CHARACTERIZATION OF WILD-TYPE SALMONELLA AND THEIR SUSCEPTIBILITY TO "MIST ENTERICA" AN ANTI-TYPHOID HERBAL PREPARATION

BY

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DECLARATION

The work described in this report was carried out by me at the Bacteriology unit, Noguchi Memorial Institute for Medical Research (NMIMR), College of Health Sciences, and the Department of Biochemistry, both of the University of Ghana, Legon under the supervision of Professor Marian E. Addy and Professor Patience A. Akpedonu. Part of the work was carried out at the Emporia State University (ESU), USA, in the laboratory of the Department of Biological Sciences.

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DEDICATION

I dedicate this thesis to the most Gracious and Merciful God, my Family and my three lovely children, Felix, Francis and Maame Efua Mills-Robertson.

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LIST OF ABBREVIATIONS

CSRPM – Centre for Scientific Research into Plant Medicine

DNA – Deoxyribonucleic Acid

ERIC-PCR – Enterobacterial repetitive intergenic consensus Polymerase Chain Reaction

GHAFTRAM – Ghana Federation of Traditional Medicine Practitioners Association

ISEP – International Student Exchange Programme

LPS – Lipopolysaccharide

MIC – Minimum Inhibitory Concentration

NMIMR – Noguchi Memorial Institute for Medical Research

OPD – Out-Patients Department

PCR – Polymerase Chain Reaction

REP-PCR – Repetitive Extragenic Pallindromic Polymerase Chain Reaction

ABSTRACT

The purpose of this study was to investigate the antibiotic resistance and clonal lineage of *Salmonella* isolated from patients suspected of suffering from typhoid fever and the susceptibility of these *Salmonella* strains to "Mist Enterica", a herbal preparation used at the Centre for Scientific Research into Plant Medicine (CSRPM) out-patients' department, Mampong-Akwapim, Ghana, to treat typhoid fever. Other strains of *Salmonella* isolated from food sources were also included in this study.

A total of 115 *Salmonella* strains were examined for drug/multiple resistance, using first-line antibiotics used to treat typhoid fever, namely, ampicillin (Am), chloramphenicol (Cm), tetracycline (Te) and trimethoprim-sulphamethoxazole (Ts). Streptomycin (St), which is a commonly used antibiotic, was also included. These strains were isolated from blood, cerebrospinal fluid, stool, urine, food, and other sources. The minimum inhibitory concentrations (MICs) for the antibiotics were studied using the 115 *Salmonella* strains. The genetic location of those with resistant genes was also investigated. Genetic fingerprinting by plasmid profiling, enterobacterial repetitive intergenic consensus (ERIC) -PCR, and repetitive extragenic pallindromic (REP)-PCR were performed to determine the diversity among the isolates. The efficacy of "Mist Enterica" as an anti-typhoid agent and the contributions made by each of the components of "Mist Enterica" were also investigated. The MICs for "Mist Enterica", and three of the component plants, namely *Cnestis ferruginea, Hoslundia opposita* and *Psidium guajava* were also determined.

The number of organisms isolated from blood alone was 82 (71.3%). Eight serological groups were identified and the most common isolates were groups D (57.4%) and B (33%), with the least found in groups A, G and I. Seventy-four percent of the *Salmonella* strains (85 out of 115) were resistant to one or more of the five antibiotics used and of the 85 resistant strains, 37 (43.53%) were resistant to all five antibiotics, 32 (37.65%) resistant to four, 7 (8.24%) to three, 1 (1.18%) resistant to two and 8 (9.41%) were resistant to one antibiotic. Thus, 76 *Salmonella* strains out of the total 115 (66.09%) were found to possess multi-drug resistance (resistance to three or more antibiotics). The MIC for ampicillin was found to be equal to or greater than

1280mg/L for 93% of the resistant *Salmonella*, whilst trimethoprim-sulphamethoxazole had MIC of 1280mg/L for 78% and 80mg/L for 22% of the strains. The MIC for chloramphenicol was equal to or greater than 1280mg/L for 48% of the strains and 20mg/L for 46%. Tetracycline had MIC of 320mg/L or lower for all the 85 resistant *Salmonella* strains tested. Two groups, Groups B and D, totaling 104 out of the 115 strains (90%), showed high level of resistance. The percentage of resistant strains in Group B was 97.3 and that in Group D was 63.6. In all, majority of the Group B strains (71.05%) were resistant to all five antibiotics whilst majority of the Group D strains (37.88%) were resistant to four.

Eighty-one out of the 85 resistant strains (95.29%) possessed conjugable plasmids which conferred multi-drug resistance on 74 of the 81 strains (91.36%). These multi-drug resistant strains belonged to the incompatibility group *Inc*HI. Out of the 85 wild-type resistant *Salmonella* strains only 15 (17.65%) could transform recipient *E. coli* strains, with all these transformants being resistant to ampicillin. Plasmid profiling discriminated 5 unique groupings, while ERIC-PCR and REP-PCR resulted in 2 and 3 groupings, respectively.

"Mist Enterica" and decoctions made from *Hoslundia opposita*, *Cnestis ferruginea* and *Psidium guajava*, three of the components of "Mist Enterica" were found to be very active against all the standard strains as well as the wild-type *Salmonella* strains with zones of inhibition ranging from 9mm to 25mm. These herbal preparations also inhibited growth of other pathogenic microorganisms such as *Staphylococcus aureus*, *Staphylococcus saprophyticus*, *Escherichia coli*, *Candida albicans* and *Neisseria gonorrhoea*.

Thus, antibiotic resistance is associated with the *Salmonella* strains whose genes are located on conjugative plasmids and appear to be minimally diversified. The results also indicate that "Mist Enterica" and a combination of *Hoslundia opposita*, *Cnestis ferruginea* and *Psidium guajava* (3-in-1) are very efficacious and could be used for the management of the type of disease caused by the strains of bacteria studied.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. GENERAL INTRODUCTION:

Salmonellosis is a disease of animals and humans, caused by different species of the genus *Salmonella* that exist in nature primarily as pathogens of humans and animals. The salmonellae may be divided into two main groups based on their pathogenicity, that is, the enteric fever (typhoid and paratyphoid) group found mainly in humans for whom they have a high degree of pathogenicity, and the food-poisoning group, members of which are essentially pathogens of animals from whom humans are frequently infected (Agbodaze *et al.*, 1988).

According to Cheesbrough (2003), typhoid fever is a septicaemic disease associated with signs and symptoms that include fever, headache, anorexia and rose-spots on light skinned people (pink papules which fade on pressure). It is caused by members of the *Salmonella* serotype *typhi* and *paratyphi* A, B, C, which are flagellated, non-spore bearing, Gram negative bacilli belonging to the family Enterobacteriaceae. They are pathogenic in man and other lower primates. Most salmonellae are found in the intestines of animals especially pigs, cows, goats, sheep, rodents, hens, ducks and other poultry, but *S. typhi* and *S. paratyphi* are usually found only in humans and excreted in the faeces and urine of infected patients. *S. typhi* and *S. paratyphi* are also present in the gall bladders of chronic carriers. The long duration of the carrier state enables the enteric fever bacilli to survive in the community at non-specific times and to persist in small, relatively isolated communities or to infect families as it happened in the case of the cook

called "Typhoid Mary" (Mandal, 1996). Infection is from contaminated hands or by ingesting the organisms in contaminated food or water. *S. typhi* is spread mainly by water and *S. paratyphi* by food. Raw fruit and vegetables are important vehicles in some countries where human faeces are used as manure. In schistosomiasis endemic areas there is a high incidence of chronic *S. typhi* and *S. paratyphi* infections where the *Salmonella* is known to adhere to the adult schistosome flukes. Salmonellae are not killed by dryness and therefore survive in products such as dried egg or bone meal fertilizers (Cheesbrough, 2003).

The number of officially recorded cases of human salmonellosis has increased significantly in many countries all over the world (Petit and Wamola, 1994; Oosterom, 1991; Ryder *et al.*, 1980). In Ghana, typhoid fever is of public health concern in urban slums and rural communities where its prevalence is highest in children and young people (National Symposium on Enteric fever/salmonellosis for the Southern sector, 1998; Voros *et al.*, 1974). Older people often seem to possess partial immunity, probably following exposure to frequent sub-clinical infective doses of typhoid bacillus (Mirza *et al.*, 1995). Globally, it is estimated that there are over 20 million cases annually, resulting in greater than 700,000 deaths (Thong *et al.*, 1994). The incidence of this disease in the industrialized world is rare primarily due to proper sanitary facilities. The sporadic outbreaks that occur are oftentimes due to transmission from an endemic region or country. The disease in most developing countries is linked to poor water supply, inadequate sewage disposal and unhygienic conditions as revealed by research conducted

in rural areas of three African countries namely Ghana, Zambia and Kenya in 1994 (Petit and Wamola, 1994).

Treatment of typhoid fever is usually with antibiotics and the drug of choice has been chloramphenicol, a bacteriostatic broad-spectrum antibiotic. However, it has recently been reported that chloramphenicol is becoming increasingly ineffective in treating typhoid cases. Additionally, chloramphenicol-resistant *Salmonella* strains have been documented abroad (Islam *et al.*, 1993) and in Ghana (Newman, 2000). Many strains of *Salmonella* have also become resistant to ampicillin, tetracycline and trimethoprim-sulphamethoxazole, which are considered appropriate alternatives to chloramphenicol (Ling and Chau, 1984). The emergence of these resistant strains could be disturbing because it has been reported that about 12-16% of patients die within four weeks of the disease if they are not well managed (Mims *et al.*, 1998).

Relapses are common unless the drug is given in adequate doses and for a sufficient length of time (http://www.merck.com/pubs/mmanual/section13/chapter153/153f.htm; Mandal, 1996; Keusch, 1994). The administration of chloramphenicol for a long period of time, however, may result in aplastic anaemia and destruction of the bone marrow. It may also lead to central and peripheral circulatory failure and mental derangement, probably due to large scale destruction of typhoid bacilli and release of large amounts of typhoid endotoxin (Manson-Bahr, 1987). Thus the use of multi-drug therapy and/or third generation drugs is recommended.

Multi-drug resistance, however, is now common among these pathogenic microorganisms, which show both *in vivo* as well as *in vitro* resistance to the four first-line antityphoid antibiotics namely, ampicillin, chloramphenicol, tetracycline and trimethoprim-sulphamethoxazole (Newman, 2000; Rowe *et al.*, 1997; Panigrahi *et al.*, 1996; Smith *et al.*, 1984; Sajjad, 1996). Resistance to each of these first line antibiotics is often plasmid encoded (Datta *et al.*, 1981; Olarte and Galindo, 1973) and strains harbouring plasmid encoding resistance to all four antibiotics have been isolated by many researchers (Panigrahi *et al.*, 1996). In most cases, plasmids responsible for the resistance belong to the incompatibility complex *Inc*HI (Rowe *et al.*, 1990). Thus, multiple drug resistant (MDR) *S. typhi* exist and are on the increase worldwide (Sajjad, 1996; Panigrahi *et al.*, 1996; Olarte and Galindo, 1973). However, these organisms are poorly documented in Ghana and therefore further investigations are needed to determine the actual situation prevailing in Ghana.

