of *Salmonella* isolates

In order to determine the clonal relatedness of the ciprofloxacin-resistant *Salmonella* isolates, molecular subtyping was performed using pulsed-field gel electrophoresis (PFGE). It was carried out according to PulseNet method (Ribot *et al.,* 2006)**.** Test isolates were streaked on Luria-Bertani agar and incubated at 37°C/18hrs. Then bacterial suspension of each isolate with OD_{620nm} of 0.12 was made in cell suspension buffer inside a test tube. The plug moulds were assembled and cleaned with 70% alcohol. Then 400µl of the suspension was transferred to an eppendorf tube; 20 µl of proteinase K (20mg/ml) was added and mixed gently with the pipet tip. Then 400 µl of molten 1% agarose solution (clean cut; Bio-rad, Hempstead, United Kingdom) (maintained at 54°C in a waterbath) was added to the 420 µl cell suspension/proteinase K, and mixed by

gently pipetting up and down a couple of times, avoiding creating air bubbles.The mixture was then immediately transfered into the plug moulds by using a pipette; the plugs were allowed to solidify at room temperature for 10 to 15mins. A mastermix of lysis-buffer solution was prepared by mixing 5mL of lysis buffer (0.5mM EDTA, 1% N-laurylsarcozine (sigma, poole, United Kingdom)) with 25µl of proteinase k (20mg/mL) (sigma) per plug. Then 5 ml of the Lysis-buffer/Proteinase K–mix was transferred to a 50 ml screw-cap tube. The plug moulds were then disassembled and each plug was then gently transferred using plastic inoculating loop to a screw-cap tube containing the lysis-buffer solution and incubated at 54°C for 2hrs in shaker water bath at 150 rpm.After incubation, the lysis-buffer was decanted and the plug washed four times with 10ml of warm (54°C) sterile TE buffer (10mM Tris-Hcl, 1mM EDTA, pH 8). Then 10ml of warm (50°C) sterile distilled water was added and incubated at 5O^{o}C /10min in a shaker water bath.

The plug was then washed four times with 10ml of TE buffer at 5O^{o}C /10min and transferred to a cryo-tube and covered with TE buffer. The plug was then ready for digestion with Xbal endonuclease or stored in the refrigerator for the digestion next day. A 1:10 dilution of the 10X concentration enzyme buffer was made in sterile distilled water and 200µl was placed in an eppendorf tube. The plug was removed from TE buffer and placed on a clean glass to cut a plug slice of 2mm broad and 10mm wide with a scapel and the remaining plug was returned into the TE buffer and stored at 4°C. The plug slice was transferred into the eppendorf tube containing 200µl enzyme buffer and incubated at 37°C/10min. Then the enzyme buffer was carefully removed with a pipette not damaging or loosing the plug slice. Then a master mix of XbaI restriction enzyme (Promega, Southampton, United Kingdom) was made by mixing 20 µl of enzyme buffer 10X Tango, 40 U of restriction enzyme Xba I (10 U/µl), 2 µl of BSA for

restriction reactions and 174 µl sterile double distilled water (per plug). Then 200µl of the restriction enzyme mixture was added to the plug slice, mixed by tapping gently and incubated at 37°C/2hr. While digestion is going on a 1% agarose (PFGE-certified agar Bio-rad) was prepared in TBE solution and maintained at 50°C in a waterbath. Then 2.5L of freshly prepared 0.5X TBE buffer (130mM Tris, 45mM boric acid, 2.5mM EDTA) was added to the pulsed-field gel electrophoresis chamber; then the pump was turned on with a flow of 1 L/min (pump setting = 70) and the cooling module set to 14°C. After end digestion of the plugs the enzyme mixture was removed from the tubes with a pipette, and 200µl 0.5X TBE buffer added and incubated at room temperature for 5 min. The gel tray was then assembled after cleaning with alcohol; the 1% agarose was then poured into the gel with the comb in place and allowed to solidify for 1hr. The comb was removed after the gel had solidified. The plug slices were then gently removed from the tubes and loaded carefully into the wells of the gel. Excess agarose was trimmed off the gel; the gel was then taken with the black platform and inserted into the black platform of the gel electrophoresis chamber. The lid of the chamber was then closed. The PFGE was performed with a CHEF DRIII System with pulse times ramped from 5 to 60s during a 48h run at 5.1v/cm. When the run was complete the power was turned off and the gel removed from the chamber. The gel was then stained in ethidium bromide solution (1µg/mL) for 20 to 30min in a non-transparent container. The gel was then destained by placing it inside 500mL distilled water for 90min. The gel was then visualized under a UV transilluminator. The *Salmonella* isolates HN-GSS-2006-0 and HN-GSS-2007-0 were used as controls. Dendrogram of the band patterns was generated by BioNumerics software version 4.1 (Applied Maths, Sint-Martens-Latem, Belgium), and banding patterns were compared by using dice coefficients with a 1.5% band position tolerance. Percentage similarity scores were used to determine categories of strain relatedness. A cluster

was defined as a group of ≥2 isolates of *Salmonella enterica* that shared a unique PFGE restriction pattern.

Statistics

SAS version 9.1.3 (SAS Institute Inc.) was used to compare proportions using a Fisher's Exact test. The criteria for evaluating the significance level in the model was a P-values <0.05.

CHAPTER FOUR

RESULTS

Prevalence of *Salmonella enterica* from different sources

A total of 709 isolates of *Salmonella enterica* were obtained: 49 (febrile patients); 251 (healthy humans); 165 (chicken); 26 (cow); 75 (goats); 64 (pigs); 35 (sheep) and 44 (environment). Prevalence of *Salmonella* was 5.0% (febrile patients) and 8.6% (healthy humans). In the animals, carriage rates ranged from 6% in cattle to 15% in goats (Table 6). A typical isolate of *Salmonella enterica* with the characteristic black centre on Salmonella-Shigella agar is shown on Plate 1.

Prevalence of *Salmonella enterica* from febrile patients

Of the 984 febrile patients from whom blood samples were collected and cultured, 215 (22%) had bacteremia associated with different bacteria. *Salmonella* was the second commonest cause of bacteremia, accounting for 23% (49 of 215) of the bacteremic cases, after *Staphylococcus aureus* (Table 7). Forty-five (92%) of the bacteremia associated with *Salmonella* were in patients less than 15 years old (children). Among the children, more than 50% of the *Salmonella* cases of bacteremia were in patients less than 2 years old with the majority of the *Salmonella* bacteremia found among 12 – 23 months age group (Table 8). Of the eight *Salmonella* bacteremic cases in children less than 12 months old, four (50%) were in children less than 1month old.

Thirty-three of the *Salmonella* bacteremic cases were observed in the male patients (more than twice the number in the females).

Initial clinical diagnosis of *Salmonella* bacteremia

The initial clinical diagnosis of the febrile patients were divided into twelve groups including pyrexia of unknown origin (A), infection of urinary tract (B), infection of gastrointestinal tract (C), typhoid (D), infection of respiratory tract (E), infection of central nervous system (F), infection of cardiovascular system (G), infection of musculoskeletal system (H), neonatal sepsis and septicemia (I), septicemia in children and adults (J), infection associated with malignancies (K) and other infections (L). *Salmonella* bacteremia was observed in patients with eight of the initial clinical diagnoses. Most, 24.5% (12 of 49), of the *Salmonella* bacteremic patients were initially diagnosed of other infections followed by septicemia in children and adults (22.4%) and pyrexia of unknown (20.4%) (Figure 1).

Local government area of residence of the *Salmonella* bacteremic patients

The forty-nine *Salmonella* bacteremic patients came from six different local government areas of Ibadan. Majority of the patients resided in Ibadan South East (32.6%) followed by Ibadan South (28.6%), Ibadan North (12.2%) and Oluyole (12.2%).

Fatality of *Salmonella* bacteremia

Overall, 8.4% (18 of 215) of the bacteremic patients died. *Salmonella* accounted for 33% (6 of 18) of the fatal cases. Fatality rate among the *Salmonella* bacteremic patients was 12.2% (6 of 49) with *Salmonella* been the fourth common cause of fatality. Five of the fatal *Salmonella* bacteremic cases (83.3%) were in children less than 2years old. The initial clinical diagnoses in the fatal *Salmonella* cases include septicemia (two), other infection (two) and diarrhea (two).

Carriage rates of *Salmonella enterica* in food animals

The highest carriage rate of *Salmonella enterica* in the food animals was 15% in goat followed by 13.7% in chicken and 11% in pigs. Chicken isolates were obtained from thirteen different poultry farms in five different local government areas. The carriage rates in chicken varied with poultry farms of origin. The carriage rates varied from 1.7% in poultry farm M to 38% in poultry farm J (P = 0.001) (Table 9).

The antibiotics used in the poultry farms included ampicillin, tetracycline, streptomycin, sulfonamides and enrofloxacin.

Table 6: Prevalence of *Salmonella enterica* from different sources

Sources	No. of samples tested	No. (%) positive
Febrile patients	984	49 (5.0)
Asymptomatic patients	2910	251 (8.6)
Chicken	1201	165 (13.7)
Cattle	500	26 (6)
Goat	500	75 (15)
Pig	560	64 (11)
Sheep	500	35 (7)
Environment	18	18 (100)

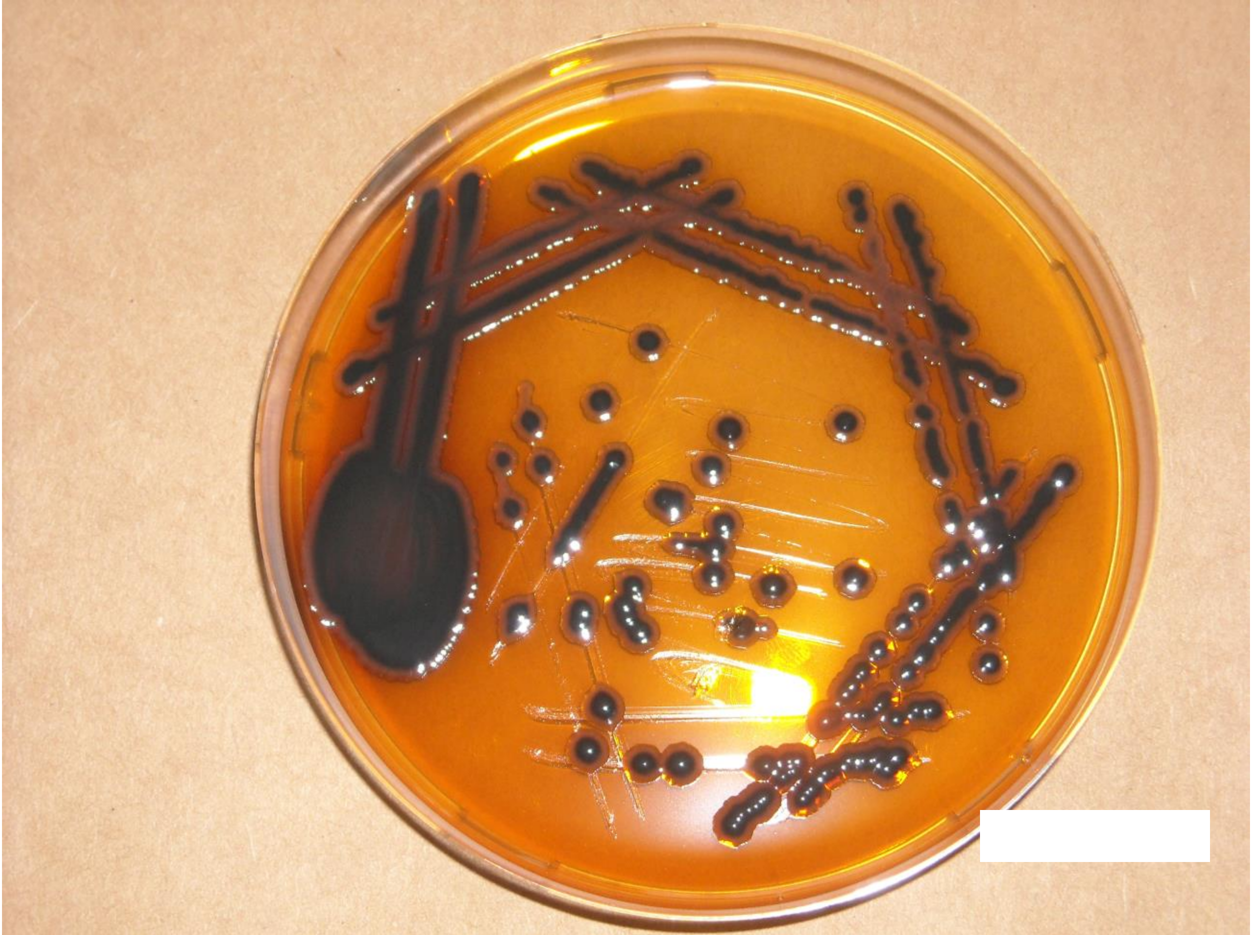

Plate 1: Colonies of *Salmonella enterica* on Salmonella-Shigella agar

Table 7: Role of *Salmonella enterica* in bacteremia

Age	No. Febrile patients	No. (%) Positive	No. (%) positive for the following bacteria											
			Salmonella enterica	*S. aureus*	*S. pneumoniae*	*Enteroccus faecalis*	*S. pyogenes*	*Klesiella spp.*	*E. coli*	*Serratia spp.*	*Enterobacter spp.*	*Citrobacter spp.*	*Pseudomonas spp.*	*Haemophilus spp.*
<1 month	173	62 (35.5)	4 (2.3)	36 (20.8)	0	2 (1.6)	0	13 (7.5)	4 (2.3)	1 (0.6)	1 (0.6)	0	1 (0.6)	2 (1.2)
1-11 months	126	45 (38.7)	4 (3.7)	29 (23.0)	2 (1.6)	2 (1.6)	0	1 (0.8)	4 (3.2)	1 (0.8)	2 (1.6)	0	0	0
1-14yrs	410	95 (23.2)	37 (9.0)	33 (8.0)	5 (1.2)	5 (1.2)	0	12 (2.9)	0	1 (0.2)	1 (0.2)	2 (0.5)	4 (1.0)	0
15-49 yrs	249	12 (4.8)	4 (1.6)	3 (1.2)	2 (0.8)	0	1 (0.4)	2 (0.8)	0	0	0	0	0	0
≥50 yrs	26	1 (3.8)	0	0	0	0	0	0	0	0	0	0	1 (3.8)	0
Total	**984**	**215 (21.8)**	**49 (5.0)**	**101 (10.3)**	**9 (0.9)**	**2 (0.2)**	**1 (0.1)**	**28 (2.8)**	**8 (0.8)**	**3 (0.3)**	**4 (0.4)**	**2 (0.2)**	**6 (0.6)**	**2 (0.2)**

Table 8: Role of *Salmonella enterica* in bacteremia in children

Age (Months)	**No. of Febrile Patients**	**No. (%) positive**	***Salmonella* (%)**	***S. aureus* (%)**	**Pneumococci (%)**	***S. feacalis* (%)**	**Klebsiella (%)**	***E. coli* (%)**	**Serratia (%)**	**Enterobacter (%)**	**Citrobacter (%)**	**Pseudomonas (%)**	**Haemophilus (%)**
<12	314	109 (34.7)	8 (2.5)	65 (20.7)	2 (0.6)	2 (0.6)	15 (4.8)	8 (2.5)	2 (0.6)	3 (1.0)	-	2 (0.6)	2 (0.6)
12-23	**89**	**40 (44.9)**	**18 (20.3)**	**15 (16.9)**	**3 (3.4)**	**-**	**3 (3.4)**	**-**	**-**	**-**	**-**	**1 (1.1)**	**-**
24-47	125	21 (16.8)	8 (6.4)	5 (4.0)	1 (0.8)	-	4 (3.2)	-	1 (0.8)	1 (0.8)	1 (0.8)	-	-
48-71	44	6 (13.6)	1 (2.3)	4 (9.1)	-	-	1 (2.3)	-	-	-	-	-	-
>72	125	22 (17.6)	12 (9.6)	4 (3.2)	1 (0.8)	-	3 (2.4)	-	-	-	1 (0.8)	1 (0.8)	-
Total	697	198 (28.4)	47 (6.7)	93 (13.3)	7 (1.0)	2 (0.2)	26 (3.7)	8 (1.1)	3 (0.4)	4 (0.6)	2 (0.3)	4 (0.6)	2 (0.2)