Quinolone derivatives such as ciprofloxacin or pefloxacin and third generation cephalosporins such as ceftriaxone or cefotaxin are very effective for treating diseases caused by multi-drug resistant *S. typhi* strains, particularly ciprofloxacin given by oral route in a 7-day course of therapy (Wallace *et al.*, 1993; Asperilla *et al.*, 1990; Eykyn and Williams, 1987). However, drug resistance to fluoroquinolones has emerged (Chitis *et al.*, 1999; Kapil *et al.*, 1999; Rowe *et al.*, 1995).

In Ghana, although these drugs are effective, they are expensive and out of reach of the poor in the endemic areas, hence, chloramphenicol is still prescribed in many health

facilities in Ghana and the resistance to the drug has led to the general notion that typhoid is difficult to treat. There is therefore the need to search for an alternative medicament that is effective and cheaper in the management of typhoid fever and other salmonellosis. In a country where over 70% of the populace rely on herbal medicine, the source of such alternative medicaments should include herbal preparations. However, thorough research into the biological and molecular characterization of the causative agent, *Salmonella*, and the prevalence of MDR strains will have a bearing on the efficacy of the alternative medicinal product. Conduction of such a research forms an integral part in the search for such alternative herbal medicaments that are cheap, easy to produce and very effective with little or no side effects. This is because there is minimal documentation of MDR *S. typhi* in Africa where some of the first cases of chloramphenicol-resistant strains were reported. The thorough research will also enable better interpretation of results obtained with the herbal medicaments and correct inferences made.

The World Health Organization (WHO) defines traditional medicine as "*the total combination of the knowledge and practices, whether explicable or not, used in diagnosing, preventing or eliminating physical, mental or social diseases and which may rely exclusively on past experience and observation handed down from generation to generation, verbally or in writing*" (WHO, 2004). In practice, the term 'traditional medicine' refers to acupuncture, the practice of traditional birth attendants, mental healing and herbal medicine with the latter being the oldest form known. These approaches to health belong to the traditions of each country (http://www.holistic-online.com/ Herbal.Med/hol_herb-intro.htm).

In Africa, research on traditional medicine dates from the colonial periods. Studies have focused primarily on ethnobotanical and phytochemical aspects, but more recent research is examining how traditional health practices and herbal medicines can be integrated into modern medical systems (http://www.idrc.ca/media/commplants_e2.html)

Ghana today has a dual system of medical practice that recognizes both traditional and modern medical practices in law and promotes their co-existence in order to reach the greatest number of citizens (http://www.unep-wcmc.org/species/plants/ghana). Traditional medicine, for instance, plays a very important role in Ghana where approximately 70% of the population makes use of its services. These services are often affordable and accessible to the vast rural populace and therefore serve as a forerunner in the primary medical care of the population (http://www1.cfiks.org/healthmed.htm).

The nation has an extremely rich biodiversity and rich tradition of plant medicine in various forms and has an enormous number of indigenous medicinal plants. Each village has its traditional practitioner who uses local plant remedies and in many cases ritual and no doubt non-plant medicine as well (http://www.nimh.btinternet.co.uk/eihm/1_1_hw3.htm).

Even though a few herbal medicines have withstood scientific testing, a lot more need to be studied scientifically. Most people believe that herbs are safer and less expensive than most orthodox medicines. However, they can be dangerous if they are not used appropriately. Herbal medicines have been reported to be responsible for 0-35% of cases

of acute renal failure (ARF) in some African countries (http://www.uninet.edu/cin2001/paper / ibanez/fovaca.html). Addae-Mensah (1992) has reported that even though *Croton membranaceous* has diterpens and alkaloids which possess anticancer and anti-ulcer properties, the same plant also has phorbol esters which are known to be co-carcinogenic and therefore can cause cancer of the oesophagus. He also reports that *Crotalaria, Senecio, Cynoglossum* and *Heliotropium* genera contain pyrrolizidine alkaloids found to be highly hepatotoxic. Scientific investigations into herbal medicines must therefore involve the therapeutic effects as well as the possible long-term deleterious effects which may not manifest themselves until it is too late.

The WHO estimates that, 80% of the world's population, presently use herbal medicine for some aspect of primary healthcare, and that about 74% of the 119 plant-derived pharmaceutical medicines are used in modern medicine in ways that correlate directly with their traditional uses as plant medicine in native cultures (http://www.who.int/inffs/en/fact 134.htm; WHO, 2003). Principe (1989) reported that pharmaceutical companies have shown decreasing interest in the development of new plant products in favour of molecular biology and biotechnology application to microorganisms. However, some others, including this researcher, believe that, plant based resources will regain their importance. This is because even though research based on microorganisms to synthesize therapeutically active chemical compounds is promising, several limitations do exist. The steps of identifying the chemical structure required to achieve a given effect and creating a proper genetic code for this structure, are the most difficult stages of drug development for which plant based genetic material

appears to be better than microorganisms. Genetically engineered microorganisms can substitute only for some of the plant based chemicals. The vast majority of plant based chemicals have not been successfully synthesized (http://www.indmedplants-kr.org /ECONOMIC_VALUE_OF_MEDICINAL 2.HTM).

In 1960, the late President of Ghana, Osagyefo Dr. Kwame Nkrumah, suggested the formation of an association of traditional healers and as a result the Ghana Psychic and Traditional Healers Association was formed. More recently, with the support of the Ministry of Health, the Ghana Federation of Traditional Medicine Practitioners Association (GHAFTRAM) was formed and now forms the authoritative voice on traditional medicine in Ghana (http://www.unep-wcmc.org/species/plants/ghana). In November 1975, the Government of Ghana established the Centre for Scientific Research into Plant Medicine (CSRPM) at Mampong-Akwapim, as a result of the dream and vision of Dr Kwaku Oku Ampofo. Dr Oku Ampofo saw the therapeutic importance of herbal medicines especially on himself and his father. It is therefore no wonder that he became an apostle of the noble profession of traditional medicine in general and herbal medicine in particular at a tender age. The Centre originally started as a small OPD under his able leadership in the present day Mampong-Akwapim community centre.

Currently, the Centre conducts and promotes scientific investigations relating to the improvement of plant medicine, ensures the safety of the drugs extracted from plants, co-operates and liaises with GHAFTRAM, Universities, Research Institutions and commercial organizations world-wide, collaborates in the publication and dissemination

of the results and establishes arboreta for medicinal plants. The Centre also encourages traditional healers to bring their herbal preparations for analysis (CSRPM Annual report, 2002; http://www.unepwcmc.org/species/plants/ghana).

Undoubtedly, traditional methods of healing have successfully served the majority of Ghanaians for years. Unfortunately, knowledge about plants used in the art of healing has not been recorded in written text, unlike those found in China and India (http://www.unepwcmc.org/species/plants/ghana). The knowledge has been passed on from one generation to the next, mainly by oral tradition and most of these herbal preparations used for treating diseases are used without the patient or the traditional medicine practitioner paying attention to their standardization, quality control and safety. One such herbal preparation is "Mist Enterica", used for the treatment of typhoid fever. "Mist Enterica" was introduced in 1997 by an herbalist, Mr Sulley Mante, who still works at the Centre as at the time of writing this manuscript. This herbal preparation is made from twelve plants, mostly leaves, and dispensed at the out-patients' clinic. It is claimed to reduce fever within an hour and treat both sensitive and multiple resistant typhoid fever without any relapse (CSRPM Annual report, 2002). Developing, improving and promoting such medicinal plants will not only supplement western-type medicine, but could be a better approach to meeting the health needs of a majority of the populace including those who may never have the facilities of a modern hospital, or are unable to afford the high cost of imported drugs. In addition, it is important to undertake scientific investigations into the therapeutic potentials of our medicinal plants so as to ensure their safety, efficacy and quality. This will enable more of our health

professionals use them, thereby reducing dependency on imported drugs which could be expensive for most people.

The main aim of this study was to isolate and characterize serovars of *S. typhi,* the causative agent of typhoid fever, and other *Salmonella* species especially those resistant to the first-line anti-typhoid drugs, and to investigate the effect of "Mist Enterica" and its component plants on the isolated strains.

The specific objectives were to

i. isolate the groups/types of *Salmonella* species found in Ghana as well as determine their prevalence;
ii. identify and characterize the resistant *Salmonella* species;
iii. investigate the efficacy of "Mist Enterica" as an anti-typhoid agent on the isolated strains; and,
iv. establish the contributions from each of the twelve plants making up "Mist Enterica" to the anti-typhoid activity.

1.2. SALMONELLOSIS:

Salmonellosis is an infection of bacteria belonging to the genus *Salmonella*. In *Salmonella* gastroenteritis, there is abdominal pain of a gripping nature, but it is not usually severe and similarly vomiting, if it occurs, is not severe. Pyrexia is common and is an evidence of the severity of infection, for example, general body pain and shivering. The incubation period for *Salmonella* gastroenteritis depends on the dose of bacteria

ingested but the symptoms usually begin 6 to 48 hours after ingestion of contaminated food or water and usually take the form of nausea, vomiting, diarrhoea and abdominal pain. However, the cardinal manifestation is diarrhoea and together with fever and chills may last for about 2 to 7 days (Ohl and Miller, 2001; Miller, 2000). Typhoid fevers, on the other hand, are severe systemic forms of salmonellosis with incubation period spanning between 10 and 14 days. The symptoms of enteric fevers are non-specific and include fever, anorexia, headache, myaglias and constipation. Without treatment, mortality is 10-15% (Ohl and Miller, 2001).

1.2.1. Causative organism:

Salmonellae (Fig. 1) are widely distributed in nature, being isolated from the gastrointestinal tracts of humans, animals, reptiles, birds and insects (Agbodaze *et al.*, 1988; Sackey *et al.*, 2001) and usually cause a self-limited enteritis in humans (Ohl and Miller, 2001). Some *Salmonella* species such as *S. typhi* and *S. paratyphi*, are highly adapted to humans and other lower primates with about 2-5% of infected patients becoming chronic carriers and serving as reservoirs. Other species such as *S. typhimurium*, have a broad host range and can infect a wide variety of animal hosts and humans. Sources of gastroenteritis (non-typhoidal) *Salmonella* for human infection are largely contaminated water and food products, with person-to-person transmission not considered an important route of transmission. *S. enteritidis* and *S. agona* can pass transovarially from chicken to eggs, with infection acquired by consuming raw or partially cooked eggs. Poultry, particularly, hen, duck and turkey are the most significant reservoirs of *Salmonella* food-poisoning (Cheesbrough, 2003; Caygill *et al.*, 1994).