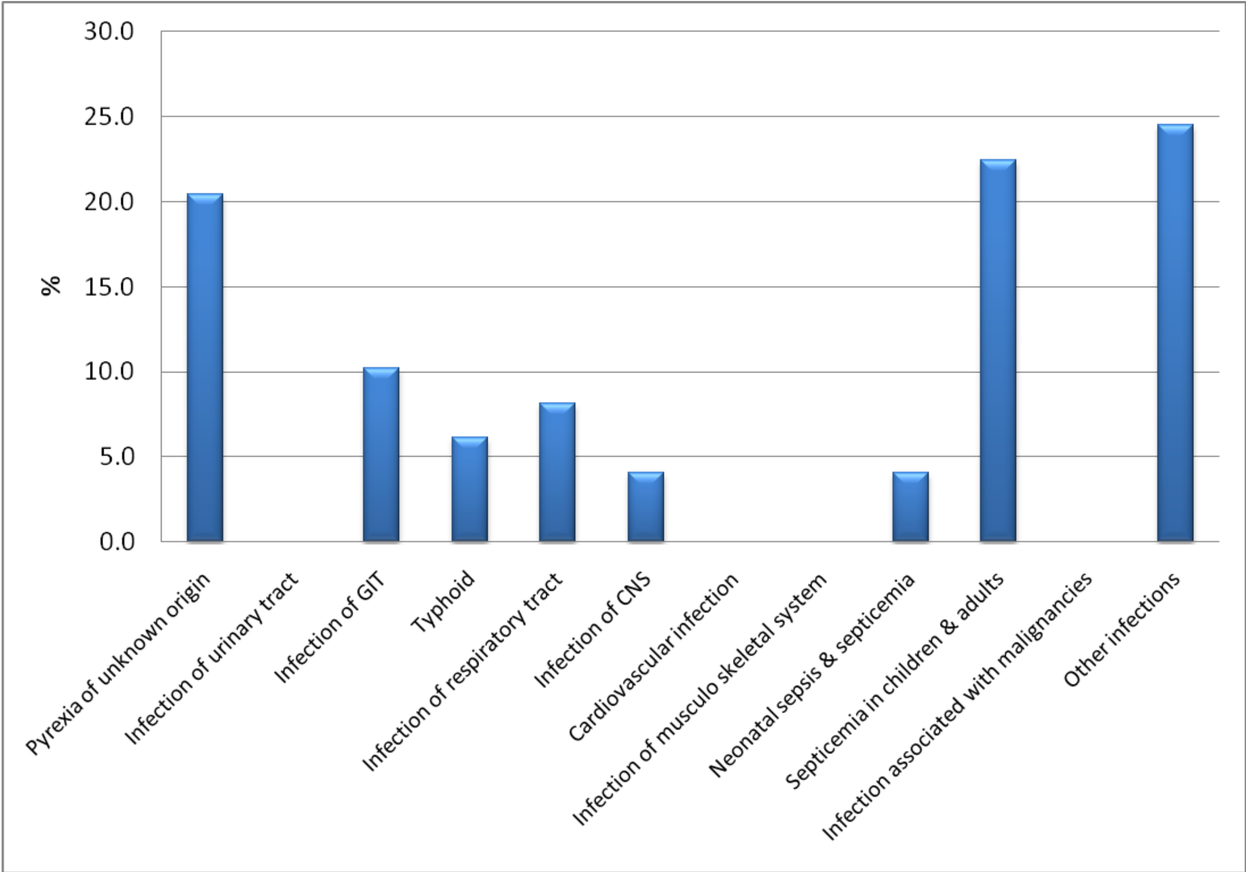

Figure 1: Initial clinical diagnosis of *Salmonella* bacteremic patients

Table 9: Prevalence of *Salmonella enterica* from poultry farms

Poutry Farms	Local Gov. Areas	No. of samples	No. Positive	% positive
A	South west	35	12	34.2
B	South west	38	8	21.1
C	South west	41	5	12.2
D	Ido	38	8	21.1
E	Ido	44	4	9.1
F	Ibadan North	44	6	13.6
G	South East	47	1	2.1
H	Ibadan south	42	4	9.5
I	Egbeda	80	12	15
J	Oluyole	92	35	38
K	Oluyole	80	26	32.5
L	Oluyole	60	18	30
M	Akinyele	60	1	1.7
Total		**701**	**140**	**20**

Serotyping of *Salmonella enterica* isolates

Only the bacteremic and chicken isolates could be serotyped due to financial constraints. Out of the bacteremic and chicken isolates, only 39 and 114 isolates respectively, could be serotyped. Non-typhoidal *Salmonella* predominated, 32 (82.1%), among the serotyped bacteremic isolates. *S*. Typhi accounted for 7 (17.9%). Overall, nine serovars were obtained from the bacteremic isolates. The predominant serovars were *S*. Enteritidis 13 (33.3%), *S*. Dublin 7(17.9%), and *S*. Typhimurium 7 (17.9%) (Table 10). Some uncommon serovars including *S*. Jukestown 2.6% (1), *S*. Monschaui 2.6% (1), *S*. Oritamerin 2.6% (1), and *S*. Apapa 2.6% (1) were also obtained (Table 10).

The obtained serotypes differed in the age of bacteremic patients from which they were isolated. The median age of *S*. Typhi bacteremic patients was 11years; all the patients were 6years and above. On the other hand, the median age of non-typhoidal bacteremic patients were 1yr 7months, 1yr 6months and 1yr 6montsh, for *S*. Enteritidis, *S*. Dublin and *S*. Typhimurium respectively. While the median age for the bacteremic patients of the uncommon serotypes was 10years.

Furthermore, the serotypes also differed in the initial clinical diagnosis of the *Salmonella* bacteremic patients. It is noteworthy that all the seven isolates of *S*. Typhi were obtained from patients with only initial clinical diagnosis of pyrexia of unknown origin. On the other hand, *S*. Enteritidis isolates were obtained from patients with six different initial clinical diagnosis; majority, 30.8% (4 of 13), had initial clinical diagnosis of septicemia. Patients with bacteremia caused by the uncommon serotypes had three different initial clinical diagnosis of septicemia,

typhoid and infection of the central nervous system (Figure 2). The local government area distribution of the serovars is shown in Figure 3

Males were more likely to be infected than females by each of the serovars with the exception of the uncommon serotypes. Ten of the thirteen S. Enteritidis were males; while four of the seven S. Typhi bacteremic patients were males (Figure 4). The overall prevalence between male and female differed with 5.0% among males in contrast to 2.8% among females (not significant p=0.17).

Five of the six fatal *Salmonella* bacteremic cases were associated with various *Salmonella* serotypes including S. Enteritidis (two patients), S. Typhimurium (one patient), S. Dublin (one patients) and S. Apapa (one patient). The isolate from the sixth fatal case could not be revived.

All the 114 (100%) serotyped chicken isolates were non-typhoidal *Salmonella*. Nine serovars were obtained. The most predominant serovar was S. Virchow, 50 (43.9%). S. Virchow was found in 11 (84.6%) of the thirteen poultry farms; this serovar predominated in farms J and K, 22 (73.3%) and 10 (47.6%). Moreover, *S*. Virchow was the only serovar found in farms D, E and F. Furthermore, two uncommon serovars including *S*. Haifa and *S*. Onireke were also obtained (Table 11). The local government area location of the poultry farms is shown in figure 5.

Table 10: Role of *Salmonella enterica* serovars in bacteremia

Sample site.	No. samples per sample site	Number and percentages of isolates per serovars:									Total no. (%) serovars per sample site
		S. Apapa	*S.* Dublin	*S.* Enteritidis	*S.* Infantis	*S.* Jukestown	*S.* Monschaui	*S.* Oritamerin	*S.* Typhi	*S.* Typhimurium	
Hospital A	204	0 (0.0)	2 (22.2)	7 (77.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	9 (23.1)
Hospital B	119	0 (0.0)	2 (16.7)	3 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (8.3)	0 (0.0)	6 (50.0)	12 (30.8)
Hospital C	120	0 (0.0)	2 (40.0)	2 (40.0)	0 (0.0)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	5 (12.8))
Hospital D	102	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (75.0)	1 (25.0)	4 (10.3)
Hospital E	104	1 (33.3)	0 (0.0)	1 (33.3)	1 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (7.7)
Hospital F	107	0 (0.0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	0 (0.0)	2 (5.1)
Hospital G	157	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	0 (0.0)	0 (0.0)	2 (66.7)	0 (0.0)	3 (7.7)
Diagnostic lab.	78	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	1 (2.6)
Total no. (%)	39 (3.9)	1 (2.6)	7 (17.9)	13 (33.3)	1 (2.6)	1 (2.6)	1 (2.6)	1 (2.6)	7 (17.9)	7 (17.9)	39 (100.0)

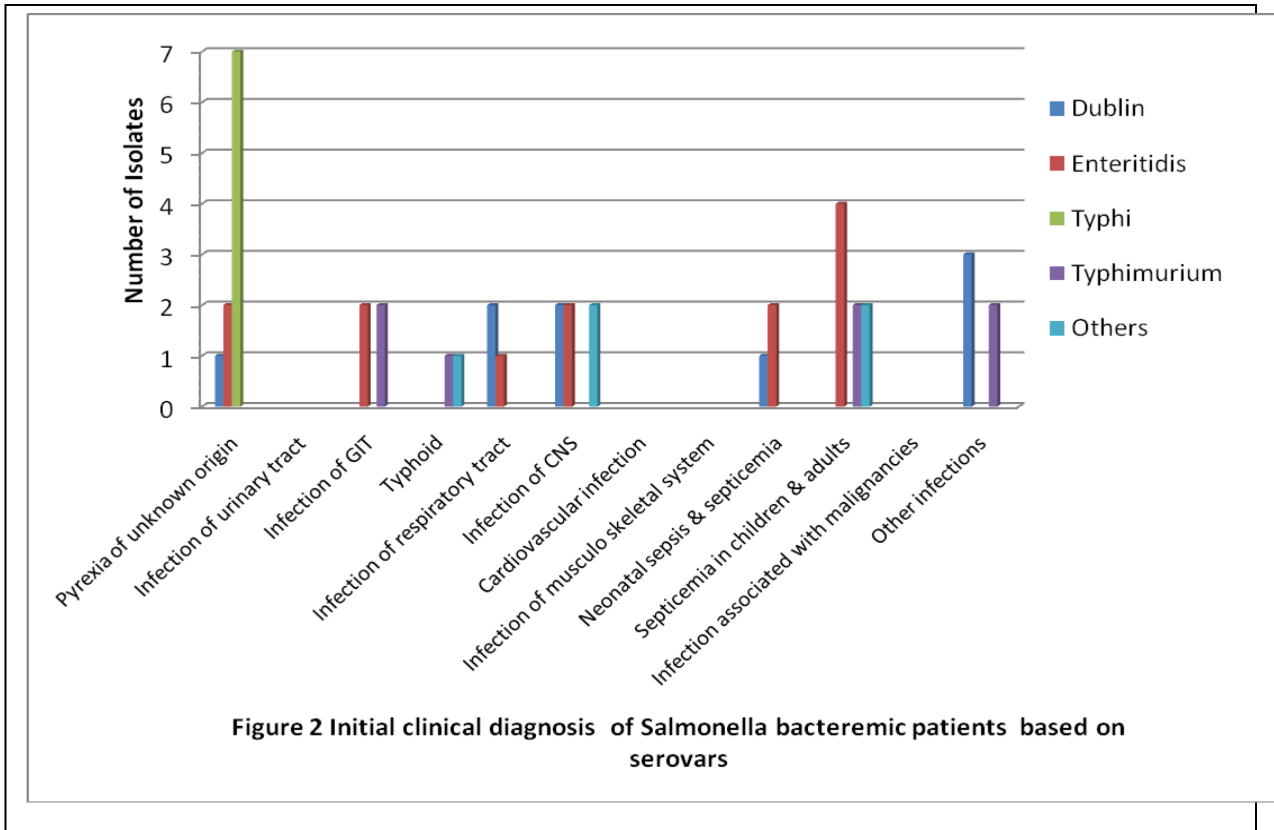

Figure 2: Initial clinical diagnosis of *Salmonella* bacteremic patients based on serovars

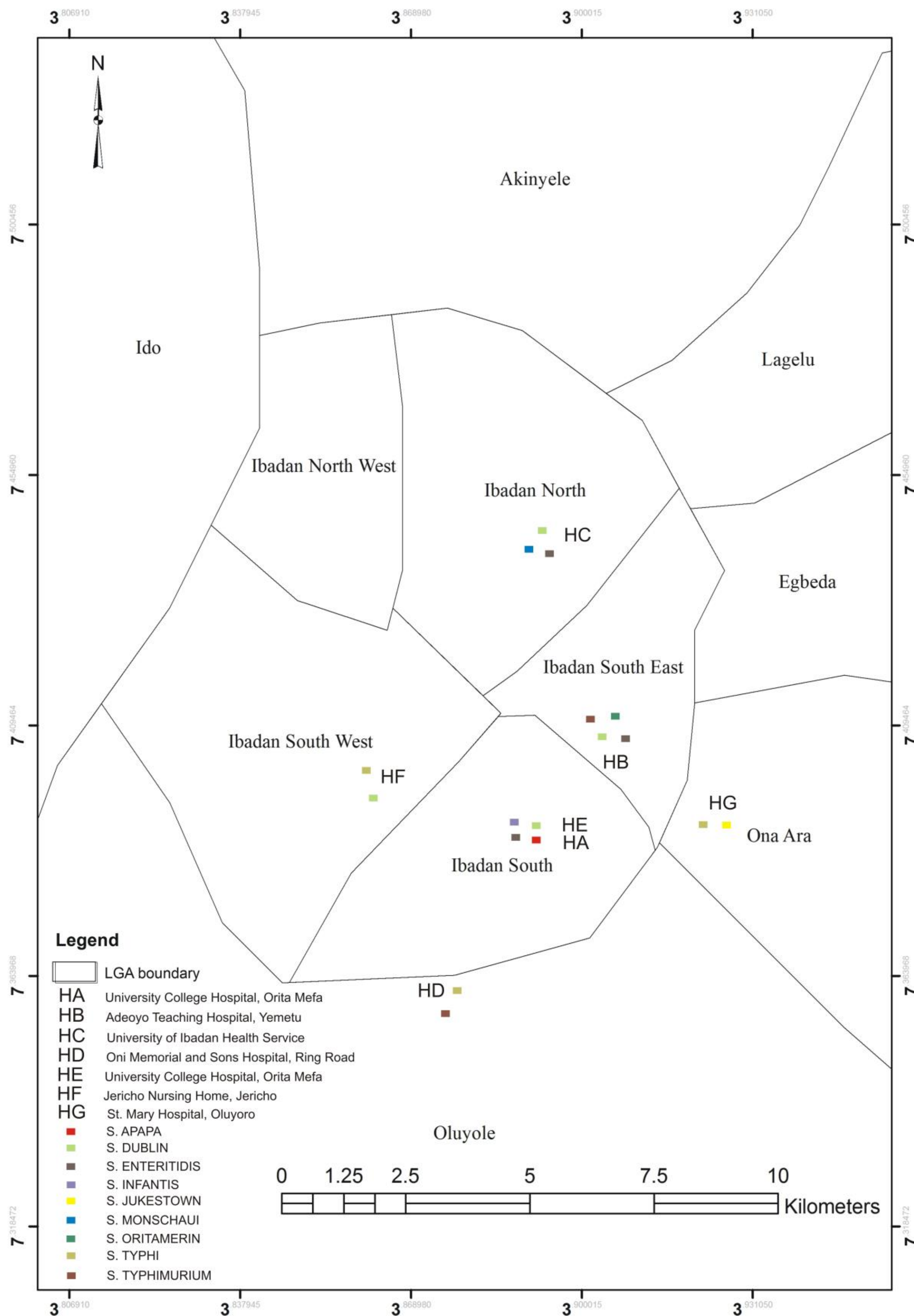

Figure 3: Local government distribution of *Salmonella* bacteremia and Salmonella serovars

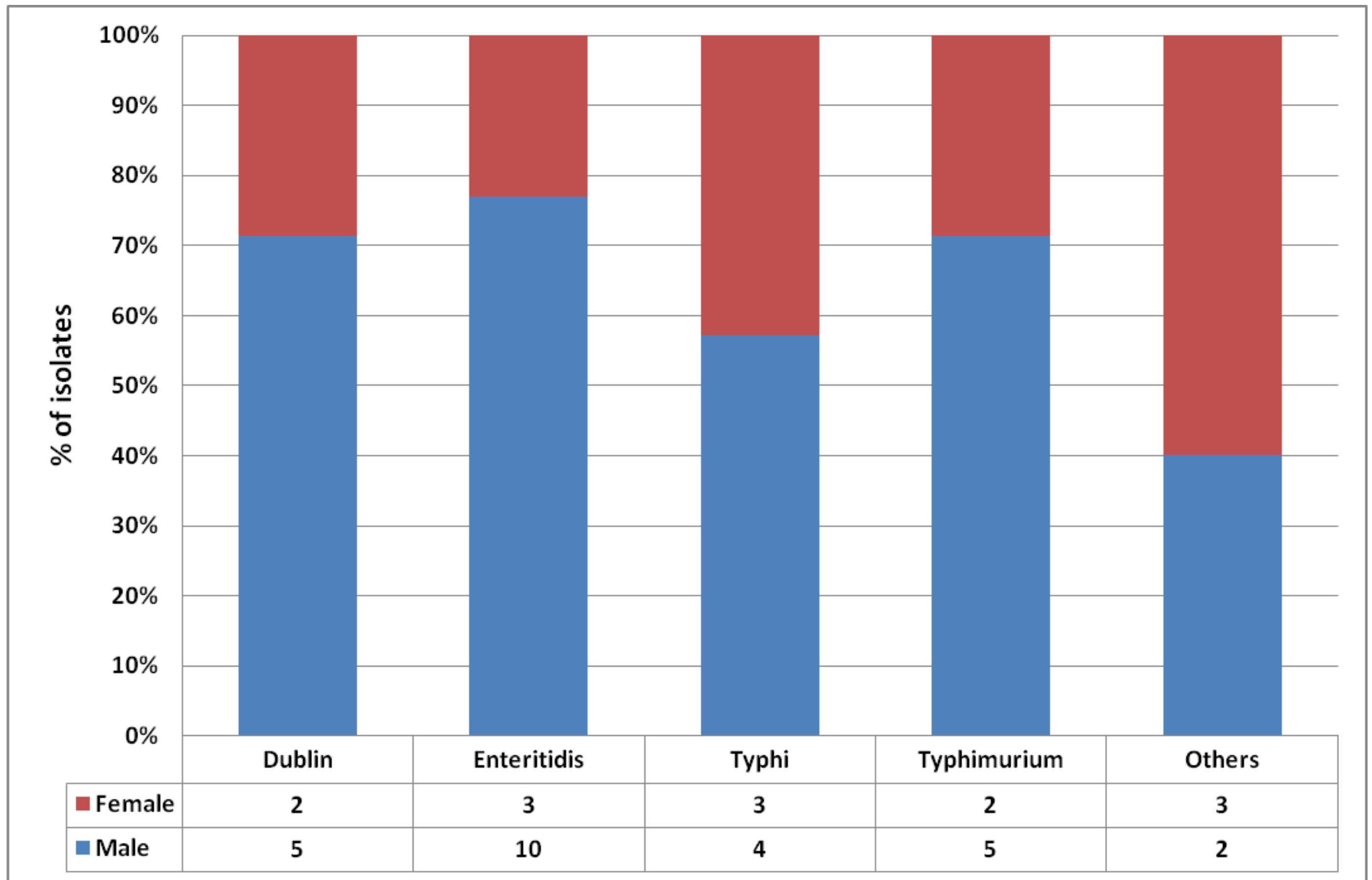

Figure 4: Sex distribution of *Salmonella* serovars from the *Salmonella* bacteremic patients

Table 11: Carriage rates of *Salmonella* serovars in the poultry farms

Farm no.	No. samples per farm	Number and percentages of isolates per serovars:									Total no. Serovars per farm(%)
		S. Bredney	*S.* Derby	*S.* Haifa	*S.* Havana	*S.* Muenster	*S.* Mbandaka	*S.* Onireke	*S.* Kentucky	*S.* Virchow	
A	35	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (11.1)	1 (11.1)	0 (0.0)	5 (55.6)	2 (50.0)	9 (7.9)
B	38	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (75.0)	1 (25.0)	5 (4.4)
C	41	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	1 (50.0)	2 (1.8)
D	38	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1(20.0)	4 (80.0)	5 (4.4)
E	44	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (75.0)	4 (5.7)
F	44	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	1 (16.7)	0 (0.0)	0 (0.0)	0 (0.0)	4 (66.7)	6 (8.6)
G	47	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.4)
H	42	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)	2 (50.0)	0 (0.0)	0 (0.0)	1 (25.0)	4 (5.7)
I	80	0 (0.0)	2 (20.0)	0 (0.0)	1 (10.0)	2 (20.0)	0 (0.0)	0 (0.0)	4 (40.0)	1 (10.0)	10 (8.8)
J	92	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.7)	0 (0.0)	6 (20.0)	22 (73.3)	30 (26.3)
k	80	1 (4.8)	1 (4.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	9 (42.9)	10 (47.6)	21 (18.4)
L	60	0 (0.0)	0 (0.0)	1 (6.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (6.3)	13 (81.3)	1 (6.3)	16 (14.0)
M	60	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1(100.0)	0 (0.0)	3 (2.6)
Total no. Per serovar	641	1 (0.9%)	4 (3.5%)	2 (1.8%)	1 (0.9%)	5 (5.3%)	6 (8.6%)	1 (0.9%)	44 (38.6%)	50 (43.9%)	114 (100.0%)

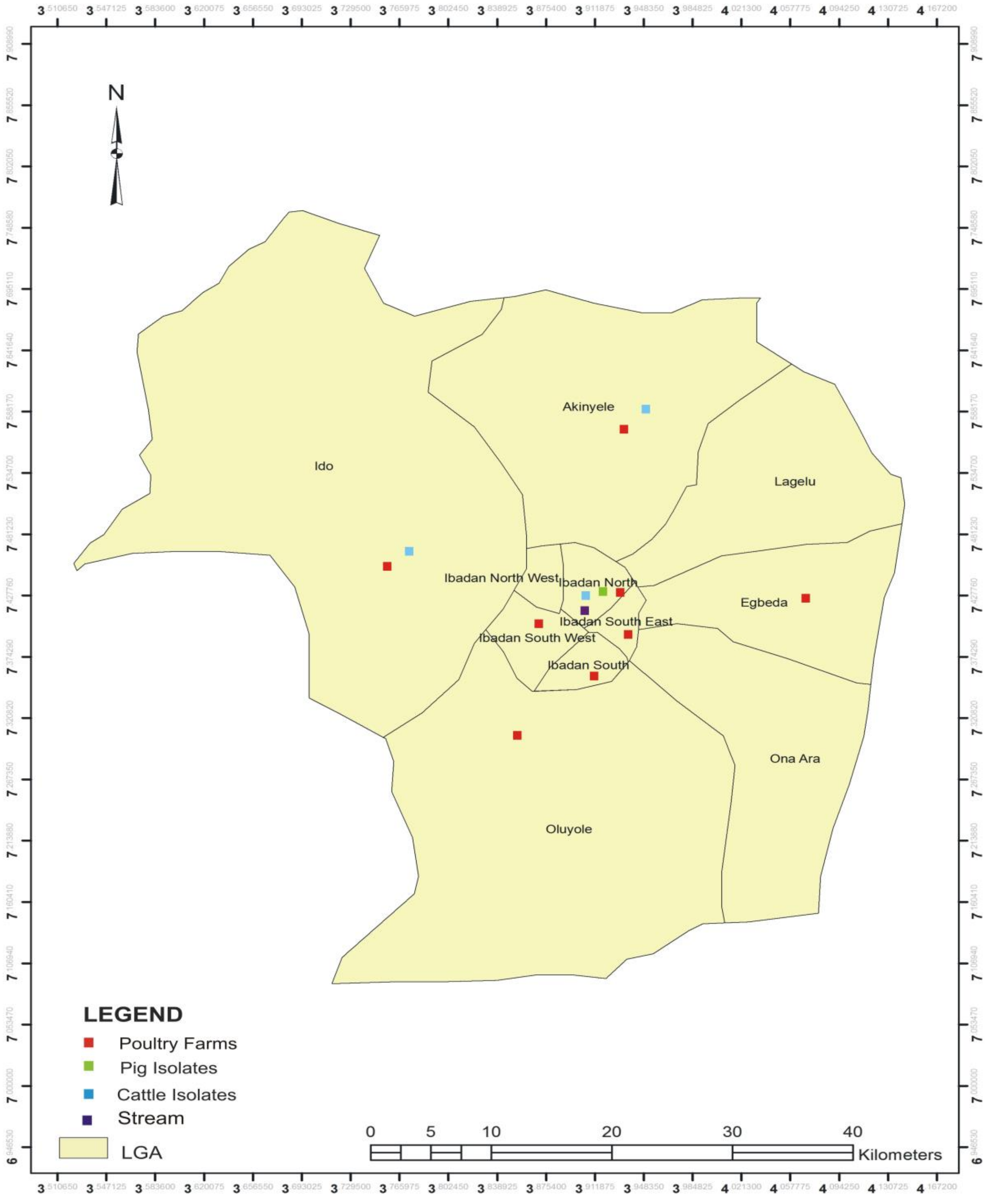

Figure 5: Local government area location of food animals and streams

Carriage of *Salmonella enterica* in Healthy Humans

The overall prevalence rate in the healthy humans was 8.6%. Among the pregnant women sub-population, the rate was 7.2% and the rates varied with age from 5.6% (age group 32-36years) to 9.1% (37-41years). The rate in pregnant women in the second trimester was 6.3% compared to 7.8% in those in the third trimester. The rate was 11.3% in pregnant women who had history of diarrhea compared to 6.0% in those with no history (P = 0.0001). Furthermore, the rate varied with the occupation of the pregnant women from student (0%) to hairdressing (29%) (P = 0.001)(Table 12). There was also variation of rate with local government area of residence from Northwest (0%) to Lagelu (14%) (P = 0.003)(Table 13).

Table 12: Carriage of *Salmonella enterica* in pregnant women based on occupation

Occupation	Number Tested	Number of Positive for *Salmonella* (%)
House wives	36	3(8)
Student	21	0(0)
Civil Service	96	9(9.4)
Hair Dressing	42	12(29)
Trading	612	39(6)
Fashion Designing	135	6(4)
Others	18	0(0)
Total	960	69 (7.2)

Table 13: Local government area variation of carriage of *salmonella enterica* in pregnant women

Local Government Area	**Number Tested**	**Number Positive for *Salmonella* (%)**
Lagelu	21	3(14)
South-West	99	6(6)
North-East	318	24(8)
North-West	54	0(0)
South-East	138	12(9)
North	252	24(10)
Other	78	0(0)
Total	960	69 (7.2)

Antibiotic resistance profiles of isolates of *Salmonella*

All the *Salmonella enterica* isolates were generally susceptible to amoxicillin-clavulanic acid, cefotaxime, ceftazidime, ceftriaxone, ceftiofur, imipenem, florfenicol, colistin, apramycin, kanamycin and amikacin. Resistance rates to other antibiotics ranged from 7.0 % (ciprofloxacin) to 21.8% (ampicillin) (Table 14). Resistance of the *Salmonella* isolates also varied with sources.

The bacteremic isolates were generally more resistant to antibiotics compared to those from healthy humans. The bacteremic isolates showed high resistance to ampicillin (46.9%), cotrimoxazole (46.9%), sulfamethoxazole (42.9%), trimethoprim (42.9%), streptomycin (34.7%), chloramphenicol (28.6%), tetracycline (24.5%), and low resistance was found to spectinomycin (14.3%). While the resistance of healthy human isolates to ampicillin, tetracycline, streptomycin, cotrimoxazole, sulfamethoxazole, trimethoprim and chloramphenicol range from 2.4% (to each of cotrimoxazole, sulfamethoxazole and trimethoprim) to 26.8% (to ampicllin) (Table 14).

Isolates of *Salmonella enterica* from animals showed resistance to more antibiotics and generally higher resistance rates compared to bacteremic and healthy human isolates. The isolates from chickens generally showed higher resistance than those from other animals. The chicken isolates showed high resistance to, tetracycline (66.1%), nalidixic acid (64.8), sulfamethoxazole (63.6%), streptomycin (36.4), gentamicin (35.2%), ampicillin (34.6%), trimethoprim (32.7%), cotrimoxazole (32.1%), ciprofloxacin (30.3%), spectinomycin (28.1%), while low resistance was seen toward neomycin (5.5%). The isolates of *Salmonella enterica* from pigs, goat, cow and sheep showed low resistance to antibiotics (Table 14). The isolates from environment showed low resistance to tetracycline (13.6%), ampicillin (9.1%) and sulfonamides (4.5%).

Resistance to quinolones (nalidixic acid and ciprofloxacin): Overall, 108 (15.2%) isolates of *Salmonella enterica* were resistant to nalidixic acid; these include one clinical isolate and 107 isolates from chickens. Resistance to nalidixic acid was low, 2.0% (1 of 49), in the bacteremic isolates compared to high resistance, 64.8% (107 of 165), amongst the chicken isolates (Table 14).

Overall, only 50 (7.0%) isolates of *Salmonella enterica* were resistant to ciprofloxacin; all of which were from chicken. Resistance rate to ciprofloxacin amongst the isolates from chicken was 30.3% (50 of 165) (Table 14).

Resistance of the *Salmonella enterica* isolates to any one antibiotic was highest in chicken (87.9%) followed by clinical isolates (51%), asymptomatic isolates (26.8%), sheep isolates (18.2%), goat isolates (10%), pig isolates (8.9%), cow isolates (7.1%) (Table 15).

Resistance to more than two antibiotics (multi-drug resistance) was found mainly among isolates from chicken (71.5%), clinical isolates (71.5%) and asymptomatic isolates (4.9%) (Table 16).

Table 17 shows the major resistance phenotypes; these are resistance phenotypes exhibited by at least two isolates of *Salmonella*. The major resistance phenotypes amongst the clinical isolates were multidrug resistance to ampicillin, streptomycin, tetracycline, and cotrimoxazole (5 isolates); ampicillin, chloramphenicol, spectinomycin, streptomycin, tetracycline and cotrmoxazole (4 isolates); ampicillin, chloramphenicol and cotrimoxazole (4 isolates); ampicillin, chloramphenicol, spectinomycin, streptomycin and cotrimoxazole(3isolates); ampicillin, chloramphenicol, streptomycin and cotrimoxazole (2 isolates); and resistance to ampicillin (2 isolates). The major resistance phenotypes amongst the isolates from asymptomatic

humans were: resistance to only ampicillin (24 isolates); multidrug resistance to ampicillin and streptomycin (18 isolates); ampicillin, chloramphenicol, streptomycin and tetracycline (6 isolates); ampicillin, chloramphenicol and streptomycin (6 isolates); and ampicillin and tetracycline (6 isolates).

The major resistance phenotypes amongst the isolates from chickens were multidrug resistance to nalidixic acid, tetracycline and cotrimoxazole (31 isolates); ampicillin, ciprofloxacin, gentamicin, streptomycin, nalidixic acid, spectinomycin, sulfamethoxazole and tetracycline (30); ciprofloxacin, gentamicin, streptomycin, nalidixic acid, spectinomycin, sulfamethoxazole and tetracycline (15); streptomycin and tetracycline (9); ampicillin, gentamicin, nalidixic acid, tetracycline and cotrimoxazole (8 isolates); streptomycin, sulfamethoxazole and tetracycline (7 isolates); nalidixic acid, streptomycin, tetracycline, and cotrimoxazole (5 isolates); ampicillin, gentamicin, nalidixic acid, neomycin, tetracycline and cotrimoxazole (4 isolates); ampicillin, ciprofloxacin and nalidixic acid (3 isolates); streptomycin, tetracycline and nalidixic acid (2 isolates); and ampicillin and tetracycline (2 isolates) (Table 17).

Multidrug resistance to ampicllin, chloramphenicol and cotrimoxazole

Multi-drug resistance to antibiotics commonly use in treatment and the most readily available (ampicillin, chloramphenicol and cotrimoxazole) was observed only in clinical, asymptomatic and chicken isolates of *Salmonella enterica*. The multi-drug resistance phenotypes encompassing these antibiotics that were observed in the clinical isolates were: ampicillin, chloramphenicol, and cotrimoxazole (26.5%); ampicillin and cotrimoxazole (14.3%); and ampicillin and chloramphenicol (2.0%). Only multidrug resistance to ampicillin and chloramphenicol (4.8%)

was found in the isolates from asymptomatic isolate*s*. Multidrug resistance to ampicillin and cotrimoxazole (7.9%) was found in the chicken isolates (Table 18).

Susceptibility to extended-spectrum β-lactams

All the isolates were susceptible to the tested extended-spectrum β-lactam antibiotics (ceftriazone, ceftazidime and imipenem).