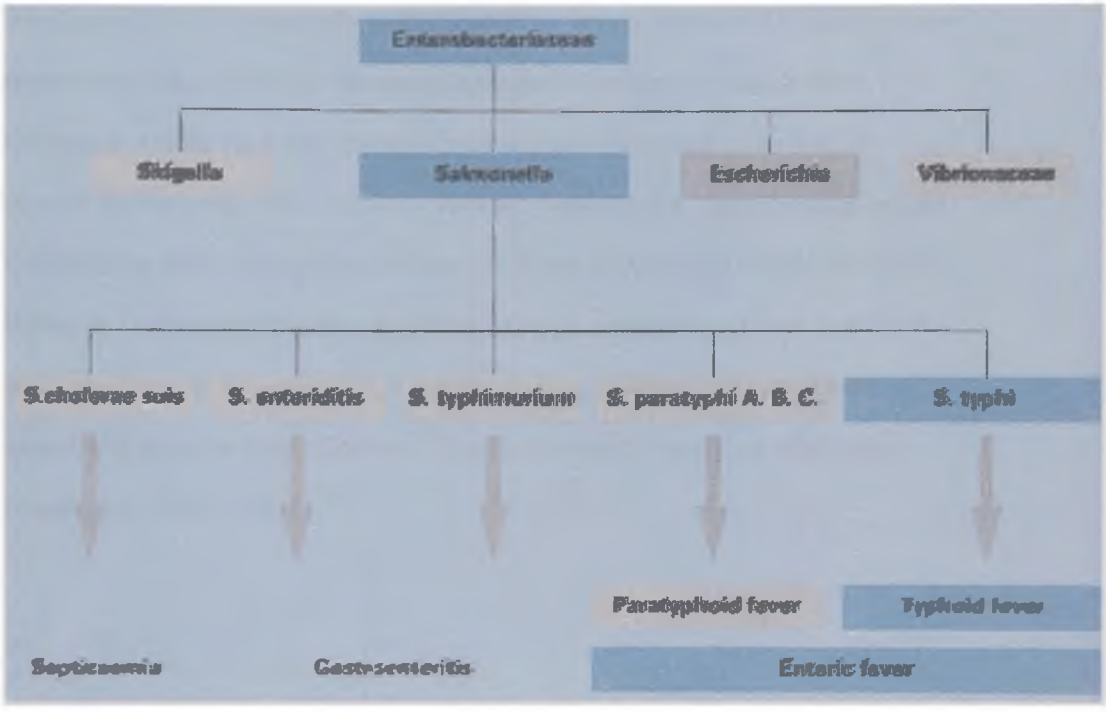

Figure 1. ***Salmonella*** **species and the disease they cause**

Salmonellae are Gram-negative, flagellated, facultatively anaerobic rods possessing three major antigens (Fig. 2); H or flagellar antigen, O or somatic antigen and V_1 antigen, possessed by only a few serovars. H antigen may occur in either or both of two forms, called phase 1 and phase 2. The organisms tend to change from one phase to the other. O antigens occur on the surface of the outer membrane and are determined by specific sugar sequences on the cell surface. Vi antigen is a superficial antigen overlying the O antigens; it is present in a few serovars, the most important being *S. typhi* (Fig. 3) (Keusch and Acheson, 1999; Gianella, 1996 http://www.hc-sc.gc.ca/pphb-dgspsp/msds-ftss/msds132e.html). Salmonellae are subdivided into groups by the Kauffmann-White scheme and represented by alphabets. For example, *S. paratyphi* A, *S. nitra and S. kiel* belong to Group A; *S. paratyphi* B, *S. agona*, and *S. typhimurium* belong to Group B; *S. paratyphi* C belong to Group C whilst *S. Dublin, S. enteritidis* and *S. typhi* belong to Group D (Le Minor, 1984).

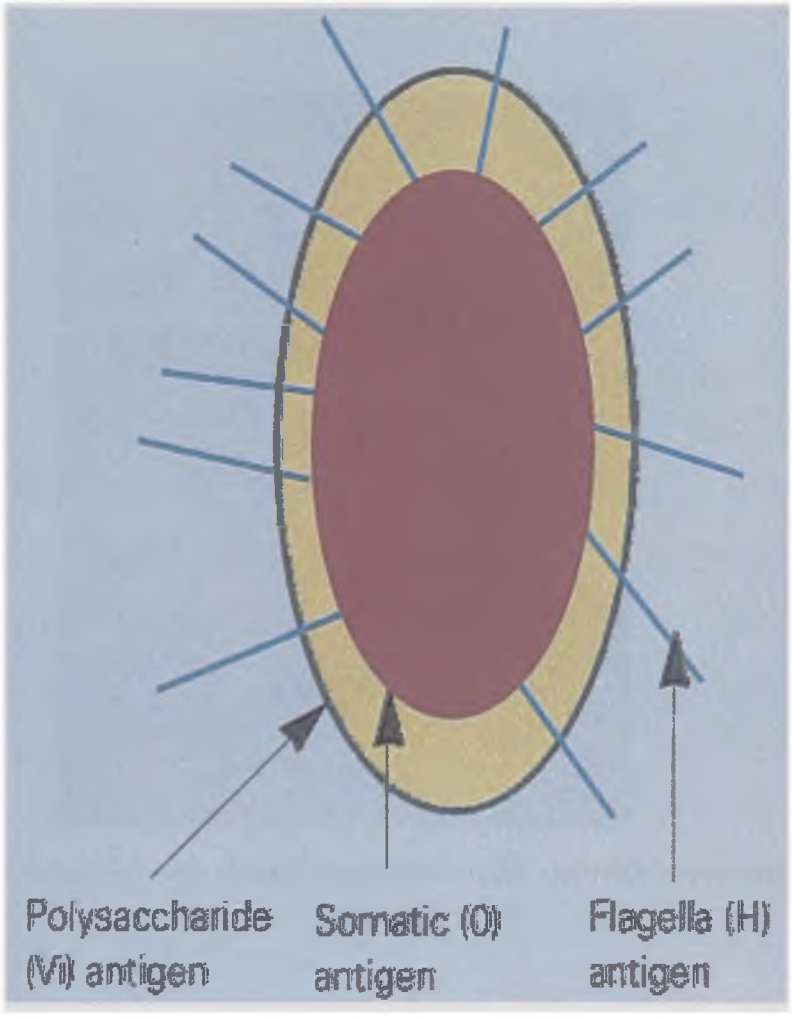

Figure 2. "Antigenic structures of *Salmonella typhi*"

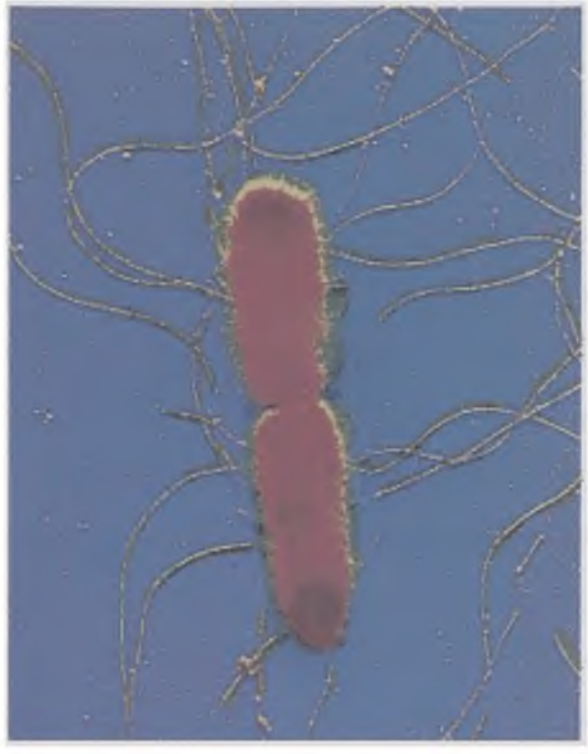

Figure 3. ***Salmonella typhi*** **(Science Photo Library, UK. Copyright protected.)**

The cell envelope of salmonellae contains a complex lipopolysaccharide (LPS) structure that is liberated on lysis of the cell and, to some extent, during culture. The lipopolysaccharide moiety may function as an endotoxin, and may be important in determining virulence of the organisms. This macromolecular endotoxin complex consists of three components, an outer O-polysaccharide coat, a middle portion called the R core, and an inner lipid A coat. The lipopolysaccharide structure is important for several reasons. Firstly, the nature of the repeating sugar units in the outer O-polysaccharide chains is responsible for O antigen specificity; and may also help determine the virulence of the organisms. Salmonellae lacking the complete sequence of O-sugar repeat units are called rough because of the rough appearance of the colonies and are usually avirulent or less virulent than the smooth strains that possess a full complement of O-sugar repeat units. Secondly, antibodies directed against the R-core (common enterobacterial antigen) may protect against infection by a wide variety of Gram-negative bacteria sharing a common core structure or may moderate their lethal effects. Thirdly, the endotoxin component of the cell plays an important role in the pathogenesis of many clinical manifestations of Gram-negative infections. Endotoxins evoke fever, activate the serum complement, kinin and clotting systems, depress myocardial function, and alter lymphocyte function (htt:/gsbs.utmb.edu/microbook/ch021.htm.). Circulating endotoxin may be responsible in part for many of the manifestations of septic shock that can occur in systemic infections (Miller *et al*, 1995: http://pathology5.pathology.ihmi.edu/micro/v16n02.htm)

1.2.2. Pathogenesis of salmonellosis:

Salmonellae are common members of the normal flora of many animals, including chickens, cattle and reptiles. The strains that cause gastroenteritis are usually transmitted by chicken meat, eggs and diary products and unless care is taken in poultry farms, chicken eggs often become contaminated, both on their surface and within (Cheeesbrough, 2003; Ohl and Miller, 2001). Outbreaks are most often related to contaminated eggs or chicken salad. Typhoid fever, on the other hand, is traceable to a human carrier, such as the infamous Typhoid Mary, although the routes of transmission often involve contaminated water or food. Like other enteric pathogens, salmoneilae must be excreted in faeces and passed on from the reservoir to the recipient (Keusch and Acheson, 1999).

When food or drink contaminated with the *S. typhi* is ingested, many of the organisms are normally inactivated by acid in the stomach. However, if a large number of bacteria are ingested, a substantial proportion of them may reach the small intestine. The time from the ingestion of *S. typhi* to the appearance of the first symptoms is usually 10-14 days, however, it could be as short as three days or as long as two months depending on the size of the ingested dose (Keusch, 1994). For paratyphoid fever, the incubation is usually 1–10 days.

Conditions that increase gastric pH reduce the *Salmonella* infectious dose, which suggests that gastric acidity represents a significant initial barrier to infection (Giannella, 1972; 1973). Salmoneilae interestingly exhibit an adaptive acid-tolerance response on

exposure to low pH, possibly promoting survival in acidic host environments such as the stomach (Garcia-del Portillo *et al.,* 1993). Strains that successfully escape being killed in the stomach pass through the duodenum to the distal ileum and colon and then penetrate the mucosal barrier, by microbe-directed phagocytosis, into phagocytic vesicles where they remain for many hours. The bacteria-containing vesicles eventually travel to the basal membrane, and the organisms are released into the lamina propria. Salmonellae are usually resistant to the lysosomal contents of cells or to cryptins, the antibacterial peptides made by intestinal epithelial cells (Ohl and Miller, 2001; Garcia-del Portillo *et al.,* 1993).