Reduced susceptibility to ciprofloxacin (ciprofloxacin MIC 0.125 to 1.0μg/mL)

Only one clinical isolate of *Salmonella enterica* (2.0%) showed reduced susceptibility to ciprofloxacin; while 36.9% (61 of 165) *Salmonella* isolates from chicken showed reduced susceptibility to ciprofloxacin.

Resistance of ciprofloxacin-resistant isolates to other antibiotics: The ciprofloxacin-resistant isolates also showed high resistance to ampicillin, gentamicin, streptomycin, spectinomycin, tetracycline, sulfamethoxazole and nalidixic acid with rates ranging from 76% to 100% (Table 19). Forty-nine (98%) of the ciprofloxacin-resistant isolates showed multidrug resistance (resistance to more than two other antibiotic*s)*. The most common resistance phenotypes were multidrug resistance to ampicillin, ciprofloxacin, streptomycin, nalidixic acid, spectinomycin, sulfamethoxazole and tetracycline (60%, 30 of 50 isolates); and ciprofloxacin, streptomycin, nalidixic acid, spectinomycin, sulfamethoxazole and tetracycline (30%, 15 of 50 isolates) (Table 20).

Antimicrobial susceptibility of *Salmonella* serovars from bacteremic patients

Antibiotic susceptibility varied with serovar*s*. Antibiotic resistance was found mainly amongst the isolates of serovars Dublin, Enteritidis and Typhimurium. Three of the seven *S*. Dublin isolates were showed multidrug resistance to ampicillin, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim; the remaining four isolates were pansusceptible (Tables 22 and 24)

Antimicrobial resistance to more than one antibiotic was observed in eleven of the 13 *S*. Enteritidis isolate*s*. Four *S*. Enteritidis isolates conferred resistance to ampicillin, chloramphenicol, sulfamethoxazole, trimethoprim and two isolates additionally also conferred resistance to streptomycin. One of the remaining five *S*. Enteritidis isolates was resistant to ampicillin, trimethoprim, streptomycin, nalidixic acid, and tetracycline. None of the 13 *S*. Enteritidis isolates were pansusceptible. All the *S*. Typhimurium isolates conferred resistance to ampicillin, chloramphenicol, spectinomycin, streptomycin, sulfamethoxazole and trimethoprim. Six of seven *S*. Typhi isolates were pansusceptible while the remaining one isolate was resistant to only streptomycin.

Antibiotic profiles of *Salmonella* serovars from chicken

All of the nine *Salmonella* serovars obtained from chicken were susceptible to apramycin, ceftiofur, chloramphenicol, colistin, florfenicol, and cefotaxime. In contrast, a high level of antimicrobial resistance was observed to tetracycline (n=105, 92.1%), nalidixic acid (n=101, 88.6%), sulfamethoxazole (n=101, 88.6%), Gentamicin (n=54, 47.4) and trimethoprim (n=53, 46.5%), respectively (Table 22).

High level resistance to ciprofloxacin (MIC ≥ 2μg/mL) was observed in 44 (43.6%) isolates; all of which were *S.* Kentucky. Furthermore, 87.1% (n=61) of the isolates exhibited reduced susceptibility to ciprofloxacin. The reduced susceptibility to ciprofloxacin was observed to all of the following serovars: *S.* Virchow (n=50, 100%), *S.* Derby (n=4, 100%), *S.* Haifa (n=2, 100%), *S.* Bredney (n=1, 100%), *S.* Onireke (n=1, 100%), *S.* Mbandaka (n=2, 33.3%), and *S.* Muenster (n=1, 20.0%), respectively (Table 22). However, the four isolates of *S.* Derby were all susceptible to nalidixic acid but exhibited reduced susceptibility to ciprofloxacin.

Antimicrobial susceptibility revealed that the three most common serovars, *S.* Virchow (n=50), *S.* Kentucky (n=44), *S.* Mbandaka (n=6), and *S.* Muenster (n=5), to be the most resistant. Those serovars showed resistance to ten (*S.* Virchow), ten (*S.* Kentucky), nine (*S.* Muenster) and eight (*S.* Mbandaka) of the seventeen tested antimicrobials, respectively (Table 24).

Six resistance patterns were observed to *S.* Virchow; most (n=31, 62.0 %,) were resistant to nalidixic acid, sulfamethoxazole, tetracycline and trimethoprim; while only 8.0% (n=4) showed multidrug resistance to seven antibiotics ampicillin, gentamicin, nalidixic acid, neomycin, sulfamethoxazole, tetracycline, and trimethoprim (Table 24).

Table 14: Antibiotic resistance of *Salmonella enterica* isolates to antibiotics

Sources	No. of isolates	No. (%) resistance to												
		Ampicillin	Amoxillin-clavulanic	Tetracyline	Streptomycin	Spectinomycin	Cotrimoxazole	Chloramphenicol	Sulfamethoxazole	Gentamycin	Trimethoprim	Nalidixic acid	Ciprofloxacin	Neomycin
Febrile patients	49	23 (46.9)	0	12 (24.5)	17 (34.7)	7 (14.3)	21(42.9)	14 (28.6)	21(42.9)	0	21(42.9)	1(2.0)	0	0
Asymptomatic humans	251	67 (26.8)	0	12 (4.9)	42 (17.1)	0	6 (2.4)	12 (4.9)	6(2.4)	0	6(2.4)	0	0	0
All animals	367	61 (16,6)	0	109 (29.7)	66 (17.9)	45 (12.3)	55 (14.9)	1 (0.3)	107 (29.2)	58 (15.8)	54 (14.7)	107 (29.2)	50 (13.6)	9 (1.4)
Chicken	165	57 (34.6)	0	109 (66.1)	60 (36.4)	45 (28.1)	53 (32.1)	0	105 (63.6)	58 (35.2)	54 (32.7)	107 (64.8)	50 (30.3)	9 (5.5)
Pigs	64	0	0	0	4 (6.3)	0	0	0	0	0	0	0	0	0
Goat	75	0	0	0	2 (2.7)	0	2 (2.7)	0	2(2.7)	0	2(2.7)	0	0	0
Cattle	28	1 (3.6)	0	0	0	0	0	1 (3.6)	0	0	0	0	0	0
Sheep	35	3 (8.6)	0	0	3 (8.6)	0	0	0	0	0	0	0	0	0
Enviroment	44	4 (9.1)	0	6 (13.6)	0	0	2 (4.5)	0	2(4.5)	0	2(4.5)	0	0	0
All sources	711	155 (21.8)	0	139 (19.5)	115 (16.2)	52 (7.3)	84 (11.8)	27 (3.8)	130 (18.3)	58 (8.2)	77 (10.8)	108 (15.2)	50 (7.0)	0

Table 15: Antibiotic resistance of *Salmonella enterica* isolates to at least one antibiotic

Sources	No. Resistant	% resistant
Febrile patients (n=49)	25	51
Asymptomatic (n=251)	67	26.8
Chicken (n=165)	145	87.9
Cattle (n=28)	2	7.1
Sheep (n=35)	6	18.2
Goat (n=75)	8	10
Pig (n=64)	6	8.9
Environment (n=44)	10	22.7

Table 16: Antibiotic resistance of *Salmonella enterica* isolates to more than two antibiotics

Sources	No. resistant	% resistant
Febrile patients (n=49)	35	71.4
Asymptomatic (n=251)	12	4.9
Chicken (n=165)	118	71.5
Cattle (n=28)	0	0
Sheep (n=35)	0	0
Goat (n=75)	0	0
Pig (n=64)	1	1.6
Enviromental (n=44)	0	0

Table 17: Major resistance phenotypes of *Salmonella enterica* isolates

Resistance phenotypes	No. with resistance phenotypes							
	Febrile patients (n=49)	Asymptomatic (n=251)	Cattle (n=28)	Pig (n=64)	Goat (n=75)	Sheep (n=35)	Chicken (n=165)	Enviroment (n=44)
Am	2	24	1	0	8	3	0	3
Sm	1	6	0	4	0	3	1	0
Te	0	0	0	0	0	0	5	5
AmSm	0	18	0	0	0	0	0	0
AmTe	0	6	0	0	0	0	2	1
SmTe	0	0	0	0	0	0	9	0
SmTeNa	0	0	0	0	0	0	2	0
AmCmSm	0	6	0	0	0	0	0	0
AmCmSmTe	0	6	0	0	0	0	0	0
AmCmSmCot	2	0	0	0	0	0	0	0
AmCpGenSmNalSpSmxTe	0	0	0	0	0	0	30	0
AmGenNalTeCot	0	0	0	0	0	0	8	0
CpGenSmNalSpSmxTe	0	0	0	0	0	0	15	0
NalSmTeCot	0	0	0	0	0	0	5	0
NalTeCot	0	0	0	0	0	0	31	0
AmCmCot	4	0	0	0	0	0	0	0
AmCmSpSmCot	3	0	0	0	0	0	0	0
AmCmSpSmTeCot	4	0	0	0	0	0	0	0
AmCpNal	0	0	0	0	0	0	3	0
AmGenNalNeoTeCot	0	0	0	0	0	0	4	0
AmSmTeCot	5	0	0	0	0	0	0	0
SmSmxTe	0	0	0	0	0	0	7	0

Keys: Am (ampicillin); Cm (chloramphenicol); Sm (streptomycin); Cot (cotrimoxazole); Te (tetracycline); Gen (gentamycin); Sp (spectinomycin); Nal (nalidixic acid); Cp (ciprofloxacin); Smx (sulfamethoxazole); Neo (neomycin)

Table 18: Mutidrug resistance to ampicillin, chloramphenicol and cotrimoxazole

Resistance phenotypes	No. (%) with resistance phenotypes							
	Febrile patients (n=49)	Asymptomatic (n=251)	Cattle (n=28)	Pig (n=64)	Goat (n=75)	Sheep (n=35)	Chicken (n=165)	Enviroment (n=44)
AmCm	1 (2.0)	12 (4.8)	0	0	0	0	0	0
AmCot	7 (14.3)	0	0	0	0	0	13 (7.9)	0
CmCot	0	0	0	0	0	0	0	0
AmCmCot	13 (26.5)	0	0	0	0	0	0	0

Table 19: Resistance of ciprofloxacin resistant *Salmonella enterica* to other antibiotics

Antibiotics	No. (%) resistance
Ampicillin	38 (76)
Gentamicin	44 (88)
Streptomycin	44 (88)
Spectionomycin	44 (88)
Tetracycline	44 (88)
Sulfamethoxazole	44 (88)
Nalidixic acid	50 (100)
Neomycin	1 (2)

Table 20: Resistance phenotypes of ciprofloxacin-resistant *Salmonella enterica* isolates from chicken

Resistance phenotypes[a]	No. of isolates (%)
AmCpGnSmNalSpSuTe	30(60)
CpGnSmNalSpSuTe	15(30)
AmCpNal	3(6)
AmCpGnSmNalNeSpSuTe	1(2)
CpNal	1(2)

Table 21: Frequency of antimicrobial resistance in isolates of different *Salmonella* serovars from bacteremic patients

	Number and (percentages) of antimicrobial resistance to the following antimicrobials:																
Serovars	Amoxicillinþ+ Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Cliprofloxacin	Colistin	Florfenicol	Cefotaxime	Gentamicin	Nalidixic acid	Neomycin	Spectinomycin	Streptomycin	Sulfamethoxazole	Tetracyclin	Trimethoprim
S. Apapa (n=1)	0 (0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0)	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)
S. Dublin (n=7)	0 (0)	3 (42.9)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	3 (42.9)	3 (42.9)	3 (42.9)	3 (42.9)
S. Enteritidis (n=13)	0(0)	12 (92.3)	0 (0.0)	0 (0.0)	7 (53.8)	1 (7.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (7.7)	0 (0.0)	0 (0.0)	5 (38.5)	10 (76.9)	4 (30.8)	10 (76.9)
S. Infantis (n=1)	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. Jukestown (n=1)	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. Monschaui (n=1)	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. Oritamerin (n=1)	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. Typhi (n=7)	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)	0 (0.0)	0 (0.0)
S. Typhimurium (n=7)	0 (0)	7 (100.0)	0 (0.0)	0 (0.0)	7 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	7 (100.0)	7 (100.0)	7 (100.0)	4 (57.0)	7 (100.0)
Total (n=39)	0 (0)	23 (58.9)	0 (0.0)	0 (0.0)	14 (35.9)	1 (2.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (2.6)	0 (0.0)	7 (17.9)	17 (43.6)	21 (53.8)	12 (30.8)	21 (53.8)

Table 22: Frequency of antimicrobial resistance of isolates of *Salmonella* serovars from chicken

		Number (percentages)of antimicrobial resistance to the following antimicrobials:																
Serovars	Amoxicillinp+ Clavulanic acid	Ampicillin	Apramycin	Ceftiofur	Chloramphenicol	Cliprofloxacin[a]	Ciprofloxacin[b]	Colistin	Florfenicol	Cefotaxime	Gentamicin	Nalidixic acid	Neomycin	Spectinomycin	Streptomycin	Sulfamethoxazole	Tetracyclin	Trimethoprim
S. Bredney (n=1)	0 (0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0))	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)
S. Derby (n=4)	0 (0)	3 (75.0)	0 (0.0)	0 (0.0)	0 (0.0)	4 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (25.0)	0 (0.0)	3 (75.0)	0 (0.0)
S. Haifa (n=2)	0 (0)	1 (50.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (50.0)	2 (100.0)	1 (50.0)	0 (0.0)	1 (50.0)	2 (100.0)	2 (100.0)	2 (100.0)
S. Havana (n=1)	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. Kentucky (n=44)	0 (0)	32 (72.7)	0 (0.0)	0 (0.0)	0 (0.0)	NA[c]	44 (100)	0 (0.0)	0 (0.0)	0 (0.0)	40 (90.9)	44 (100.0)	1 (2.3)	40 (90.9)	40 (90.9)	40 (90.9)	40 (90.9)	0 (0.0)
S. Muenster (n=5)	0 (0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (20.0)	1 (20.0)	0 (0.0)	0 (0.0)	4 (80.0)	4 (80.0)	4 (80.0)	1 (20.0)
S. Mbandaka (n=6)	0 (0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (33.3)	1 (16.7)	1 (16.7)	5 (83.3)	6 (100.0)	5 (83.3)	1 (16.7)
S. Onireke (n=1)	0 (0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
S. Virchow (n=50)	0 (0)	12 (24.0)	0 (0.0)	0 (0.0)	0 (0.0)	50 (100.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	12 (24.0)	50 (100.0)	6 (12.0)	0 (0.0)	5 (10.0)	49 (98.0)	50 (100.0)	49 (98.0)
Total (n=114)	0 (0)	51 (44.7)	0 (0.0)	0 (0.0)	0 (0.0)	61 (87.1)[d]	44 (43.6)	0 (0.0)	0 (0.0)	0 (0.0)	54 (47.4)	101 (88.6)	9 (7.9)	41 (36.0)	56 (49.1)	101 (86.6)	105 (92.1)	53 (46.5)

[a]Reduced susceptibility to ciprofloxacin (MIC, >0.125μg/mL)

[b]Full resistance to ciprofloxacin (MIC, >2μg/mL); [c]Not applicable; [d]Excluding 44 ciprofloxacin resistant *S.* Kentucky isolates

Table 23: Resistance phenotypes of serovars of *S. enterica* isolates from bacteremic patients

Serovars	Resistance phenotypes	No of isolates
Dublin (n=7)	AMP, STR, SMX, TET, TMP	4
	Pansusceptible	3
Enteritidis (N=13)	AMP	2
	AMP,CHL	1
	STR, SMX, TET, TMP	1
	AMP, SMX, TET, TMP	1
	AMP, STR, SMX, TET, TMP	1
	AMP, NAL, STR, SMX, TET,TMP	1
	AMP, CHL, SMX, TMP	4
	AMP, CHL, STR, SMX, TMP	2
Typhimurium (n=7)	AMP, CHL, SPE, STR, SMX,TET, TMP	4
	AMP, CHL, SPE, STR, SMX, TMP	3
Typhi (n=7)	STR	1
	Pansusceptible	6
Others (n=5)	AMP, STR, SMX, TET, TMP	1
	Pansusceptible	4

Table 24: Resistance phenotypes of serovars of *S. enterica* isolates from chicken

Serovars	Resistance phenotypes	No. of Isolates
Virchow (n=50)	NAL, SMX, TET, TMP	31
	AMP, GEN, NAL, SMX, TET, TMP	8
	NAL, STR, SMX, TET, TMP	5
	AMP, GEN, NAL, NEO, SMX, TET, TMP	4
	NAL, NEO, SMX, TET, TMP	1
	NAL, NEO, TET	1
Kentucky (n=44)	AMP, CP, GEN, STR, NAL, SPE,SMX, TET	28
	CP, GEN, STR, NAL, SPE, SMX, TET	11
	AMP, CP, NAL	3
	AMP, CP, GEN, STR, NAL, NEO, SPE, SMX, TET	1
	CP, NAL	1
Mbandaka (n=6)	STR, SMX, TET,	4
	NAL, NEO, SPE, STR, SMX, TET, TMP	1
	NAL, SMX	1
Muenster (n=5)	STR, SMX, TET,	3
	AMP, GEN, NAL, STR, SMX, TMP	1
	Pansusceptible	1
Derby (n=4)	AMP, TET	2
	AMP, STR, TMP	1
	Pansusceptible	1
Haifa (n=2)	AMP, GEN, NAL, SMX, TET, TMP	1
	NAL, NEO, STR, SMX, TET, TMP	1
Others	AMP, NAL, TET	1
	AMP, NAL	1
	Pansusceptible	1

Molecular investigation of antbiotic resistant *Salmonella enterica* isolates

Identification of β-lactamase genes: All the tested 155 ampicillin resistant *Salmonella enterica* isolates: clinical (23 isolates); healthy human (67 isolates); chicken (57 isolates); cattle (1 isolate); sheep (3 isolates) and environmental (4 isolates) were positive for β-lactamase enzyme production by nitrocefin test. But all the isolates were negative for extended-spectrum β-lactamase (ESBL) enzyme production by double disk synergy test. Forty of the isolates representative of the ampicillin-resistant *Salmonella enterica* isolates from all the sources: clinical (10), healthy human (10), chicken (12), sheep (3), cow (1) and environment (4) that were further subjected to iso-electric focusing (IEF) to determine the β-lactamase enzyme type*s* produced a β-lactamase enzyme that co-focused with *Tem*-type β-lactamase enzyme. Moreover, all the 40 IEF positive isolates yielded amplicons of expected size (770bp) by polymerase chain reaction using the specific *Tem* primers (Figure 6). Two of the Tem amplicons were sequenced and the two sequences were confirmed to be *Tem* type genes.