After invading the epithelium, the salmonellae multiply intracellularly and then spread to mesenteric lymph nodes and throughout the body via the systemic circulation. However, they are rapidly taken up by the phagocytic cell system and effectively killed (Finlay *et al.*, 1992). Thus, they normally do not cause sustained bacteremia. Nevertheless, depending on the serotype and the effectiveness of the host defenses against that serotype, some of the organisms, spread systemically and may infect the liver, spleen, gallbladder, bones, meninges, and other organs (Finlay *et al.*, 1992). Fortunately, most serovars are killed promptly in extraintestinal sites, and the most common human *Salmonella* infection, gastroenteritis, remains confined to the intestine (Giannella, 1979).

The interaction of gastroenteritis-producing salmonellae with epithelial cells activates the inflammatory response and results in ulceration or damage to the intestinal mucosa initially. The interaction involves the assembly of nonpili appendages by the organism

within 15 minutes of contact with the host cell (Mishu *et al.*, 1994). By 30 minutes, ruffles form on the host cell, and the bacterial appendages disappear. Salmonellae that are pathogenic are able to assemble these appendages, and also able to shed them. During the invasion, many biochemical events are activated by signals of which mitogen-activated protein kinase (MAP-kinase) may be the first. MAP-kinase is linked to a receptor on the cell surface, and the binding of the organisms to the receptors lead to the activation of phospholipase A_2 (PLA_2), release of arachidonic acid, production of eicosanoids, including prostaglandins and leukotrienes, and a sharp increase in intracellular calcium concentration. These events underlie the induction of ruffles and subsequent bacterial uptake, but a number of the mediators involved are also capable of altering electrolyte transport and consequently, diarrhoea as a result (Galan, 1996; Hobbie *et al.*, 1997; Pace *et al.*, 1993).

In the case of typhoid fever, passage of the organisms into the small intestines is followed by invasion across the mucosa and their rapid uptake by mononuclear cells in regional lymph nodes. An initial bacteremia carries the organisms to the liver and spleen for further growth. The importance of typhoid-causing serovars is their ability to survive and grow within the liver and spleen, in contrast to the gastroenteritis-causing salmonellae. Patients are found to be asymptomatic as the organisms multiply in macrophages of liver, spleen and mesenteric lymph nodes (Ohl and Miller, 2001; Giannella, 1973; 1975). When the number of intracellular organisms reaches a threshold, they are released into the bloodstream, initiating a continuous bacteremia characteristic of typhoid fever. This event signals the start of clinical illness, manifested by daily high fever that continues for

4–8 weeks in untreated cases. This second bacteremia may lead to invasion of the gall bladder, kidney and reinvasion of the gut mucosa, especially at the Peyer's patches. At this stage, *S. typhi* can be isolated not only from blood, but also from stool and urine (Alpuche-Aranda *et al.*, 1994; Fields *et al.*, 1986).

Invasion of the gall bladder by *S. typhi* may be temporary or may result in the long-term colonization that characterizes the typhoid carrier state, especially in the presence of gallstones. Occasionally, acute necrotizing cholecystitis may result. *S. typhi* survive well in gallstones and can be recovered from the centre of a stone and also viable organisms may be obtained even after dipping stones in antibiotics (Keusch and Acheson, 1999). Gallstones are thus a source of prolonged asymptomatic carriage and excretion of the organism in stool.

The source of secondary gut invasion may be *S. typhi* from bloodstream during the prolonged secondary bacteremia or *S. typhi* shed in the bile. Such secondary reinvasion leads to severe bleeding and/or perforation attributable to the marked inflammatory response induced in the Peyer's patches. It is not known why the invasion of the gut at this stage results in more extensive damage to the intestinal mucosa than the primary invasion, but severity could be immunologically mediated. Invasion of liver, spleen and kidney can result in hepatitis, splenitis that makes the spleen prone to rupture, or glomerulonephritis.

Perforation of the intestine and haemorrhage are two of the most feared complications of typhoid fever occurring in 0.5-1% of cases (Levine, 1999). Relapses are not uncommon. Paratyphoid fever is similar to typhoid fever but is usually much milder and is caused by the organisms *S. paratyphi* A, B and C.

1.3. TYPHOID FEVER

1.3.1. Epidemiology:

WHO estimates that there are about 16 million cases of clinical typhoid fever annually and many sub-clinical infections worldwide, resulting in about 600,000 deaths every year throughout the world (WHO, 1996). The incidence of the disease increases wherever developments in sanitation are unable to keep pace with a rapid growth in population. The cultivation of rice in paddy fields, raw fruits washed with contaminated river water, vegetables fertilized with waste products, dairy products contaminated by the hands of carriers and, in some countries, shellfish harvested from contaminated coastal waters, have been associated with the occurrence of typhoid fever. Canned food and bottled water are usually safe, although outbreaks have occurred through faulty processing (http://www.worldwidevaccines.com/public/diseas/typ13.asp).

The global distribution of typhoid fever (Fig. 4) is very similar to that of hepatitis A (DuPont and Steffen, 1997). As with hepatitis, typhoid fever is endemic in all developing countries, where children and the youth aged 5-12 years are mostly affected, but relatively high incidence rates are also observed in young adults (Mermin *et al.*, 1996). It is generally accepted that acquired immunity explains the decreased incidence in adults

living in endemic areas. For instance, sero-epidemiological studies in Peru and China have shown that by 15-19 years of age 50-80% of teenagers have serological evidence of past infection with *S. typhi* (Levine, 1999). In endemic areas, typhoid fever is a major cause of absenteeism from school and employment and the direct expenditures for hospitalization and medication further raise the public health costs of this disease.

Figure 4. The geographical distribution of typhoid fever cases

1.3.2. Diagnosis:

It is very difficult to diagnose typhoid fever based on the clinical features of the disease since similar signs and symptoms are mimicked by malaria parasite infestation and many other conditions. However, if the fever is accompanied by a slow heart rate (bradycardia) and an abnormally low level of white cells in the blood (leucopenia), typhoid fever is considered as a possible diagnosis with appropriate bone marrow or blood culture in the early stages of the infection or stool culture later on in the course of the disease (http://gsbs.utmb.edu/microbook/ch021.htm; Keusch, 1994). Thus, the disease is generally diagnosed in the laboratory by culture on selective media and combination of serological and biochemical tests to identify the individual serovars. The gold standard of bacteriological confirmation of typhoid fever is, however, the bone marrow culture, which is positive in 85-90% of cases, even when the patient has received antibiotics (Levine, 1999). In the case of the stool, isolation of *S. typhi* only confirms the diagnosis if characteristic clinical features are present since the patient may be a chronic carrier. Prompt processing of stool specimen is important, since a drop in pH, which occurs with a decrease in temperature, can inhibit the growth of *Salmonella* species (http://pathology5 pathology.jhmi.edu/micro/v16n02htm).

The Widal agglutination test, which is used in diagnosing typhoid fever in most developing countries, measures the antibodies against flagellar (H) and somatic (O) antigens of *S. typhi*. However, this test can only support the diagnosis of typhoid if there is a fourfold rise in the titre of antibody to the O antigen. The H antibodies appear shortly after O antibodies and persist for more than a few months. Hence, a rise or high

O antibody titre generally indicates acute infection, while a rise in H antibody helps to identify the type of enteric fever. H antigen also provides a useful epidemiological tool with which to determine the source of infection and its mode of spread. Agglutination test, however, may often be misleading since raised antibodies may result from typhoid immunization or from earlier infection with salmonellae or other Gram-negative bacteria sharing common antigens. A high Vi capsular antibody is suggestive of a carrier state, but there are high rates of false-positives and of false-negatives. Thus, serological evidence alone is not sufficient for diagnosis (DuPont and Steffen, 1997).

Diagnosis can also be based on EnteroTest, which is used to obtain bile cultures for *S. typhi* from the bowel. It consists of a nylon string affixed to a gelatin capsule with the end of the string taped to the cheek and the gelatin capsule swallowed. After a meal or overnight, the string is removed and mucus scraped from the string cultured for *S. typhi*. An alkaline pH of the string should confirm a duodenum location of the organism. Recently, indirect haemagglutination, indirect fluorescent Vi antibody, ELISA for IgM and IgG antibodies to *S. typhi* polysaccharide, monoclonal antibodies and DNA probes have been evaluated with promising results, but these tests are not yet routinely used (DuPont and Steffen, 1997).

1.3.3. Drugs used in treatment and prevention of typhoid fever:

Treatment of typhoid fever is usually by antibiotics. However, the early use of antibiotics is associated with a relatively high rate of relapse (Keusch, 1994). In some cases the relapse rate may be as high as 20% compared to 5-12% in most untreated cases. This is

because prompt therapy does not allow adequate immune response. Nevertheless, effective therapy potentially reduces suffering, complications and loss of life. Most clinical responses are usually seen within 1-2 days during treatment. The mortality rate may fall from more than 10% to less than 1% in most cases after antibiotic therapy (Hornick, 1991). Ampicillin, chloramphenicol, tetracycline and trimethoprim-suphamethoxazole, either used individually or in combination, have been the first-line drugs until recently, due to the appearance of resistant strains to these antibiotics (Newman, 1996; 2000; Rowe *et al.*, 1997). More recently quinolones, such as ciprofloxacin, and third-generation cephalosporins, such as ceftriaxone, are now recommended as the first line treatments for multi-drug resistant typhoid fever.

Ampicillin:

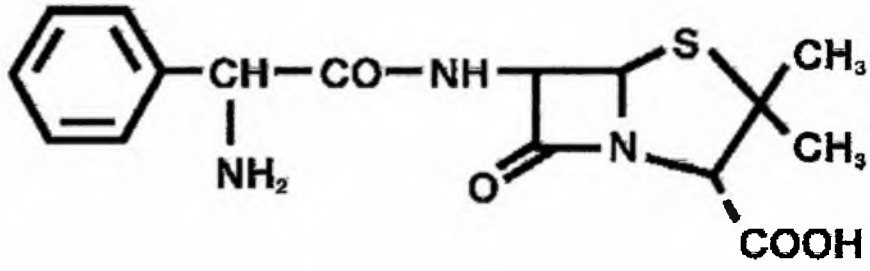

Figure 5. Chemical structure of ampicillin

Ampicillin (Fig. 5) is a semi-synthetic penicillin derived from the penicillin nucleus, 6-amino-penicillanic acid. It is an antibiotic with a broad spectrum of bactericidal activity against most of the bacteria that show sensitivity to penicillin G. Ampicillin is indicated

primarily for infections with certain Gram-negative organisms and for enterococcal infections. Ampicillin is effective in typhoid fever caused by sensitive organisms and, with probenecid, it has been effective in treating some chronic typhoid carriers.