Transferability of the *Tem* gene was evaluated by conjugation and Tem-polymerase chain reaction in all the 40 isolates positive for *Tem* type β-lactamase; transconjugants were only obtained in 17 (42.5%) of the forty isolate*s*. The 17 transconjugants included: 5 of 10 (50%) (bacteremic patients), 4 of 10 (40%)(healthy humans), 4 of 12 (33.3%)(chicken), 2 of 3 (sheep), 1 of 1 (cow) and 2 of 4 (environment) isolate*s*. The transconjugants from bacteremic patients included S. Dublin (one isolate); *S*. Enteritidis (six isolates) and *S*.Typhimurium (two isolates). The transconjugants from chicken included *S*. Kentucky (three isolates from poultry farms J, K and L) and *S*. Virchow (one isolate from farm J).

All the *Salmonella enterica* isolates were negative for SHV and OXA-1 β-lacatamase enzymes by polymerase chain reaction.

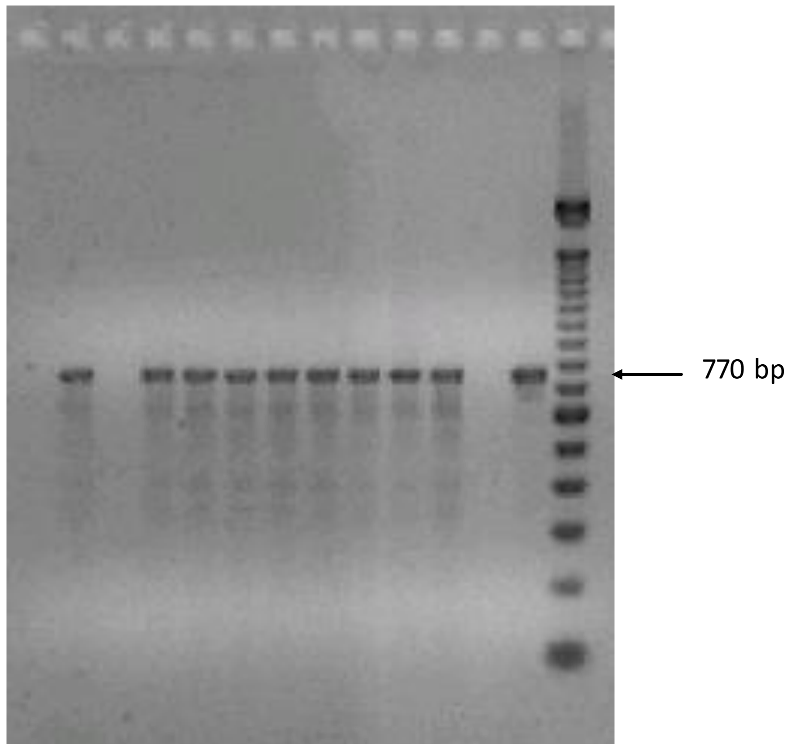

Figure 6: Amplification of bla_{Tem} gene from selected *Salmonella* isolates using TEMprime1F and TEMprimeEndR primers.

Lanes: 1 and 12 (negative controls); 3 (ampicillin sensitive *Salmonella* strain); 2, 4 to 6 (clinical isolates); 7 to 11 (chicken isolates); 13 (Positive control strain)

Prevalence of Integrons: Thirty one of the tested 48 (64.6%) antibiotic resistant *Salmonella* isolates were positive for class 1 integrons by polymerase chain reaction, while none was positive for class 2 and class 3 integrons. High prevalence rates were obtained in isolates from the bacteremic patients (75 %), chicken (76.6%) and healthy humans (100%) (Table 25). The positive isolates from bacteremic patients included serovars *S*. Enteritidis, *S*. Typhimurium and *S*. Dublin. While the positive isolates from chicken included serovars *S*. Kentucky and *S*. Virchow.

Correlation of the integron positive *Salmonella enterica* isolates with their resistance phenotypes showed that they were resistant to one to eight different antibiotics including ampicillin, chloramphenicol, streptomycin, spectionomycin, gentamicin, cotrimoxazole, tetracycline, nalidixic acid and ciprofloxacin (Tables 27 and 28).

Two amplicons of class 1 integrons of sizes 800bp and 1600bp were obtained from the positive *Salmonella enterica* isolates (Figures 9 and 10) but none of the isolates yielded the two amplicons simultaneously. The 1600bp amplicon was obtained in 77.4% (24 of 31) of the positive isolates including isolates from bacteremic patients, chickens (from different farms), goat and cow. The 800bp amplicon was obtained in 35.3% (6 of 17) of positive isolates from the bacteremic patients and 33.3% (1 of 3) of positive isolates from the healthy humans while the amplicon was not obtained in any of the positive animal isolates.

Endonuclease restriction of the 1600bp amplicons was only achieved by *Hinf1* endonuclease. While partial or no restriction was obtained with other endonucleases (*Pst1, BCN and HindIII*) (Figures 11, 12 and 13). All the 1600bp amplicons from the bacteremic isolates yielded similar restriction patterns that were different from those of animals (Figure 9).

The sequencing of 1600bp amplicon from an isolate of *Salmonella* Kentucky to know the integrated resistance gene cassettes yielded *aaCA5 and aadA7* as the integrated resistance gene cassettes.

Transfer of integrons from positive *Salmonella* isolates to E. coli J53.2 as recipient was obtained in 25.8% (8 of 31) of the integron positive isolates. The eight isolates that transferred their integrons included three isolates from febrile patients with serovars *S*. Enteritidis (two isolates) and *S*.Typhimurium (one isolates). The other five isolates were from chicken from different farms with serovars *S*. Kentucky (three isolates including the sequenced isolate) and *S*. Virchow (two isolates).

Table 25: Relative occurrence of class 1 integrons in *Salmonella* entrica from different sources

Sources	No. positive (%)
Febrile patient (n=16)	12 (75)
Chicken (n=17)	13 (76.5)
Asymptomatic (n=3)	3 (100)
Cattle (n=5)	2 (40)
Goat (n=1)	1(100)
Pig (n=2)	0 (0)
Environment (n=2)	0 (0)
Total(n=48)	31(64.6%)

Table 26: Resistance phenotypes of integron positive *Salmonella enterica* isolates that yielded PCR amplicons of size 1600bp

Resistance phenotypes[a]	Total isolates pos.	Source (number)/serovars
Sm	1	Cow (1)
SmTe	3	Chicken (3)
AmSm	1	Cattle (1)
AmCmSm	3	Febrile patient(1); Asymptomatic (2)
AmSmTe	1	Febrile patient (1)
AmCmSpeSmSuTeTmp	1	Febrile patient (1)/Typhimurium
AmCmSpeSmSuTmp	1	Febrile patient (1)/Typhimurium
AmCmSmCot	2	Febrile patient (2)/Enteritidis
AmCpGnSmNalSpSuTe	4	Chicken (4)/Kentucky
AmGenNalSmxTeTmp	3	Chicken (3)/Virchow
CpGnSmNalSpSuTe	3	Chicken (3)/Kentucky

[a]Am (ampicillin); Cp(ciprofloxacin); Cot(cotrimoxazole); Gn(gentamicin); Sm(streptomycin); Nal(nalidixic acid); Sp(spectionmycin); Su(sulfamethoxazole); Te(tetracycline)

Table 27: Resistance phenotypes of integron positive *Salmonella enterica* isolates that yielded PCR amplicons of size 800bp

Resistance phenotypes[a]	Total isolates pos.	Source (number)/serovar
Am	1	Febrile patient (1)/Enteritidis
AmCmSm	2	Febrile patient (2)/Enteritidis
AmSmTeSuTmp	1	Febrile patient (1)/Dublin
AmCmSmTe	2	Asmptomatic (1); Febrile patient (1)/Enteritidis
AmSmTeNalSuTmp	1	Febrile patient (1)/Enteritidis

[a]Am (ampicillin); Cm(chloramphenicol); Sm(streptomycin); Nal(nalidixic acid; Te(tetracycline)

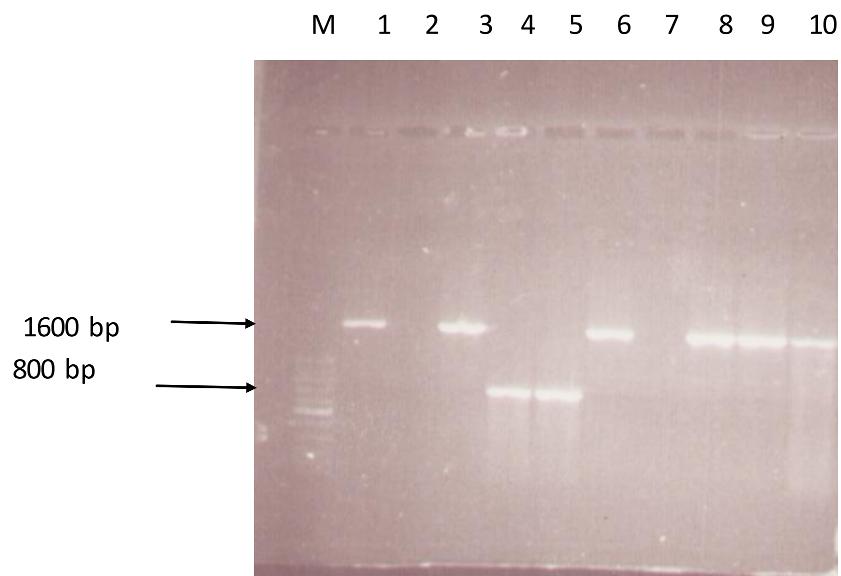

Figure 7: Polymerase chain reaction amplification of class 1 integron from antibiotic resistant *Salmonella enterica* isolates

Lanes: M (100bp ladder); 1 to 10 (isolates from febrile patients).

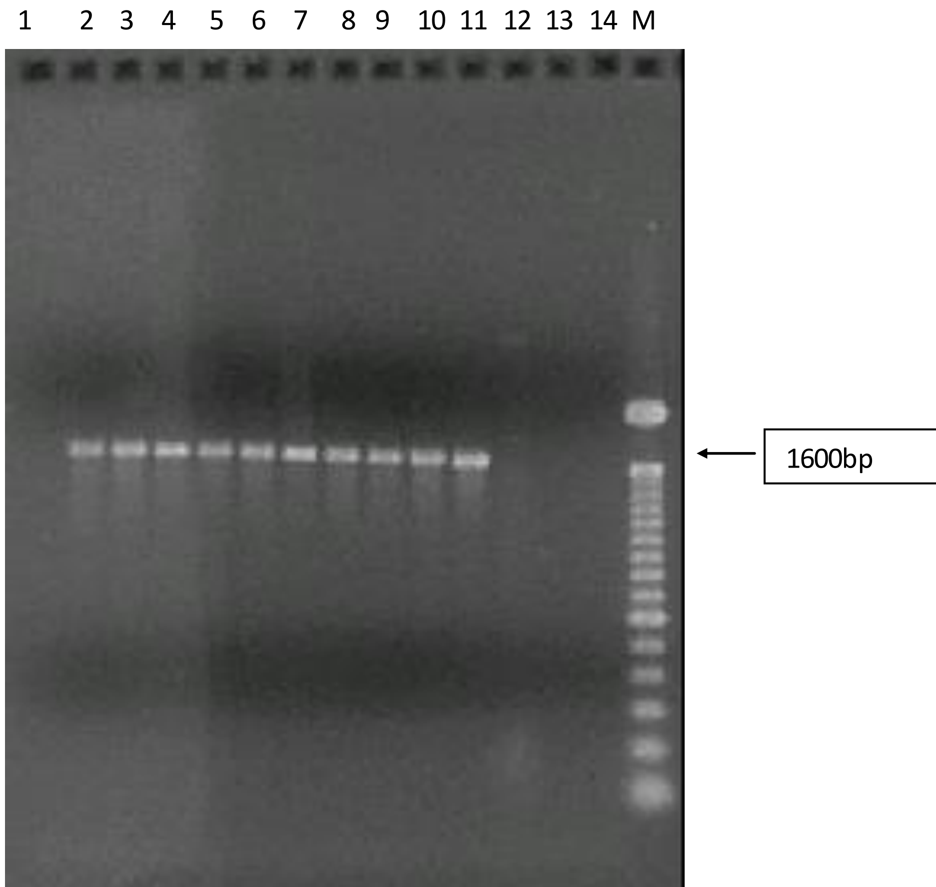

Figure 8: Polymerase chain reaction amplification of class 1 integron from antibiotic resistant *Salmonella enterica* isolates

Lanes: M (100bp ladder); 1, 12, 13 and 14 (negative controls); 2 to 11 (chicken isolates)

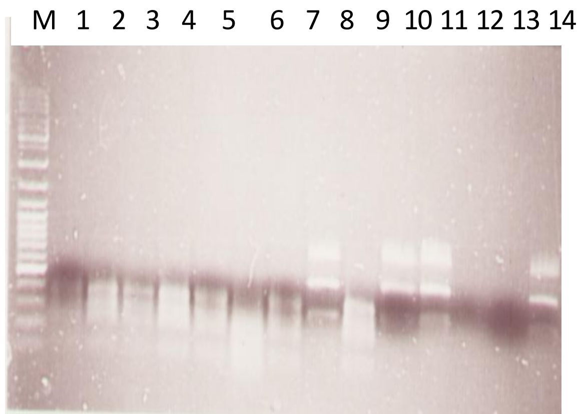

Figure 9: Restriction fragment length polymorphism of 1600bp integron amplicons from antibiotic resistant *Salmonella enterica* isolates obtained with *Hinf1*

Lanes : M (100bp ladder); 1 to 6 (clinical isolates); 7 and 9 (carrier isolates); 8,12 and 13 (chicken isolates); 10 and 11 (cattle isolates); 14 (goat)

M 1 2 3 4 5 6 7 8 9 10 11 12 13 14

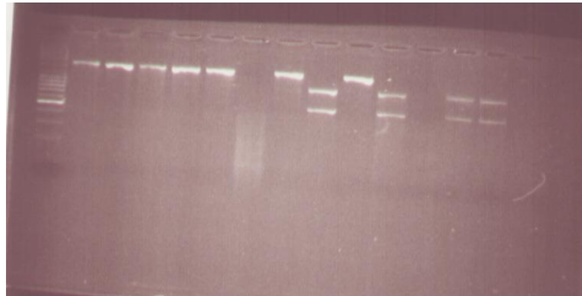

Figure 10: Restriction fragment length polymorphism of 1600bp integron amplicons from antibiotic resistant *Salmonella enterica* isolates obtained with *Pst1*