Like other penicillins, ampicillin inhibits the third and final stage of bacterial cell wall synthesis by preferentially binding to specific penicillin-binding proteins (PBPs) that are located inside the bacterial cell wall. PBPs are responsible for several steps in the synthesis of the cell wall and are found in quantities of several hundred to several thousand molecules per bacterial cell. PBPs vary among different bacterial species. Thus, the intrinsic activity of ampicillin, as well as the other penicillins, against a particular organism depends on their ability to gain access and bind with the necessary PBP. Like all β-lactam antibiotics, ampicillin's ability to interfere with PBP-mediated cell wall synthesis ultimately leads to cell lysis. However, due to the increasing bacterial resistance, the efficacy of ampicillin against strains of *E. coli*, *Salmonella*, and *Shigella* has been declining (http://www.medicine.mcgill.ca/cai/meded/drugdb/ampicillin).

Ampicillin may be given orally, intramuscularly or intravenously. Oral absorption is variable and may be decreased when the drug is taken with food. Peak blood levels occur about two hours after oral or one hour after intramuscular administration. The usual oral dosage in adults and children weighing greater than 20 kg is 250 to 500mg every six hours. In children less than 20 kg, it is 50 to 100 mg/kg/day in divided doses. The parenteral dosage is 1-2g four to six hours in adults and 100 to 200 mg/kg/day in divided doses in children. For bacteremia, the dosage is 150 to 200 mg/kg/day intravenous for adults and 200 to 400 mg/kg/day (maximum dose 12g/day) intravenous in children.

Chloramphenicol:

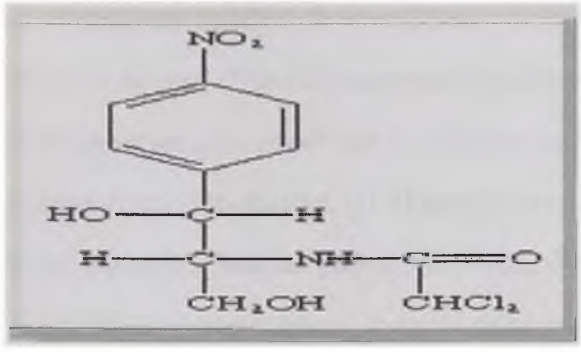

Figure 6. Chemical structure of chloramphenicol

Chloramphenicol (Fig. 6) has been the drug of choice since 1948 throughout the world, until the 1970s due to the emergence of resistant strains (Rowe *et al.*, 1997). Nevertheless, it is still the standard treatment in some developing countries such as Ghana due to its affordability. Chloramphenicol binds to peptidyl-transferase and inhibits transfer of growing peptide chain to "Acceptor" site aminoacyl-tRNA. It is therefore primarily bacteriostatic and has a wide spectrum of activity against Gram-positive and Gram-negative cocci and bacilli. The drug is well absorbed orally but not intramuscularly (IM), and intravenous (IV) route is used for parenteral therapy. It is metabolised in the liver to the inactive glucuronide which is excreted together with chloramphenicol in the urine. Due to the fact that active chloramphenicol is metabolised in the liver to inactive glucuronide the drug does not accumulate in the plasma of patients with renal insufficiency. Chloramphenicol therapy is mostly restricted to serious infections, because it may cause the potential lethal complication of aplastic anaemia (http://www.merck.com/pubs/mmanual/section13/chapter153/153f.htm).

The dosage of chloramphenicol in adults and children is 50 mg/kg/day orally or intravenous in divided doses every six hours. In other serious infections, 75 to 100 mg/kg/day in divided doses are used. To avoid the gray baby syndrome, newborns less than or equal to one month are not given greater than 25 mg/kg/day initially. Doses are adjusted to result in serum levels of 10-30µg/mL (31-93 µmol/L) to avoid toxicity, especially in newborns, premature infants, and patients with hepatic disease. Chloramphenicol is not given to women in labour. It should not be used topically because small amounts may be absorbed which can cause aplastic anaemia (http://www.merckcom/pubs/mmanual/section13/chapter153/153f.htm).

Tetracyclines:

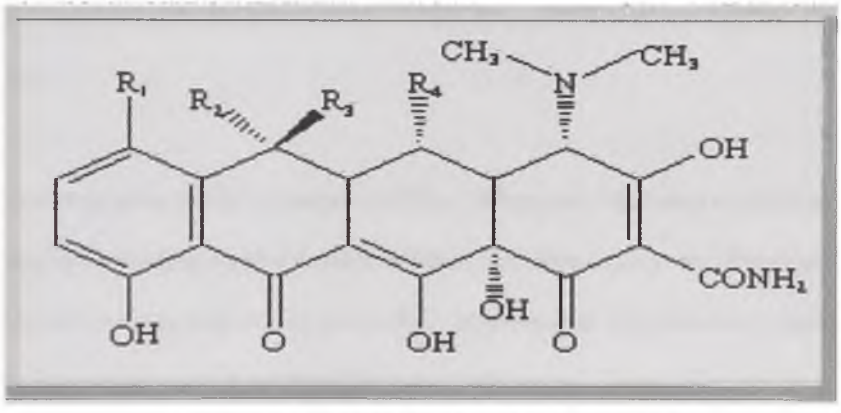

Figure 7. Chemical structure of a tetracycline

Tetracyclines (Fig. 7) bind reversibly to the 30S subunits of bacterial ribosomes where they interfere with binding of charged-tRNA to the "Acceptor" site. They are bacteriostatic rather than bacteriocidal. Tetracyclines can also inhibit protein synthesis in

the host, but are less likely to reach the concentration required because eukaryotic cells do not have a tetracycline uptake mechanism as prokaryotic cells do. They are amphoteric, and thus form salts with both strong acids and bases (http://www.vet.purdue.edu/depts/bms).

All orally administered tetracyclines produce varying degrees of gastro-intestinal (GI) adverse reactions, such as nausea, vomiting, and diarrhoea, and can cause pseudomembranous colitis (caused by *Clostridium difficile*) and *Candida* superinfections. Thrombophlebitis is common with intravenous use. Tetracyclines can cause staining of teeth, hypoplasia of dental enamel, and abnormal bone growth in children less than or equal to 8 years old and in the foetuses of pregnant women. Therefore, tetracyclines should be avoided after the first trimester of pregnancy and in children less than eight years old.

Tetracycline is given orally in dosages of 250 to 500mg every six hours to adults and 25 to 50 mg/kg/day in four divided doses to children older than eight years. Doses should be taken an hour before meals or two hours after. Intramuscular injection is very painful, and the intravenous route is preferred for parenteral therapy. Tetracycline can be given intravenously in dosages of 250 to 500mg (rarely 1g) every twelve hours to adults and, in those rare cases when required, 10 to 25 mg/kg/day in two to three equal doses to children older than eight years.

Trimethoprim-sulphamethoxazole:

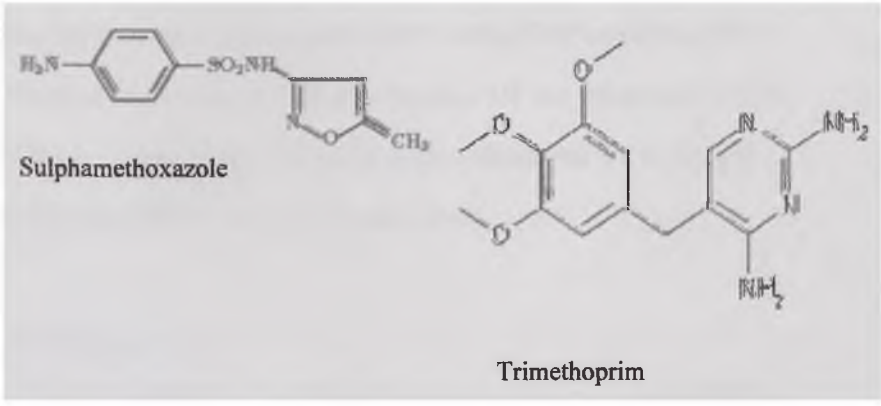

Figure 8. Chemical structures of trimethoprim and sulphamethoxazole

Trimethoprim-sulphamethoxazole (TMP-SMX) (Fig. 8) is a fixed combination (1:5) of Trimethoprim and sulphamethoxazole. It is usually bacteriostatic. The dosage ratios are set to produce a twenty to one ratio of SMX to TMP in blood and tissues, which gives maximal antibacterial activity. Both drugs block the folic acid metabolism cycle of bacteria and are much more active together than either agent alone. Sulfonamides are competitive inhibitors of the incorporation of *p*-aminobenzoic acid. TMP prevents reduction of dihydrofolate to tetrahydrofolate. Both TMP and SMX are well absorbed orally and are excreted in the urine. They have similar half-lives of about nine hours in plasma and penetrate well into tissues and body fluids, including the CSF. TMP-SMX is useful in treatment of typhoid fever, especially when ampicillin and chloramphenicol cannot be used. The adverse reactions are usually nausea, vomiting, rash, and folate deficiency resulting in macrocytic anaemia.

The usual oral dosage in adults is two regular-strength tablets (each tablet contains 80mg TMP and 400mg SMX) or one double-strength tablet (160mg TMP and 800mg SMX) twice daily. The usual oral dosage in children is 8mg/kg TMP and 40mg/kg SMX daily in two divided doses. The intravenous dosage in adults and children is 8 to 12mg/kg TMP and 40 to 60mg/kg SMX daily in four divided doses.

Quinolones and fluoroquinolones:

The quinolones and fluoroquinolones are bacteriocidal and inhibit the activity of DNA gyrase. The older quinolones, nalidixic acid and cinoxacin, are active only against Enterobacteriaceae, with no activity against Gram-positive organisms, *Pseudomonas aeruginosa,* or anaerobes. The fluoroquinolones have much greater activity against Enterobacteriaceae and are also active against staphylococci, *P. aeruginosa, Mycoplasma, Chlamydia,* and some streptococci, but with the exception of trovafloxacin, are not reliably active against anaerobes. Resistance to one fluoroquinolone generally means resistance to all. Fluoroquinolones with the exception of norfloxacin are better absorbed orally, resulting in blood levels adequate for treating systemic infection.

Except for ciprofloxacin, ofloxacin, trovafloxacin, and levofloxacin, the quinolones are available only orally. After administration, they are widely distributed to most body fluids and tissues. However, norfloxacin does not reach concentrations adequate for treatment of systemic infection. The quinolones are variably metabolized in the liver and excreted in the urine. All the quinolones and fluoroquinolones are useful in urinary tract

infections. The fluoroquinolones are effective in bacterial diarrhoea except that caused by *Campylobacter difficile*.

Serious adverse reactions to quinolones are uncommon. About 5% of patients experience gastro-intestinal side effects such as nausea, vomiting and anorexia. Diarrhoea, leucopenia, anaemia, rash and photosensitivity are uncommon. There is some concern that tendonitis, including rupture/Achilles tendon, is associated with fluoroquinolone use. Nephrotoxicity is rare with side effects on the central nervous system (CNS) occurring in less than 5% of patients. These are usually manifested by mild headaches, sleep disturbance or mood alteration. Seizures are rare, but these drugs should be avoided in patients with convulsive or other CNS disorders. The fluoroquinolones are currently contraindicated for use in children and pregnant women, but further research is ongoing. Ciprofloxacin is given orally in dosage of 250 to 750 mg twice daily in adults and intravenous in a dosage of 200 to 400mg every twelve hours.

Third-generation cephalosporins:

These drugs have excellent activity against Enterobacteriaceae. Of the parenteral drugs, cefotaxime, ceftizoxime, and ceftriaxone have very similar *in vitro* activity. Ceftazidime is more active than cefoperazone against Enterobacteriaceae and *P. aeruginosa*. In general, Enterobacteriaceae can be treated with cefotaxime, ceftriaxone, ceftazidime, or ceftizoxime.

Ceftriaxone is given intramuscularly or intravenously in a dosage of one to two grams once or twice per day to adults. In children, 50 to 75mg/kg/day (not to exceed 2.0 g) is given in one to two equally divided doses.

1.4. ANTI-MICROBIAL MEDICINAL PLANTS:

There are claims of a number of medicinal plants used for the treatment of a variety of ailments caused by microorganisms, from stomachache to diarrhoea, malaria and skin infections. *Ocimum gratissimum* leaf or whole plant is known to be very effective against diarrhoea. The use of *Azadirachta indica* (neem) to treat malaria has been in existence since time immemorial along the Western, Central and Eastern African regions (Sofowora, 1993; Mshana *et al*., 2000). Chewing sticks used in many African homes to clean teeth have been shown to possess antimicrobial activity against oral microbial flora (Asuquo and Montefiore, 1977; Moran *et al.*, 1988; Al.lafi and Jordan, 1995). In Tanzania the root or root bark of *Zanha africana* is used to treat various skin diseases and the Traditional Medicine Research Unit (TMRU) of the Muhimbili Medical Centre in Dar-es-Salaam has demonstrated that the methanolic extract of *Z. africana* root possesses strong antifungal activities when tested against *Trichophyton rubrum* and *Trichophyton mentagraphytes* using Sabouraud dextrose agar medium and mycobiotic agar medium (Kokwaro, 1993). Addae-Mensah (1992) has reported the treatment of gonorrhoea, syphilis as well as other bacterial and viral infections using *Piper guineense*. Methanolic extract of *Bridelia ferruginea* is known to be active against *S. dysenteriae*, *P. aeruginosa*, *E. coli*, *S. typhimurium*, and *P. vulgaris* (Akinpelu and Olorunmola, 2000; Muanza, *et al.*, 1994).

The dried aerial parts of *Hoslundia opposita* is used to treat gonorrhoea, cystitis, hookworm, cough, fevers, colds, wounds, and bilharzia in Tanzania (Chhabra and Uiso, 1991). In Cameroon and East Africa, the hot water extract of the dried entire *H. opposita* plant is used for snakebites, herpes, conjunctivitis, yellow fever, stomach troubles, liver diseases, mental disorders and as an antibiotic, (Ngadjui *et al.*, 1991; Hedberg, *et al.*, 1983). In Ghana, the hot water extract from the leaves of *H. opposita* is known to reduce fever possibly due to malaria (Boye, 1989) whilst in Ivory Coast, it is used for dysmenorrhoea (Bouquet and Debray, 1974). In Nigeria, the hot water extract is used for convulsions, to treat bad breath accompanied by aching chest and head (Aka and Nwambie, 1993). In Ivory Coast, hot water extract of the entire plant of *Cnestis ferruginea* is used as an aphrodisiac (Bouquet and Debray, 1974), whilst in Senegal, it used for conjunctivitis, syphilis, gum pain, wounds/sores, diarrhoea and dysentery (Le Grand, 1989). *Psidium guajava* is used to treat acute and chronic diarrhoea as well as *Herpes zoster*, a virus infection (Ghana Herbal Pharmacopoiea, 1992; Mshana, 2000).

1.5. CHARACTERIZATION OF PATHOGENIC MICROORGANISMS:

1.5.1 Polymerase chain reaction (PCR):

PCR is a technique that amplifies a specific DNA segment from a gene (Coen, 1994). Amplification of the target DNA sequence is accomplished in three basic steps. The first step, which is denaturation, involves separating the double stranded DNA molecule into single strands. The second step, annealing, refers to the ability of gene-specific primers to bind to specific sequences of the DNA sequence now being used as a template, and the final step, elongation, involves extension of primers by *Thermos aquaticus* (Taq) DNA

polymerase. These three steps are repeated approximately 30 times, resulting in the amplification of the desired gene sequence. There are several applications of PCR including direct cloning of amplified fragments, detection of mutagenesis, and genetic fingerprinting of complex genomes (Belkum, 1994; Coen, 1994). PCR typing methods are simple, fast, and provide a comparative way to differentiate organisms (Liu *et al.*, 1994).

Randomly amplified polymorphic DNA (RAPD) PCR:

This is based on decreased annealing temperatures resulting in low stringent amplification of random DNA fragments within the genome of an organism (Sechi *et al.*, 1999). RAPD requires no previous knowledge of the DNA that is to be amplified and involves primers that are randomly selected and vary in length. The primers are generally shorter than primers used in conventional PCR. Since the number of annealing sites vary between strains, amplification results in variable DNA patterns (Power, 1996). Fingerprints generated from RAPD provide the highest level of taxonomic resolution achieved by PCR methods (Vinuesa *et al.*, 1998).

Enterobacterial repetitive intergenic consensus sequence (ERIC) PCR:

This is based on a consensus sequence found in the Enterobacteriaceae family (Power, 1996). ERIC sequences are repetitive elements that are 126 base pairs long and contain a highly conserved central inverted repeat. They appear to be restricted to transcribed regions of the chromosome and their position seems to be variable in different species. ERIC-PCR has been used to successfully fingerprint numerous organisms including, *S.*

enterica and *S. typhimurium* (Versalovic *et al.*, 1991; Beyer *et al.*, 1998; Sechi *et al.*, 1999).

Repetitive extragenic palindromic (REP) PCR:

This is based on short extragenic repeat sequences found throughout the genome of bacteria (Power, 1996). These sequences, which have a consensus 38 base pair sequence, appear to be highly conserved among many members of the family Enterobacteriaceae. Functions proposed for REP elements include roles in transcription termination, mRNA stability, and chromosomal domain organisation. No examples of the REP sequence coding for a protein have been reported. Even though the functions of REP sequences are unclear, they are a significant part of the bacterial genome. REP-PCR is simple, rapid, and sensitive for discriminating between closely related strains. It has been used in the typing of many organisms such as *S. enterica* and *S. typhimurium* (Stern *et al.*, 1984; Versalovic *et al.*, 1991; Wood *et al.*, 1992; Beyer *et al.*, 1998).

1.5.2 Plasmid incompatibility:

Plasmids are small circular, double-stranded DNA molecules that replicate independently of the cell's chromosomes and found mainly in bacteria and some eukaryotic microorganisms, such as *Saccharomyces cerevisiae*. It has been found that not all plasmids are able to coexist in the same cell and any two plasmids known to bear this trait are described as incompatible. Plasmids which have the same replication control functions are incompatible, and those plasmids which share the trait of a similar nature are assigned to the same incompatibility group (*inc* group). Thus, plasmids of one

incompatibility group are related to each other, but cannot survive together in the same bacterial cell, as only different kinds of plasmids are compatible. In many cases, incompatible plasmids belonging to the complex group *Inc*HI have been implicated in bacterial resistance to antibiotics by *S. typhi* (Rowe *et al.*, 1990) and therefore could be used to characterize different strains of *S. typhi*.

1.5.3 Antibiotic susceptibility patterns:

Laboratories involved in epidemiological studies frequently perform antibiotic susceptibility tests to bacteria. Most of these tests are qualitative in terms of categorizing strains as susceptible, intermediate, or resistant to various antibiotics (Pfaller and Cormican, 1996). When the method is used to determine minimal inhibitory concentrations (MIC) it is considered quantitative. Common approaches to antibiotic susceptibility testing include the Kirby-Bauer test (Tortora *et al.*, 1995), in which paper disks impregnated with different concentrations of antibiotics are placed in contact with bacterial lawns (Hunt and Sandham, 1969).

Most strains of pathogenic bacteria isolated from the same hospital often share similar susceptibility patterns, resulting in poor discrimination of the isolates (Debast *et al.*, 1995). The information acquired from these tests, however, may be helpful in the recognition of different bacterial strains and in detecting early trends of elevating MIC among groups of organisms (Pfaller and Cormican, 1996). Generally, determination of MIC is not suitable for epidemiological typing by itself. If used in combination with other typing methods, it can further discriminate between strains (Larose *et al.*, 1990) and

be useful as an epidemiological tool (Acar, 1986). Antibiotic susceptibility testing is relatively inexpensive and generally adequate in most clinical settings which have the facilities (Pfaller and Cormican, 1996).

CHAPTER TWO

MATERIALS AND METHODS

2.1. MATERIALS:

2.1.1. Chemicals, media and reagents:

Ampicillin, chloramphenicol, rifampicin, tetracycline, trimethoprim-sulphamethoxazole, Mueller Hinton agar and Mueller Hinton broth were obtained from Becton Dickinson Microbiology System, Cockeysville, MD, USA. Dimethylsulfoxide (DMSO) was from the British Drug House (BDH), UK whilst the *Salmonella* antisera was obtained from Denka Seiken Co., Ltd., Japan. Agarose gel, glycerol, Luria-Bertani (LB) broth and sterile swabs were obtained from Fisher Scientific, Maryland, USA whilst the *E. coli* DH5α and *E. coli* HMS 174 were from Genetic Stock Center, Yale University, New Haven, USA. From Oxoid, Maryland, MD, USA the following were obtained; blood agar, desoxycholate agar (DCA), Kovac's reagent, MacConkey agar, nutrient agar, *Salmonella/Shigella* (SS) agar, selenite F broth, sulphur indole motility (SIM) agar, triple sugar iron (TSI) agar, trypton soya agar (TSA), trypton soya broth (TSB), urea agar and 40% urea solution. The wizard genomic DNA purification kit was obtained from Promega, Maryland, USA whilst the QIAprep spin miniprep kit was obtained from QIAGEN, Valencia, USA. Acetic acid, deoxynucleoside triphosphate (dNTP), ethidium bromide, ethylenediaminetetraaceticacid (EDTA), calcium chloride, magnesium chloride, potassium chloride, *Thermos aquaticus* (*Taq*) polymerase, tris-hydrochloric acid (Tris-HCl) and triton-X100 were from Sigma-Aldrich, Germany.