Lanes : M (100bp ladder); 1 to 6 (clinical isolates); 7 and 9 (carrier isolates); 8,12 and 13 (chicken isolates); 10 and 11 (cattle isolates); 14 (goat)

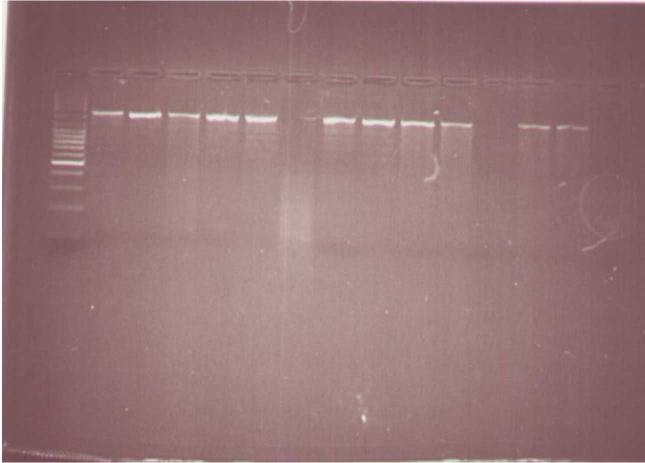

Figure 11: Restriction fragment length polymorphism of 1600bp integron amplicons from antibiotic resistant *Salmonella enterica* isolates obtained with *BCN*

Lanes : M (100bp ladder); 1 to 6 (clinical isolates); 7 and 9 (carrier isolates); 8,12 and 13 (chicken isolates); 10 and 11 (cattle isolates); 14 (goat)

Genotyping of ciprofloxacin-resistant *Salmonella enterica* isolates: Genotyping of the 44 ciprofloxacin-resistant *S.* Kentucky isolates by pulsed-field gel electrophoresis (PFGE) revealed a total of 10 PFGE patterns (P1a, P1b, P1c, P1d, P1e, P1f, P1g, P1h, P1i and P2). According to the criteria of Tenover *et al.,* 1995, the ten patterns can be broadly divided into two distinct PFGE types (P1 and P2) with the patterns P1a, P1b, P1c, P1d, P1e, P1f, P1g, P1h and P1i been subtypes of PFGE 1 since they differed by less than three bands. The dice correlation coefficient of all the PFGE patterns was 79% (Fig 10). PFGE P1a was the predominant (n=16, 36.4%). The predominant PFGE pattern (P1a) was observed in five different farms (B, I, L, M and K)(Figure 12, Table 28). The PFGE patterns P1b, P1d, P1e, P1g, P1i and P2 were observed in only one farm each: farms L, C, J, K, K and I respectively.

Only one PFGE pattern was found in the farms C, D and M; these were patterns P1d (in farm C), P1g (farm D), and P1a (farm M). At least two PFGE patterns were observed in the other six farms (Table 29). Four PFGE patterns were observed in each of farms J and K: PFGE patterns P1c, P1e, P1f, and P1g, and P1a, P1f, P1h and P1i respectively.

Forty of the 44 (90.9%) ciprofloxacin-resistant *Salmonella* isolates formed 6 different clusters (i, ii, iii, iv, v and vi) of indistinguishable PFGE patterns (100% similarity) (Figure 12). The clusters consistituted by different number of isolates: cluster i (16 isolates), cluster ii (8 isolates), cluster iii (2 isolates), cluster iv (9 isolates), cluster v (3 isolates) and cluster vi (2 isolates). Cluster i was the most predominant and constituted by isolates from five different farms: farm B (1 isolates), I (2 isolates), L (9 isolates), M (1 isolates) and K (3 isolates). Only cluster vi was constituted by isolates from only a single farm (K); other four clusters were constituted by isolates from two to five farms.

Correlation of pulsed-field gel electrophoresis (PFGE) patterns of the ciprofloxacin-resistant *Salmonella enterica* isolates with the antibiotic resistance phenotypes: As shown above all the 44 ciprofloxacin-resistant *Salmonella* isolates could also be divided into only five different resistance profiles which corroborated the limited genetic diversity observed by pulsed-field gel electrophoresis. Moreover, the two predominant and apparently similar resistance profiles R1 (resistance to ampicillin, ciprofloxacin, gentamicin, streptomycin, nalidixic acid, spectinomycin, and tetracycline) and R2 (ciprofloxacin, gentamicin, streptomycin, nalidixic acid, spectinomycin and tetracycline) accounted for 90% suggesting high degree of clonality.

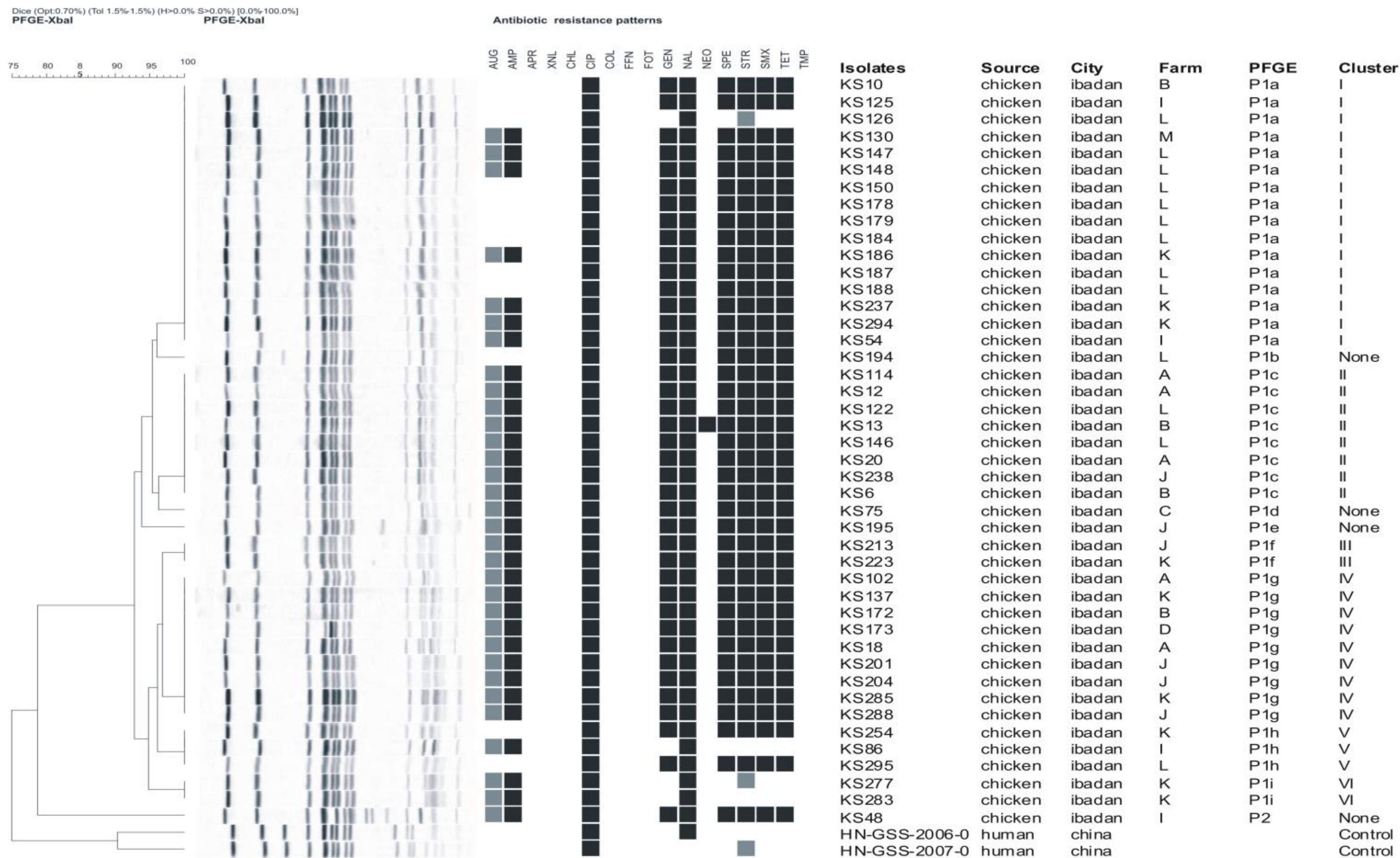

Figure 12: Pulsed-field gel electrophoresis analysis of ciprofloxacin-resistant isolates of *Salmonella enterica* showing generated dendrogram, PFGE patterns, antibiogram, farm distribution and cluster analysis.
Am (ampicillin); Cp(ciprofloxacin); Gn(gentamicin); Sm(streptomycin); Nal(nalidixic acid); Ne(neomycin); Kn(kanamycin); Sp(spectionmycin); Su(sulfamethoxazole);Te(tetracycline), Apr (apramycin); TMP (trimethoprim).
Black boxes(resistance); grey boxes or blank spaces (susceptible)

sulfamethoxazole and tetracycline) and R2 (resistance to ciprofloxacin, gentamicin, streptomycin, nalidixic acid, spectinomycin, sulfamethoxazole and tetracycline) which together accounted for 88% of the isolates and differed only by susceptibility to ampicillin was associated with nine of the observed PFGE patterns (P1a, ,P1b, P1c, P1d, P1e, P1f, P1g, P1h and P2) (Table 30) indicating limited variation in the ciprofloxacin-resistant isolates both phenotypically and genotypically.

Table 28: Poultry farm distribution of pulsed-field gel electrophoresis patterns of ciprofloxacin resistant isolates of *Salmonella enterica*

PFGE Patterns	Farm where observed (no of isolates)	Total isolates (%)
P1a	B(1), I(2), L(9), M(1), K(3)	16 (36.4)
P1b	L(1)	1 (2.3)
P1c	A(3), B(2), L(2),J(1)	8 (18.2)
P1d	C(1)	1(2.3)
P1e	J(1)	1(2.3)
P1f	J(1), K(1)	2 (2.6)
P1g	A(2), B(1), D(1), J(3), K(2)	9 (20.5)
P1h	I(1), K(2)	3 (6.8)
P1i	K(2)	2 (2.6)
P2	I(1)	1(2.3)

Table 29: Pulsed-field gel electrophoresis patterns observed in each of the poultry farms

Poultry farms	PFGE patterns observed (no of isolates)	Total isolates (%)
A	P1a(3), P1g(2)	5 (11.4)
B	P1a(1), P1c(2), P1g(1)	4(9.1)
C	P1b(1)	1(2.3)
D	P1g(1)	1(2.3)
I	P1a(2), P1h(1), P2(1)	4(9.1)
J	P1c(1), P1e(1), P1f(1), P1g(3)	6(13.6)
K	P1a(3), P1f(1), P1g(2),P1h(2), P1i(2)	10(22.7)
L	P1a(9), P1b(1), P1c(2)	12(27)
M	P1a(1)	1(2.3

Table 30: Antibiotic resistance profiles (antibiograms) of the ciprofloxacin-resistant *Salmonella enterica* isolates, associated pulsed-field gel electrophoresis patterns and farm distribution

Resistance phenotypes[a]	Farms where observed (number of isolates)	Associated PFGE patterns	Total isolates (%)
AmCpGnSmNalSpSuTe	A(5), B(2), D(1), I(2), J(7), k(6), L(4), M(1)	P1a, P1b, P1c, P1e, P1f, P1g, P2	28(63.6)
CpGnSmNalSpSuTe	B(1), I(2), K(1), L(7)	P1a, P1b, P1h, P1g	11(25.0)
AmCpNal	I(1), K(2)	P1h, P1i	3(6.8)
AmCpGnSmNalNeSpSuTe	B(1)	P1c	1(2.3)
CpNal	L(1)	P1a	1(2.3)

[a]Am (ampicillin); Cp(ciprofloxacin); Gn(gentamicin); Sm(streptomycin); Nal(nalidixic acid); Ne(neomycin); Kn(kanamycin); Sp(spectionmycin); Su(sulfamethoxazole); Te(tetracycline)

CHAPTER FIVE

DISCUSSION

This study shows *Salmonella* as the second commonest cause of bacteremia in Ibadan accounting for more than one-fifth of the bacteremic cases. This observation is similar to studies in other African countries that found *Salmonella* to be one of the leading causes of bacteremia (Vandenberg *et al.,* 2010).

The *Salmonella* bacteremic patients generally did not have specific clinical manifestation. About one-fifth of the non-typhoid bacteremic and all the typhoidal bacteremic patients presented as febrile illness with no specific localizing symptoms. This can lead to misdiagnosis and overuse of antibiotics. This is a common presentation of *Salmonella* bacteremia (Graham and English, 2009) and this indicates the need to strengthen laboratory infrastructures in our environment to pave the way for proper diagnosis and management of *Salmonella* bacteremia. The benefits include confidence in laboratory diagnosis and treatment of patients with the right antibiotics (Vandenberg *et al.,* 2010).

Over 90% of the *Salmonella* bacteremic patients were children (ages less than 15years old). This is a general observation in sub-Saharan African countries Graham *et al.,* 2000; Gordon *et al.,* 2008). The predisposing factors include malaria and malnutrition (Vandenberg *et al.,* 2010). *Salmonella* bacteremia is a significant marker of HIV-infection in adults. *Salmonella* was found to be the leading cause of bacteremia in HIV-infected adults patients in Lagos (Ogunsola *et al.,* 2009). The low prevalence of *Salmonella* bacteremia in adults in this study may signify low prevalence of HIV infection in the patients examined.

Salmonella bacteremia caused by S.Typhi and the uncommon *Salmonella* serovars occurred in older children compared to those caused by S. Enteritidis, S. Dublin and S. Typhimurium. This may be explained by the relative exposure and susceptibility of the different age groups to the sources of the different serovars. The sources need to be identified and targeted for control.

The overall positive isolation rate of *Salmonella* spp. from the human blood samples is comparable to the rate of 4.4% reported from Ethiopia ((Ghiorghis *et al.,* 1992) but higher than the rates of 1.0% and 2.0% from Kenya (Brent *et al.,* 2006) and Mozambique (Sigauque *et al.,* 2009) respectively. On the other hand, higher rates have been reported from other African countries such as Congo (7.06%) (Bahwere *et al.,* 2001) and Malawi (6.5% and 7.9%) (Walsh *et al.,* 2000; Gordon *et al.,* 2008). The skewed distribution of 1:0.5 between males and females are difficult to explain, but may be due to more males being examined or a higher susceptibility to infections in the male population.

The genus *Salmonella* is currently divided into 2,587 serovars (Grimont and Weill, 2007). Although all the serovars are able to infect humans, only a limited number of serovars are of significant public health importance. This study established the relative role played by different serovars of *Salmonella* bacteremia in Ibadan. Majority (51.2%) of the *Salmonella* bacteremia were caused by S. Enteritidis and S. Typhimurium, which is consistent with the findings of studies in other African countries that observed S. Enteritidis and S. Typhimurium to be the most common serovars causing human salmonellosis (Gordon, 2008; Gordon *et al.,* 2008 kingsley *et al.,* 2009; Morpeth *et al.,* 2009). It is interesting to note that the majority of the *S*. Enteritidis and *S*. Dublin were isolated from hospital A, B and C whereas the remaining hospitals found very few. *S*. Typhimurium was mostly isolated from hospital B. This observation tends to

suggest difference in prevalence rates of the serovars in different local government areas of Ibadan.

Epidemic increase in prevalence of *S.* Typhimurium has recently been linked to circulation of a particular MLST clone; ST 313 in sub-saharan African countries (Kingsley *et al.,* 2009). It is however, not determined if the *S.* Typhimurium ST313 clone has spread to Ibadan, Nigeria. A future study will reveal if this should be the case or if the isolates belong to the previously described clone of phage type U282 (Ojeniyi and Montefiore, 1986). Similarly, the clonality of invasive *S.* Enteritidis also needs to be investigated to identify if certain clones are responsible for causing bacteraemia compared to those causing mild gastroenteriti*s.*

It is also noteworthy that neither *S.* Enteritidis nor *S.* Typhimurium was isolated from chickens included in this study indicating that intensively raised chickens are not the reservoir for invasive serovars in human*s.* It should however, be noted that the samples obtained from chickens were from 2007 compared to the samples from humans which were from 2004 to 2005. *S.* Enteritidis and *S.* Typhimurium have previously in 2004 been isolated from poultry droppings and beef in Nigeria (Orji *et al.,* 2005). Other potential sources of infection by *S.* Enteridis and S. Typhimurium in Nigeria include wall geckos, snails (*Achatina achatina*) and pigeons (Obi and Nzeako, 1980; Okoh and Onazi, 1980; Oboegbulem and Iseghohimhen, 1985; Gugnani *et al.,* 1986).