2.1.2. Bacterial sample:

Majority of the *Salmonella* isolates were supplied by the Microbiology Department, Korle-Bu Teaching Hospital, whilst the others were isolated from samples collected by the staff of the Bacteriology Unit, Noguchi Memorial Institute for Medical Research (NMIMR), Legon. The sources of the samples were blood, cerebrospinal fluid, food, stool, urine, and from other miscellaneous sources.

2.1.3. Herbal preparations:

The aqueous herbal concoction and the decoctions were supplied by the Production Department of the Centre for Scientific Research into Plant Medicine (CSRPM) at Mampong-Akwapim. At the Production Department, the twelve (12) plant materials were boiled together with water to extract the juice. The extracted juice was cooled and lyophilized at the Pharmacology and toxicology Department also of CSRPM. The leaves from each plant were also individually boiled in water and cooled at the Production Department. The aqueous extracts were then filtered and subsequently lyophilized at the Pharmacology and toxicology Department.

In this study, the freeze-dried products were reconstituted in distilled water (32% w/v), dispensed into 15ml centrifuge tubes and refrigerated at 4°C until needed. Some of the herbal preparations were sterilized by autoclaving at 121°C for 15min, cooled, and centrifuged at 7,000rpm for 15min. The supernatants were then refrigerated at 4°C until needed.

Solvent-solvent extraction method was used to obtain fractions of plant extracts found to have activity against the standard *Salmonella*. The following organic solvents, petroleum ether, chloroform, ethylacetate, and n-butanol were used for the extraction. Volumes of 400ml of aqueous extract of the plant and 200ml of the organic solvent were added in a separating funnel and shaken intermittently. After 48 hours the aqueous layer was removed and the solvent from the organic layer evaporated using a rotary evaporator. The residue from each fraction was then reconstituted into 10ml solution using 20% dimethylsulphoxide (DMSO). The antimicrobial activities of the fractions were then investigated using the agar diffusion method. The plants used were *Hoslundia opposita*, *Cnestis ferruginea*, and *Psidium guajava*.

2.2. METHODS:

2.2.1. Bacterial culture, isolation and identification:

The specimens were plated out using appropriate selective media such as MacConkey agar, blood agar and desoxycholate agar and/or *Salmonella/Shigella* agar, and colonies with the characteristics of *Salmonella* isolated and purified using standard microbiological methods (Farmer, 1999). Identification of *Salmonella* from the various sources was done using the following biochemical tests; Triple Sugar Iron (TSI) agar test, Sulphur Indole Motility (SIM) agar test, and Urea agar test. The presence of *Salmonella* was confirmed and grouped using *Salmonella* antisera kit from Denka Seiken Co., Ltd., Japan. Briefly, a needle of solid bacterial growth (or two loops of broth bacterial growth) was placed in the centre of a clean sterilized slide. One drop (and only one drop) of

sterile physiological saline was added to the specimen. Where broth medium was used no saline was added. Using an inoculating loop or the tip of a toothpick, the specimen-saline mixture was completely mixed after which a drop of the *Salmonella* antisera was added. The resulting mixture was rocked for about 5 seconds and observed for coagulation.

In vitro antibiotic susceptibility against ampicillin (10µg), chloramphenicol (30µg), tetracycline (30µg), and trimethoprim-sulphamethoxazole (1.25/23.75µg) was tested according to the guidelines set by the National Committee for Clinical Laboratory Standards (NCCLS, 1998).

2.2.2. Minimum inhibitory concentration (MIC) of the antibiotics used:

The minimum inhibitory concentration for the antibiotics against the *Salmonella* isolates were determined by the agar dilution technique according to the guidelines set by the National Committee for Clinical Laboratory Standards (NCCLS, 1997). Sterile Mueller-Hinton agar in molten form was prepared and distributed in exact aliquots sufficient to dilute the starting antibiotic10-fold. The appropriate volume of the starting antibiotic was added to the molten Mueller-Hinton (MH) agar that had been allowed to equilibrate to 48-50°C in a water bath. The agar-antibiotic mixture was mixed thoroughly by gentle inversion and the mixture poured into100mm petri dishes on a level surface to result in an agar depth of about 4mm. The agar was allowed to solidify at room temperature and the plates used immediately. For growth controls, plates containing no antibiotic were prepared.

The top of a well isolated *Salmonella* colony was touched with a wire loop and transferred into 3ml MH broth. The broth culture was incubated at 37°C until it achieved the turbidity of 0.5 McFarland standard resulting in a suspension containing approximately 1-2 x 10^8 CFU/ml. By using sterile broth, a 1:10 dilution of the bacterial suspension for each of the 115 strains was made to give an adjusted concentration of 10^7 CFU/ml. An aliquot of each diluted suspension was placed in a well of a replicator inoculum block. A 1-2ul aliquot of each inoculum was applied to the agar surface by the use of an inocula-replicating device. A growth control plate (no antimicrobial agent) was inoculated first and then, starting with the lowest concentration, the plates containing the different antimicrobial concentrations were inoculated. A second growth control plate was inoculated last to ensure that there was no contamination or significant antimicrobial carryover during the inoculation. A sample of each inoculum was streaked on a nutrient agar plate and incubated overnight to detect any probable contamination and to provide freshly isolated colonies in case retesting proved necessary. The inoculated plates were allowed to stand at room temperature until the moisture in the inoculum spots had been absorbed into the agar. The plates were then inverted and incubated at 35°C for 16-20 hours.

The lowest concentration of antibiotic that allowed no more than one or two CFU or only a slight haze to grow was chosen as the MIC.

2.2.3. Storage of *Salmonella* isolates:

The salmonellae were grown overnight at 37°C in 1.5ml TSB with shaking. To each overnight culture was added 1.5ml 40% glycerol-TSB mixture and mixed gently without vortexing. Two milliliter sample of the mixed cultures was put into a 2ml cryovial under sterile conditions, labelled with name/type of organism and date and stored at -70°C until needed.

2.2.4. Isolation and profiling of plasmids:

Plasmids from the 115 strains were isolated using the QIAprep spin miniprep kit according to the manufacturer's guidelines. This procedure uses the modified alkaline lysis method of Birnboim and Doly (1979). The organisms were grown in 3ml Luria-Bertani (LB) broth for about 16 hours at 37°C and centrifuged at 13,000g for about 1 minute. The pellet obtained was resuspended in 250µl of glucose solution (making sure that no clumps were visible after resuspension), and the whole content transferred to a microfuge tube. Into this tube was added 250µl of alkaline-sodium dodecyl sulphate (SDS) buffer, gently inverted 4-6 times or until the solution became viscous and slightly clear. Potassium acetate (KAC) solution was then added and the tube immediately inverted, but gently, 4-6 times or until the solution became cloudy, care being taken to avoid local precipitation. Centrifugation of the microfuge tube with its content was done at 13,000g for 10min. The supernatant fraction was decanted into QIAprep column and centrifuged again at 13,000g for 30-60 seconds. The flow-through was discarded and the QIAprep Spin column washed with 0.75ml of 95% ethanol and centrifuged for 30-60 seconds after which the flow-through was discarded and the column centrifuged again for

30-60 seconds. The QIAprep column was placed in a clean 1.5ml microfuge tube and the DNA was eluted with pre-heated polished water after centrifugation at 13,000g for 1 minute. The eluted plasmids were stored at 4°C until needed.

Agarose gel electrophoresis was used to visualize plasmid DNA products. A 0.7% agarose gel was prepared by dissolving 0.28g of agarose in 40ml of 1X TAE (Tri-acetic acid-EDTA), which was prepared from a 50X stock (242g Tris, 57.1ml acetic acid, and 4ml 0.5M EDTA/1L). Ethidium bromide (1µl of a 10mg/ml solution) was added to aid visualization of the DNA. The electrophoretic chamber was a Minicell EC370M powered by a Bio-RAD model 250/2.5 power supply. The agarose gels were visualized using a UV Intensity Trans-illuminator and documented with Panasonic CCD Ultra Lum camera and scion image software.

2.2.5. Plasmid incompatibility testing:

Plasmid DNA was isolated from each *Salmonella* strain, transformants and transconjugates to determine if they belonged to the incompatibility group *Inc* HI. The Rep HI1A replicon, present in *Inc* HI plasmids, was amplified via the polymerase chain reaction (PCR) using the primers 5'GGTCCAACCCATTGCTTTAC3' and 5'CACGGAAAGAAATCACAAC3' on a model PT 150 MiniCycler. Reaction conditions consisted of 50ng plasmid DNA and 50nM of each primer in a buffer composed of 10mM Tris-HCl (pH 8.3), 50mM KCl, 1.5mM $MgCl_2$, 200µM dNTP mixture, and 1U of *Taq* polymerase in a final volume of 100µl. Amplification conditions were 30 cycles of 94°C/30sec, 55 °C/30sec, and 72 °C/30sec, with a final extension step

of 72°C for 10min. Amplicons of 365bp were considered positive for the Rep HI1A replicon.

2.2.6. Transfer of antibiotic resistance via conjugation:

This was carried out using the resistant *Salmonella* isolates as the donors and *E. coli* HMS 174 as the recipients. Preliminary investigations were done to establish that the *Salmonella* strains were sensitive to 100µg/ml rifampicin whilst the *E. coli* HMS 174 was resistant at this concentration. These organisms were grown separately overnight at 37°C with shaking in 3ml tryptic soy broth (TSB). Into fresh 3ml TSB was added 0.3ml overnight cultures and the mixture incubated at 37°C with shaking for about 1 hour or until 0.5 McFarland turbidity standard was obtained. Five hundred microlitres of each suspension of *Salmonella* strain was mixed with the same quantity of *E. coli* HMS 174 in a sterile tube and the suspension incubated at 37°C without shaking for 90 minutes. At 30-minute intervals, the cultures were mixed by gentle inversion. These conjugation mixtures were then plated on MacConkey agar plates containing 100µg/ml rifampicin and either ampicillin (32µg/ml), chloramphenicol (32µg/ml), or trimethoprim-sulphamethoxazole (4µg/ml; 16µg/ml) and incubated overnight at 37°C. Lactose fermenting colonies growing on the plates indicated conjugational transfer of antibiotic resistance to *E. coli* HMS 174.

2.2.7. Preparation of competent cells and transformation by heat shock technique:

Competent cells were prepared by inoculating 2ml of 2X Luria Bertani (LB) broth with a single colony from a plate culture and grown overnight at 30°C with shaking.

Aseptically, 0.5ml of the overnight culture was added to 200ml pre-warmed (30°C) 2X LB contained in a 1 litre flask. This represented 1:400 dilution. The cells were incubated at 30°C with shaking until an OD_{600} of 0.3 was obtained. Four milliliters of 1M magnesium chloride ($MgCl_2$) was then added and growth continued until the OD_{600} was 0.45-0.55. The cells were chilled on ice for 2 hours, and the content placed in a sterile centrifuge tube and spun at 3000g for 5min at 4°C. The supernatant fraction was discarded and the cells resuspended in 100ml of 1M ice cold calcium chloride ($CaCl_2$) solution using a pipetteman without vortexing. The resuspended cells were put back on ice for another 40min after which the tube was centrifuged at 3000g for 5min at 4°C. The supernatant fraction was again discarded and the cells resuspended in 5.1ml of ice-cold $CaCl_2$-glycerol media. The suspension, containing competent cells, was aliquoted into sterile microfuge tubes and stored at -70°C.