This study showed S. Dublin as playing a significant role in the causation of invasive salmonellosis in Ibadan. *S.* Dublin was not reported as an important cause of invasive salmonellosis in some sub-saharan African countries (Graham *et al.,* 2000; Galanis etal., 2006; Brent *et al.,* 2006; Kariuki *et al.,* 2006; Gordon, 2008; Gordon *et al.,* 2008; Morpeth *et al.,* 2009)

but this serovar constitutes an important human pathogen in Upper Volta (Ricosse *et al.,* 1979). *S.* Dublin is host-adapted to cattle and highly invasive; it is transmitted to humans through beef, cheese and raw milk (Maguire *et al.,* 1992; Vaillant *et al.,* 1996; Allerberger *et al.,* 2002;). In many European countries, surveillance shows that *S.* Dublin is the most commonly isolated serovar from bovine meat, exceeding the level of *S.* Typhimurium (Anonymous, 2009). Only a few publications have described *S.* Dublin in animals from Nigeria and all of them also associate the serovar to cattle (Collard and Sen, 1956; Collard and Sen, 1962). Cattle farming are a thriving business in Nigeria because beef meat is highly consumed either in soup dishes or roasted (suya); other cattle products thereof such as raw milk and cheese are heavily consumed in Nigeria without adequate control measures to prevent spread of zoonotic agents to human. It would be interesting to determine the occurrence of *S.* Dublin among cattle in Nigeria.

The study further reveals that *S.* Typhi still constitutes a significant public health importance in Ibadan, Nigeria. Therefore, appropriate control measures such as vaccination and food safety measurements need to be put in place. Overall, the study supports other publications that invasive salmonellosis caused by NTS are of greater public health importance than the *S.* Typhi (Morpeth *et al.,* 2009).

Rarely isolated *Salmonella* serovars such as *S.* Apapa, *S.* Mouschaui, *S.* Jukestown and *S.* Oritamerin were observed causing invasive salmonellosis in human*s*. Severe human salmonellosis caused by those serovars have previously been linked to the exposure to reptiles and amphibians (Carlquist and Coates, 1947; Collard and Sen, 1960; Sharma *et al.,* 1977; Milstone *et al.,* 2006; Bertrand *et al.,* 2008; Cooke *et al.,* 2009) . Several studies have shown that reptiles are reservoirs for *Salmonella* that are able to affect humans and recently constitute a

significant public health problem in many countries due to captive pet reptiles (Bradley *et al.,* 2001; Mermin *et al.,* 2004; Pedersen *et al.,* 2009) . However, in Nigeria the infections are most likely not caused by captive pet reptiles but more likely household wall lizards. Lizards are found around houses in Nigeria, most commonly in the rural areas and sometimes inside houses, thus constituting a potential health risk. In addition, in some parts of Nigeria, snake meats are special delicacies; whereas, in some places, snakes are adorned. It is interesting to note that rarely isolated serovars that were obtained in this study, with the exception of *S.* Apapa, were isolated from samples obtained at more rural sampling sites thus suggesting the sources of infections to be reptile / environment associated. Wall geckos have previously been described as reservoirs of *Salmonella* in Nigeria and constituting a public health problem (Oboegbulem and Iseghohimhen, 1985; Mascher *et al.,* 1988) which is difficult to control due to the nature of these animals. However, information on the potential risk of invasive salmonellosis due to the exposure to reptiles and amphibians should be disseminated to the people at risk in rural areas.

Mortality rate among the *Salmonella* bacteremic patients was 12.2% (6 of 49). It is interesting to note that all the fatal cases were associated with non-typhoidal *Salmonella* (NTS). The NTS mortality rate is comparable to those reported in studies from sub-Saharan African countries (Bahwere *et al.,* 2001; Vandenberg *et al.,* 2010). A high mortality rate has also been reported for *S*. Typhi (Vandenberg *et al.,* 2010).

Salmonella infection can be followed by carriage which is characterized by asymptomatic excretion of *Salmonella* for varying lengths of time. Such carriers are also important sources of *Salmonella* infections. A high carriage rate (8.6%) was observed in the healthy people including pregnant women (7.2%). The carriage rate was significantly associated with history of diarrhoea

suggesting *Salmonella* as an important cause of gastrointestinal infections. *Salmonella* most commonly causes acute gastroenteritis, with symptoms including diarrhea, abdominal cramps, and fever (Hohman, 2001). It is worthy of note that the carriage rate was also high at the third trimester of gestational period, therefore these pregnant women could act as source of *Salmonella* infection to their newborns (Morpeth *et al.,* 2009). The highest carriage rate was from pregnant women from Lagelu local government area (14%) followed by Ibadan North (10%) and South-East (9%). It is interesting to note that no carriage was observed in pregnant women from the North-West.

Salmonellae are able to colonise the elimentary tracts of several animals including food-producing animals. The infected animals may not show symptoms of infection thus acting as carriers of *Salmonella*. Such animals act as reservoirs of *Salmonella* from which these pathogens can be transmitted to humans (as zoonotic agents) through contacts and consumption of contaminated meat and other animal products (Hald *et al.,* 2007; Boyen *et al.,* 2008). Such animal carriers pose an important threat to both animal and human health. This study shows high carriage rates of *Salmonella* in all the food animals investigated with the highest rate being in goats (15%) followed by chicken (13.7%). These findings underscore food animals as potential sources of *Salmonella* infection in our environment.

It is interesting to note the very high carriage rates of 20.0% and 26.1% in chickens from poultry farms J and K. The reason for the high prevalence rate in farms J and K may be due to limited data. Similarly, a high prevalence rate has also been reported from Maiduguri, northern Nigeria (Raufu *et al.,* 2009). Similarly, a high rate (37%) of *Salmonella* contamination of broiler farms has been reported from Algeria (Elgroud *et al.,* 2009). Chicken is also an important source of

Salmonella in Europe and this warranted the initiation of targeted control measures (Anonymous 2009b).

In this study, the predominant serovar in chicken was *S.* Virchow which is one of the most common serovars in chicken (Tibaijuka *et al.,* 2003; Weill *et al.,* 2004; Bertrand *et al.,* 2006; Weinberger *et al.,* 2006; Anonymous, 2009; Elgroud *et al.,* 2009; Ammari *et al.,* 2009) . In Europe, *S.* Virchow was listed as the seventh most common serovar isolated from broiler meat in 2007 (Anonymous, 2009). *S.* Virchow was observed in all farms except G and with a high prevalence of 23.9% and 12.5% in farm J and K. Additionally, S. Kentucky, another common serovar in chicken (Anonymous, 2009) was observed as the second most common serovar in this study. Interestingly, *S.* Enteritidis was not in this study isolated from chicken. In contrast, *S.* Enteritidis is the most predominant serovar in *Gallus gallus* flocks in EU with a prevalence of 37.6% (Annonymous, 2009). This difference may be explained by differences in import patterns, production systems, climate or feed. Recently, *S.* Hiduddify; a rare serovar was found to be the predominant serovar in chickens in Maiduguri, Northern Nigeria (Raufu *et al.,* 2009) but was also not isolated in the fecal samples of chicken in this study. The northern Nigeria is generally warmer and moreover, the chickens studied by Raufu *et al.* (2009) were largely free range in contrast to the intensively reared chicken we studied; this may suggest the presence of *S.* Hiduddify in the environment where chickens probably pick the serovar up with foodstuffs as they scavenge for foods in the environment; this needs to be confirmed in free range chicken in the south or by investigation of the environment in Maiduguri, Nigeria. However, we also isolated *S.* Onireke, a rare serovar in chickens from Ibadan, Nigeria. It can be speculated that the rare serovar found in chicken also originates from reptiles or the environment just like the rare

serovars obtained from invasive *Salmonella* in humans. Further research need to explore this hypothesis.

Although, none of the serovars obtained from chicken fecal samples was isolated from the analysed human blood samples, those serovars still constitute potential serious health risks for humans in Ibadan, Nigeria. In Europe, *S.* Virchow, *S.* Kentucky and *S.* Derby, which are also found in chicken in this study, are among the ten most frequent serovars causing human salmonellosis (Anonymous, 2009) and may be found in human stool samples from Ibadan, Nigeria if investigated. A plausible explanation why we could not link any of the serovars obtained from chickens to humans may be due to analysis of human blood and chicken fecal samples at different years suggesting time based shift in prevalence of serovars; analysis of only blood samples from human could also be responsible because all serovars of NTS are not equally invasive. Moreover, it is interesting to note that some of these serovars (*S.* Virchow, *S.* Mbandaka, *S.* Bredney and *S.* Onireke) have earlier been associated with human salmonellosis in Nigeria (Collard and Sen, 1960; Mascher *et al.,* 1988; Oboegbulam *et al.,* 1995).

Antibiotic treatment is crucial for proper management of invasive salmonellosis (Parry and Threlfall, 2008). On the overall, antibiotic resistance of the *Salmonella* isolates in this study varies with sources which is probably reflective of relative usage of antibiotic*s.* High resistance rates were observed among the human clinical isolates toward ampicillin, sulfamethoxazole and trimethoprim and chloramphenicol. Multidrug resistance to these old and commonly used antimicrobials is a public health problem (Parry and Threlfall, 2008) and could facilitate usage of fluoroquinolones and third generation cephalosporins for empiric treatment. *S.* Typhimurium was the most resistant of the serovars obtained from the bacteremic patients and exhibited resistance

to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, and trimethoprim. The *S.* Typhimurium isolates in this study match in many ways the resistance profile of the epidemic multidrug resistant invasive *S.* Typhimurium ST313 clone found in sub-saharan Africa (Kingsley *et al.,* 2009). This hypothesis need to be further investigated by MLST typing before concluded.

It is interesting that all the isolates of *S.* Typhi were susceptible to all the tested antibiotics, suggesting the efficacy of old antimicrobials for the treatment of typhoid fever. This is in constrast to high resistance rates to those antibiotics in *S.* Typhi isolates from Lagos (Akinyemi *et al.,* 2007).

Salmonella isolates from chickens were the most resistant compared to isolates from other food animals. This may not be unconnected to the observed high usage of antibiotics in chicken rearing under intensive farming. The *Salmonella* isolates from chicken were more commonly resistant to tetracycline, sulfamethoxazole and trimethoprim, nalidixic acid and and ciprofloxacin; this resistance may be attributed to indiscriminate use of antibiotics at recommended doses or at subtherapeutic doses as feed additives to promote growth and as chemotherapeutic agents to control epizootics on the farms (Adetosoye, 1980). The high level of resistance to nalidixic acid coupled with high level of reduced susceptibility and full resistance to ciprofloxacin is worrisome because fluoroquinolones are strategic in the treatment of invasive salmonellosis (Parry and Threfall, 2008). It is noteworthy that resistance to nalidixic and ciprofloxacin was observed on all the poultry farms; additionally, high level of ciprofloxacin resistance has also been observed in chickens in Maiduguri, northern Nigeria (Raufu *et al.,* 2009) thus indicating a serious threat to public health in Nigeria. Moreover, over 95% of the

ciprofloxacin resistant *Salmonella* isolates were also multiply resistant to non-quinolone antibiotics including ampicillin, tetracycline, streptomycin and sulfamethoxazole, therefore, these antibiotics can co-select for ciprofloxacin resistance; this finding is similar to other reports (Hakanen *et al.,* 2001; Izumiya *et al*., 2005; Cui *et al.,* 2008). The most resistant serovars from chicken were *S.* Kentucky and *S.* Virchow; these serovars are usually multiple resistant to antimicrobials (Weinberger *et al.,* 2004; Weill *et al.,* 2004; Doublet *et al.,* 2008; ; Lee *et al.,* 2009). This calls for regulation of antibiotic usage in Nigeria to stem spread of resistance to antimicrobials.

The setting in some of the poultry farms visited could actually encourage the spread of resistant bacteria to many other food animals thus potentiating the spread to humans; this is because many of the farms keep more than one kind of animal within close range probably to minimize the cost of production. Moreover, heaps of animal faeces with hovering flies were seen unattended in some farms; such flies could serve as transfer agents of the resistant bacteria. In fact, due to urbanization, some farms are now within residential areas thus increasing exposure to foodborne pathogens including *Salmonella* spp.

All the *Salmonella* isolates from all the sources were susceptible to extended-spectrum cephalosporins, therefore these antibiotics remain potent drugs for the treatment of salmonellosis in our environment. This finding may be partly due to less indiscriminate use of the extended-cephalosporins due to prohibitive high cost. Similarly, *Salmonella* isolates from Kenya and Malawi were susceptible to extended-spectrum cephalosporins (Gordon *et al.,* 2008; Kariuki *et al.,* 2006). This finding, however, is in contrast to reports of *Salmonella* resistance to extended spectrum cephalosporins from several other African countries like Tanzania (Blomberg *et al.,*

2005), Tunisia (Bouallegue-Gdet *et al.,* 2005), Morocco (AitMhand *et al.,* 2002), Algeria (Koeck *et al.,* 1997) and South Africa (Kruger *et al.,* 2004) and Egypt (Abdelghani *et al.,* 2010). *Salmonella* isolates resistant to extended cephalosporins have also been reported from other continents like Europe (Hopkins *et al.,* 2007), United States (Carattoli *et al.,* 2002; Whichard *et al.,* 2007) and Asia (Zaidi *et al.,* 2007). It is also notewhorthy that, contrary to the finding of this study, resistance to extended-spectrum cephalosporins have been reported in isolates of several other enterobacteria including *Klebsiella* spp., *Escherichia* coli and *Enterobacter* spp. in Nigeria (Aibinu *et al.,* 2003; Fashae *et al.,* 2004; Soge *et al.,* 2006; Kasap *et al.,* 2010).

Resistance of bacteria to β-lactam antibiotics is mainly due to the production of β-lactamases and several types have been reported from resistant bacteria. Resistance to extended-spectrum cephalosporins (one of the antibiotics of last choice for treatment) is due to production of extended-spectrum β-lactamases (ESBLs), which was first reported in *Salmonella* in 1988 and have since then been increasingly reported (Arlet *et al.,* 2006). The ESBLs were however not observed in any of the isolates of *Salmonella* in this study indicating low prevalence of ESBLs and further confirming the observed susceptibility of all the isolates to extended-spectrum cephalosporin*s.* In contrast to the findings of this study, several ESBLs have been reported in *Salmonella* isolates from some Sub-Saharan African countries including Algeria, Gambia, Libya, Mali, Morocco, Senegal, South Africa, Tanzania, Egypt and Tunisia (Arlet *et al.,* 2006; Usha *et al.,* 2008; Bouchrif *et al.,* 2009). The reported ESBLs in Africa include SHV-12, TEM-63, TEM-116, TEM-131, SHV-2, CTX-M-3, CTX-M-15 and CTX-37 (Arlet *et al.,* 2006; Bouchrif *et al.,* 2009; Abdelghani *et al.,* 2010). These enzymes have also been reported in Europe, United States and Canada (Parry and Threlfall, 2008).

The lack of observation of ESBLs in the *Salmonella* isolates is contrast to high prevalence of ESBLs in other species of enterobacteriaceae (*Klebsiella pneumonia*, *E. coli* and *Enterobacter cloacae)* in Nigeria (Aibinu *et al* 2003; Fashae *et al.,* 2004; Soge *et al.,* 2006; Kasap *et al.,* 2010). This observation may suggest low prevalence of ESBls in *Salmonella enterica* or the enzymes have not been extensively sought in *Salmonella*. This is similar to the situation in Vietnam and Thailand where ESBLs (such as VEB-1) are widely disseminated in *Klebsiella pneumoniae* and *E. coli* (Bradford, 2001) but not detected in *Salmonella*. In Portugal, ESBL was not detected in *Salmonella* despite high rate of ampicillin resistant isolates (Antunes *et al.*, 2006).

Animals are considered reservoirs of ESBL producers (Carattoli, 2008) and various types ESBLs have been reported in *Salmonella* isolates from animals (Brinas *et al.,* 2005; Hasman *et al.,* 2005; Politi *et al.,* 2005). The high rate of indiscriminate use of antibiotics in animals in Nigeria (Adetosoye, 1980) may eventually lead to the emergence of ESBLs.

TEM type is the main broad-spectrum β-lactamases observed in the isolates of *Salmonella enterica* in this study. Broad-spectrum SHV-1, TEM-1 and OXA-type β-lactamases have been described in *Salmonella enterica* isolates in other studies and TEM-1 was the most common variant among the isolates (Mathew, 1979; O'Brien *et al.,* 1982; Philippon *et al.,* 1984; Shaokat *et al.,* 1987; Wiedemann *et al.,* 1989; Ling *et al.,* 1991; Kariuki *et al.,* 1996; Olsen *et al.,* 2002; Miriagou *et al.,* 2004; Batchelor *et al.,* 2005). PSE-1 β-lactamase has been rarely reported in isolates of *Salmonella enterica* (Casin *et al.,* 1999). This study also observed the transfer of TEM gene by conjugation thus suggesting the location of the gene on plasmid which may give rise to wide dissemination of this gene among bacterial population among both human and animal.

This study suggests the importance of integron in antibiotic resistance of *Salmonella enterica* in Ibadan. Integrons are a novel group of mobile DNA elements that play an important role in the development of antimicrobial resistance in enterobacteria because they are able to capture, integrate and express gene cassettes encoding proteins associated with antimicrobial resistance (Vinue *et al.,* 2008). These genetic elements were found in more than 60% of the tested isolates from diverse sources expressing resistance to one to eight antibiotics thus suggesting the importance of integrons in the development of multiresistance resistance in *Salmonella enterica* in Ibadan; therefore, further confirming the presence of different antibiotic selective pressure in our environment. The indiscriminate use of antibiotics in our environment, coupled with the unique ability of integrons to integrate and express more than one resistance gene cassettes by sites-specific recombination, signal a great danger to public health; because the lack of judicious use of many antibiotics makes our environment rife for integrons to integrate many resistance gene cassettes thus leading to development of multiple antibiotic resistance in *Salmonella enterica* .The integron associated resistance can spread among *Salmonella enterica* and to other bacteria species in Ibadan because integron can be associated with mobile genetic elements such as plasmids and transposons (Vinue *et al.,* 2008) thus facilitating spread by horizontal transfer. This is corroborated by the transfer of integrons by conjugation in some of the positive isolates in this study.

Integron associated resistance can also spread by clonal expansion when this mobile genetic element are associated with chromosome; this may be responsible for lack of conjugal transfer of integron in some of the *Salmonella enterica* isolates. The presence of integron in both human and animal isolates of antibiotic resistant *Salmonella enterica* suggests presence of antibiotic selective pressure in both populations thus the integron associated resistance can spread through

the food chain; therefore there is the need for adequate control measures to be put in place to curtail the spread. This study suggests chicken as the most important animal reservoir of integron associated resistance contrary to a report from Ethopia (Molla *et al.,* 2007) that found pig to be the most important reservoir of integron.

Considering the resistance profiles of the integron positive isolates which includes resistance to one or more of ampicillin, chloramphenicol, cotrimoxazole, streptomycin, spectinomycin, gentamicin and tetracycline suggests that the integron integrated resistance gene cassettes possibly mediate resistance against one or more of β-lactams, chloramphenicol, sulphonamides and aminoglycosides in our environment. Integron integrated resistance gene cassettes mediating resistance to these antibiotics have been identified in *Salmonella enterica* from different countries (Krauland *et al.,* 2009). This is corroborated by the sequence data from one of the chicken isolates that contained resistance gene cassettes *aaCA5* and *aadA7*; these gene cassettes mediate resistance against aminoglyccosides.

The difference between the PCR-RFLP patterns of the 1600bp amplicons of class 1 integron of human and chicken isolates suggests dissemination of different resistance gene cassettes in human and chicken, which possibly indicate restricted dissemination of specific structures in which the integrons are located. This warrants further study. The similarity of PCR-RFLP patterns among the tested human isolates suggests stability and dissemination of certain integrons in human population; this also possibly applies to the observed similar PCR-RFLP patterns in the animal isolates.

This study observed the presence of integrons in 64.6% of the tested antibiotic resistant *Salmonella enterica* isolates thus indicating high occurrence of integrons in Ibadan. This rate is

higher than the 53.1% reported from Ethopia (Molla *et al.,* 2007). The rate is comparable to those reported from other countries. For example, integrons were reported from 11 to 65% of the antibiotic resistant *Salmonella enterica* isolates from Germany, Spain, UK, Japan, china and U.S.A (Guerra *et al.,* 2000; Randall *et al.,* 2004; Zhang *et al.,* 2004; Ahmed *et al.,* 2005; Miko *et al.,* 2005; Zhao *et al.,* 2005). These illustrate worldwide spread of integrons. In fact, a recent study provided strong evidence of dissemination of integrons between different continents of the world (Krauland *et al.,* 2009); horizontal transfer and clonal expansion were showed to play significant role in the dissemination.

Diverse amplicon sizes have been reported for integrons in antibiotic resistant *Salmonella enterica* isolates which probably reflect the size and number of integrated resistance gene cassettes (Molla *et al*., 2007; Krauland *et al.,* 2009). All the amplicons in this study were below 2kb in size. This is comparable to the study from Ethopia that reported occurrence of integrons in antibiotic resistant *Salmonella enterica* from slaughter animals and foods of animal origin (Molla *et al.,* 2007). Similar amplicons were also reported in Portugal from antibiotic resistant *Salmonella enterica* from diverse sources (Antunes *et al.,* 2006). However, amplicon sizes ≥ 4.0kb were recently reported (Krauland *et al.,* 2009) in some isolates of *Salmonella enterica* in some countries.

It is noteworthy that all the integron positive antibiotic resistant *Salmonella* isolates in this study contained only one amplicon confirming the presence of only one integron in the isolates. The presence of only one integron is the most commonly reported in *Salmonella enterica* (Antunes *et al.,* 2006; Molla *et al.,* 2007; Krauland *et al.,* 2009). However, multiple integrons (≥ 2) have also

been reported in *Salmonella enterica* (Molla *et al.,* 2007). Also, Krauland *et al.,* (2009) reported ≥3 integrons in three isolates of *Salmonella enterica*.

Furthermore, only class 1 integron was found in all the positive isolates of *Salmonella enterica* in this study thus confirming wide association of class 1 integrons with antibiotic resistance (Mazel *et al.,* 2006). Various resistance gene cassettes mediating resistance to various classes of antibiotics have been detected in class 1 integron. Class 2 integrons have only been associated with resistance in few isolates of *Salmonella enterica* (Miko *et al.,* 2005; Ahmed *et al.,* 2005). Other classes of integrons have not been associated with resistance in *Salmonella enterica*. In this study, integrons were not detected in some antibiotic resistant isolates of *Salmonella enterica*; a similar observation has also been made in other studies (Randall *et al.,* 2004). This indicates that integrons do not account for resistance in these isolates thus suggesting presence of other resistance mechanisms.

The sequenced 5' – 3' region of class 1 integron in one of the chicken isolates yielded *aaCA5* + *aadA7* resistance gene cassettes. The gene *aaCA5* encodes an aminoglycoside (3) acetyltransferase which confers resistance to gentamicin while *aaAd7* encodes aminoglycoside (3'') (9) adenylyltransferase that confers resistance to streptomycin and spectinomycin (Levings *et al.,* 2005). Acetylation or adenylation of aminoglycosides by transferases is one of the major mechanisms of resistance to these drugs. The sequenced isolate was resistant to ampicillin, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, spectinonmycin and sulfamethoxazole (R-type: AmCpGnNalSmSpTe). This is the first report of these genes in Nigeria. These resistance gene cassettes have also been reported from Ethiopia in *Salmonella enterica* serotype Kentucky but from pigs thus suggesting dissemination of these resistance genes in food animals.

In addition, these resistance gene cassettes have also been found in isolates of antibiotic resistant *Salmonella* enteric serotype Kentucky in French travelers returning from various African countries (Egypt, Libya, Sudan and Kenya) (Doublet *et al.,* 2008). This indicates dissemination of these resistance gene cassettes in Africa with food animals as one of the important reservoirs. A recent report showed these gene cassettes to be associated with *Salmonella* genomic Island 1 (SGI 1). SGI 1 is an integrative mobilizable element that harbors a multidrug resistance gene clucter (Doublet *et al.,* 2005). Therefore, the SGI 1 may be responsible for the wide dissemination. Because of the prevailing lack of judicious use of antibiotics in human and animals in Nigeria as obtained in many developing countries (Okeke *et al.,* 2005), these resistance gene cassettes may spread among *Salmonella enterica* and other bacteria thus putting public health at risk. In fact, the *aacCA5* resistance gene cassette has been reported in multidrug-resistant *Vibrio fluvialis* (Ahmed *et al.,* 2004); also, the *aadA7* has been found in *Vibrio cholerae* and *E. coli* (Ahmed *et al.,* 2004) and *Proteus mirabilis* clinical and food isolates (Boyd *et al.,* 2008). This calls for effective control measures in Nigeria to stem spread of these genes.

Ciprofloxacin is an antimicrobial of last resort for the treatment of severe salmonellosis. The efficacy of this fluoroquinolone is gradually eroded due to increasing reports of resistant *Salmonella* isolates. Ciprofloxacin-resistance is a serious global public health concern and has been reported in certain *Salmonella* serovars including *S. Typhimurium, S. Schwarzengrund* and *S. Choleraesuis* (Baucheron *et al.,* 2002; Ling *et al.,* 2003; Hsueh *et al.,* 2004; Izumiya *et al.,* 2005; Baucheron *et al.,* 2005). It is interesting that all the ciprofloxacin-resistant *Salmonella* isolates in this study belong to *S.* Kentucky and obtained from chickens in different poultry farms. *Salmonella* Kentucky (SK) is a common serovar in poultry but was not recognized as important human pathogen until recently (Weill *et al.,* 2006; Majtan *et al.,* 2006; Collard *et al.,*

2007).The increasing importance of this serovar in human salmonellosis, coupled with multidrug resistance and resistance to ciprofloxacin (Doublet *et al.,* 2008) warrants studies to characterize this emerging human pathogen by molecular methods.

Molecular analysis of these ciprofloxacin resistant *S.* Kentucky revealed only two major PFGE types suggesting limited genetic diversity of this serovar. One of the PFGE type (P1) accounted for majority of the isolates, 43 of 44 (97.7%), from the different poultry farms, suggesting clonal dissemination of a particular strain of *S.* Kentucky in the poultry farm*s*. This indicates that infection probably originates from a common source which may be contaminated feeds, day-old chicks or in the environment of the poultry farms. This warranted further studies to unravel the source for implementation of specific control measure*s*.

The PFGE findings were corroborated by the results of antimicrobial susceptibility testing (AST). Similarly, the 44 isolates of ciprofloxacin-resistant *S.* Kentucky revealed few and similar AST patterns. Majority of the isolates (n=39, 89%) have very similar AST patterns (R1 and R2) further suggesting that the isolates are closely related.

Similar to the finding of this study, a clonal spread of *S.* Kentucky was observed in hospitals in two geographical different regions of Slovak republic (Majtan *et al.,* 2007). The epidemic strain in Slovak republic was associated with travel to Egypt. Furthermore, 17 cases of human salmonellosis caused by ciprofloxacin-resistant *S.* Kentucky in French patients, which were linked to travel to different African countries, also revealed that the majority of the isolates were closely related suggesting clonal spread. Unfortunately, no studies have been executed in the implicated African countries visited by the European patients to determine the sources and

spread of *S.* Kentucky. Therefore, there is paucity of data in Africa countries to compare the result of molecular analysis obtained in this study.

Nevertheless, in Ethiopia, swine has been identified as an important source of multidrug and ciprofloxacin resistant *S.* Kentucky (Molla *et al.,* 2007). In concordance with the present study, molecular analysis revealed limited genetic diversity suggesting clonal spread of *S.* Kentucky in Swine husbandry in Ethiopia (Molla *et al.,* 2007).

Although ciprofloxacin-resistant *S.* Kentucky isolate was not obtained from human samples in this study, it still constitutes potential public health importance in this environment because chicken is heavily consumed either in meat dishes or roasted (suya) particularly during festive period*s*. Moreover, poultry husbandry is not adequately regulated in Nigeria and this could potentiate the spread to human through the food chain. *S.* Kentucky is now an important human pathogen with ability to disseminate and spread (Majtan *et al.,* 2006; Weill *et al.,* 2006; Collard *et al.,* 2007).

SUMMARY OF FINDINGS IN RELATION TO OBJECTIVES:

1. To determine sources and clinical burden of *Salmonella*:

Salmonella is the second most common cause of bacteremia accounting for 23% of the bacteremic cases; majority (92%) of the *Salmonella* bacteremic cases were in children. The carriage rate of *Salmonella* in the healthy people was 8.6%. The animal carriage of *Salmonella* ranged from cattle (6%) to goat (15%).

2. Evaluate serovars of *salmonella* isolates:

The non-typhoidal *Salmonella* (NTS) accounted for majority (82.1%) of the *Salmonella* bacteremia and *S*. Typhi (17.9%). The most common NTS serovars was *S*. Enteritidis (33.3%), *S*. Dublin (17.9%), and *S*. Typhimurium (17.9%). The median age of *S*. Typhi bacteremic patients was 11years and 1year 7months for the NTS bacteremic patients. The most predominant serovar from chicken was *S*. Virchow (43.9%) followed by *S*. Kentucky (38.6%). The nine *Salmonella* serovars obtained in the bacteremic patients were different from the nine found in chicken.

3. Analyse antibiotic resistance profiles of the *Salmonella* isolates:

Resistance to extended-spectrum cephalosporins was not observed in any of the *Salmonella* isolates. On the other hand, resistance to quinolones was only 2.0% for bacteremic *Salmonella* isolates compared to 64.8% in the isolates from chicken. Leading resistant serovars in Ibadan are *S*. Typhimurium, *S*. Virchow and *S*. Kentucky.

4. Elucidate molecular determinants of resistance and clonal relationship of isolates:

Although β-lactamases (mainly TEM) were produced by the *Salmonella* isolates, none produced extended-spectrum β-lactamases that mediate resistance to the extended spectrum cephalosporin antibiotics. There was high prevalence of class 1 integrons which integrate resistance genes in the resistant isolates. Resistance gene cassette *aaCA5* that mediates resistance to gentamicin was found in an isolatate of S. Kentucky.There was limited genetic diversity among the ciprofloxacin resistant isolates from the poultry farms in Ibadan suggesting dissemination of certain strains among different poultry farms in Ibadan.

Contributions to Knowledge

- *Salmonella* accounts for approximately one-quarter of bacteremia in Ibadan with involvement of diverse serovars of *Salmonella* predominated by S. Enteritidis. The role of S. Dublin is noteworthy. Food animals: cattle, pig, goat, sheep and chicken are potential animal sources of *Salmonella* in our environment

- Chickens habour *Salmonella* serovars of potential public health hazard such as *S*. Virchow and S. Kentucky in Ibadan. Serovars found in chickens may not be associated with bacteremia

- Extended-spectrum cephalosporins and quinolones remain efficacious for the treatment of invasive salmonellosis in Ibadan. However, the high resistance to quinolones found in *Salmonella* isolates from chicken is a threat to public health. Chicken is also a potential reservoir of resistance gene cassette *aaCA5* to gentamicin. To the best of my knowledge, this is the first report of aaCA5 and the *aadA7* genes in Nigeria. Class 1 integron is important for the development of resistance in *Salmonella* in Ibadan. It further showed clonal dissemination of ciprofloxacin-resistant *S*. Kentucky in poultry farms.

RECOMMENDATION

The sources of *Salmonella* serovars causing bacteremia should be determined and appropriate control measures instituted against them and in the poultry industry to limit spread. Attention should be paid to biosecurity measures in the poultry industry to prevent *Salmonella* infection of flocks from the environment; risk factors for *Salmonella* infections in the flocks need to be idenfied. The poultry industry should be regulated to ensure food safety. The high prevalence of antibiotic resistance in the *Salmonella* isolates is a public health concern and measures should be put in place to enforce judicious use of antibiotics both in human and veterinary medicine.

CHAPTER SIX

CONCLUSION

Several serovars of *Salmonella* predominated by NTS are involved in the causation of bacteremia in Ibadan which may not be associated with chicken; the sources of these serovars need to be identified and appropriate control measures instituted against them to prevent spread. Chickens habour serovars of public health importance that may spread through the food chain but which may not be associated with bacteremia. Extentended –spectrum cephalosporins and quinolones remain efficacious for the treatment of salmonellosis in Ibadan but the high occurrence of ciprofloxacin-resistant strains which are widely disseminated in poultry farms in Ibadan and which may spread to humans through the food chain posses a threat to public health; therefore the use of antibiotics in poultry industry needs to be regulated. Poultry is a reservoir for the antibiotic resistance gene pool in Ibadan.

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