Plasmid DNA was isolated as previously described and used to transform *E. coli* DH5α according to standard procedures (NCCLS, 1997). Briefly, 2µl of the plasmid preparation was added to 100µl of the competent cell suspension and incubated on ice for 15-30min. The DNA-competent cell suspension was placed in a 42°C water bath for 90sec and then chilled on ice for 1min. Nine hundred microlitre volume of LB was added immediately and the cells incubated at 37°C for 1 hour for expression of antibiotic resistance. Selection of transformants was made on trypton soy agar (TSA) plates containing either ampicillin (32µg/ml), chloramphenicol (32µg/ml), or trimethoprim-sulphamethoxazole (4µg/ml:16µg/ml). Previous testing had proved that the *E. coli* DH5α was sensitive to all antibiotics investigated in this study.

2.2.8. Chromosomal DNA:

Isolation:

Chromosomal DNA was isolated from each *Salmonella* strain using the Wizard Genomic DNA Purification Kit. Samples of 1ml each of overnight culture of the *Salmonella* strains were dispensed into a 1.5ml microfuge tube and centrifuged at 13,000-16,000g for 2 minutes to pellet the cells. The supernatant fraction was discarded and 600µl of Nuclei Lysis Solution added and gently pipetted until the cells were resuspended. The suspension was incubated at 80°C for 5 minutes to lyse the cells. It was then cooled to room temperature after which 3µl ribonuclease (RNase) solution was added and mixed by inverting the tubes 25 times. The cell lysate was incubated at 37°C for 15-60 minutes and cooled again to room temperature. The RNase-treated cell lysate was mixed with 200µl of protein precipitation solution and vortexed vigorously at high speed for 20 seconds in order to mix the two. The protein precipitation solution/cell lysate mixture was incubated on ice for 5 minutes and then centrifuged at 13,000-16,000g for 3 minutes. The supernatant fraction containing the DNA was transferred to a clean 1.5ml microcentrifuge tube containing 600µl of isopropanol at room temperature and mixed gently by inversion until thread-like strands of DNA were visible. The mixture was centrifuged at 13,000-16,000g for 2 minutes and the supernatant fraction poured off. The tube was drained on a clean absorbent paper and 600µl of 70% ethanol at room temperature was added and gently inverted several times to wash the DNA pellets. Centrifugation at 13,000-16,000g for 2 minutes was repeated, the ethanol was carefully aspirated and the tube drained on a clean absorbent paper. The pellet was allowed to air-

dry for 10-15 minutes, 100µl of DNA rehydration solution was added and the mixture was incubated overnight at 4°C. Storage of the DNA was at 4°C.

Amplification:

Chromosomal DNA from each *Salmonella* strain was amplified using the PCR on a MJ Research Minicycler. The PCR techniques used were repetitive extragenic palindromic (REP) elements and enterobacterial repetitive intergenic consensus (ERIC) sequences (Sander *et. al.*, 1998). The primer used for ERIC was $^{5'}$GTGAATCCCCAGCAGCTT ACAT$^{3'}$ and the primers used for REP were $^{5'}$REP1R-Dt: 5'NCGNCGNCATCNGGC$^{3'}$ and $^{5'}$RE 2D:5'RCGYCTTATCMGGCC- TAC$^{3'}$ (N=A,C,G, or T; M=A or C; R=A or G; Y=C or T). For both PCR types the amplification conditions were 94°C for 1min, 52°C for 1min and 72°C for 1min, all representing one cycle. However, all the PCR conditions included an initial 94°C for 5min and final 72°C for 5min. Typical reaction mixtures consisted of 500ng DNA, 200µM deoxynucleotide triphosphates (dNTPs), 1.5µM $MgCl_2$, 0.5µl Taq polymerase, and 50nM of each primer in buffer containing 50mM TrisCl, 50mM KCl, and 0.01% Triton-X100 in a final volume of 100µl.

2.2.9. Agarose gel electrophoresis:

Agarose gel electrophoresis was used to separate the PCR products and to obtain DNA fingerprints of the *Salmonella* isolates. Preparation of agarose gel size of 60 wells was done by dissolving 3g of agarose powder in 200ml of TAE after which 6µl ethidium bromide was added and the mixture poured into the appropriate electrophoretic trays. Each electrophoresis was run at 120V for 1½h. The electrophoretic chambers included a

Minicell EC370M and Maxicell EC360M powered by a Bio-RAD model 250/2.5 power supply. Agarose gels were visualized using a UV Intensity Transilluminator and documented with Panasonic CCD Ultra Lum camera and scion image software. The TAE was prepared from a 50X stock (242g Tris, 57.1ml acetic acid, 4ml 0.5 EDTA/1L).

2.2.10 Determination of the potency of the herbal preparations:

This study was conducted to determine if the herbal preparations had any antimicrobial activity. The methods employed were as described in the British Pharmacopoeia (A147 Appendix XIVA, 1988, Vol. II)

Hundred millimeter petri dishes were filled with 25ml of molten Mueller-Hinton agar to a depth of about 4mm and allowed to solidify. Each plate was then flooded with a particular *Salmonella* strain and the plate allowed to dry at room temperature for an hour on a level surface. A sterilized borer of an internal diameter of about 6mm was used to bore holes in the medium and into these holes were added 100µl of the different herbal preparations. Chloramphenicol was always added to one hole to serve as the control. The plates were kept in the refrigerator overnight, for complete absorption of the extract or antibiotic, and then incubated at 37°C for approximately 18 hours. The zones of inhibition produced by the various preparations were measured by using a metric ruler.

2.2.11. Minimum inhibitory concentrations (MICs) of the herbal preparations:

The MIC for the herbal preparation was determined using the agar dilution method as described in the NCCLS (1997). A dilution scheme for the stock solution of herbal

preparation, with concentration 320mg/ml was prepared. Sterile Mueller-Hinton agar in molten form was prepared and distributed into universal bottles in exact aliquots sufficient to dilute the starting herbal concentration 10-fold. The agar was allowed to equilibrate in a water bath to 48-50°C. The appropriate volume of the starting herbal (heat stable) concentration that has been sterilized by autoclaving was added to the molten Mueller-Hinton agar. The agar-herbal preparation was mixed thoroughly by gentle inversion and the mixture poured into100mm petri dishes on a level surface to result in an agar depth of about 4mm. The agar was allowed to solidify at room temperature and the plates used immediately. Plates containing no herbal preparation were prepared to serve as growth controls.

The top of a well isolated *Salmonella* colony was touched with a wire loop and transferred into 5ml Mueller-Hinton broth. The broth culture was incubated at 37°C until it achieved the turbidity of the 0.5 McFarland standard resulting in a suspension containing approximately 1-2 x 10^8 colony forming units (CFU)/ml. By using sterile broth, a 1:10 dilution of the bacterial suspension was made to give an adjusted concentration of 10^7 CFU/ml. An aliquot of each well-mixed suspension was placed in the corresponding well in the replicator inoculum block. A 1-2ul aliquot of each inoculum was applied to the agar surface by the use of an inocula-replicating device. A growth control plate (no herbal preparation) was inoculated first and then, starting with the lowest concentration, the plates containing the different concentrations of the decoctions/concoctions were inoculated. A second growth control plate was inoculated last to ensure that there was no contamination or significant antimicrobial carryover

during the inoculation. A sample of each inoculum was streaked on a nutrient agar plate and incubated overnight to detect any probable contamination and to provide freshly isolated colonies in case retesting proved necessary. The inoculated plates were allowed to stand at room temperature until the moisture in the inoculum spots had been absorbed into the agar. The plates were then inverted and incubated at 35°C for 16-20 hours. The lowest concentration of agent that allowed no more than one or two CFU or only a slight haze to grow was chosen as the MIC.

CHAPTER THREE

RESULTS

3.1. BACTERIOLOGICAL CHARACTERIZATION OF THE *SALMONELLA* STRAINS

3.1.1. Bacterial culture, isolation and identification:

One hundred and fifteen (115) *Salmonella* strains were isolated from the samples obtained from the various sources, including blood, CSF, food, stool, and urine at the Microbiology Department Korle Bu Teaching Hospital and the Bacteriology unit, NMIMR. The breakdown is as found in Table 1. The *Salmonella* isolates were characterized using *Salmonella* antisera kit into eight different groups, based on the Kauffmann-White scheme, with Groups B and D forming 90.4% (Table 2).

3.1.2. Antibiotic susceptibility testing of *Salmonella*:

One hundred and fifteen *Salmonella* strains were examined for their antibiotic susceptibility. The antibiotics chosen were based on NCCLS standards as well as current treatment regimens for *Salmonella* infections in Ghana. Figure 9 shows that, 74% *Salmonella* strains (85 out of 115) were resistant to one or more of the first-line anti-typhoid antibiotics namely; ampicillin, chloramphenicol, tetracycline and trimethoprim-sulphamethoxazole as well as streptomycin. The remaining strains were found to be susceptible to all five antibiotics used. Of the eighty-five resistant strains, 37 (43.53%) were resistant to all five antibiotic, thirty-two (37.65%) resistant to four antibiotics, seven (8.24%) to three antibiotics, one (1.18%) to two antibiotic and 8 (9.41%) were resistant to

one antibiotic. Thus, 76 *Salmonella* strains out of the total of 115 (66.09%) were found to possess multi-drug resistance (resistance to three or more antibiotics).

Table 1. Number of *Salmonella* strains isolated from different sources.

SOURCE OF SAMPLE	No. OF SALMONELLA ISOLATED	PERCENTAGE (%)
Blood	82	71.3
Cerebrospinal fluid	3	2.6
Food	14	12.2
Stool	6	5.2
Urine	4	3.5
Other sources	6	5.2
TOTAL	115	100.0

Identification of *Salmonella* from the various sources was done using the following biochemical tests; Triple Sugar Iron (TSI) agar test, Sulphur Indole Motility (SIM) agar test, and Urea agar test.

Table 2. Groups of *Salmonella* strains isolated

GROUP	No. OF ORGANISMS	PERCENTAGE (%)
A	1	0.9
B	38	33.0
C_1	4	3.5
C_2	2	1.7
D	66	57.4
E_1	2	1.7
G	1	0.9
I	1	0.9
TOTAL 8	115	100.0

Salmonella were grouped using *Salmonella* antisera kit from Denka Seiken Co., Ltd., Japan. Briefly, a needle of solid bacterial growth (or two loops of broth bacterial growth) was placed in the centre of a clean sterilized slide. One drop of sterile physiological saline was added to the specimen. Where broth medium was used no saline was added. Using an inoculating loop or the tip of a toothpick, the specimen-saline mixture was completely mixed after which a drop of the *Salmonella* antisera was added. The resulting mixture was rocked for about 5 seconds and observed for coagulation.

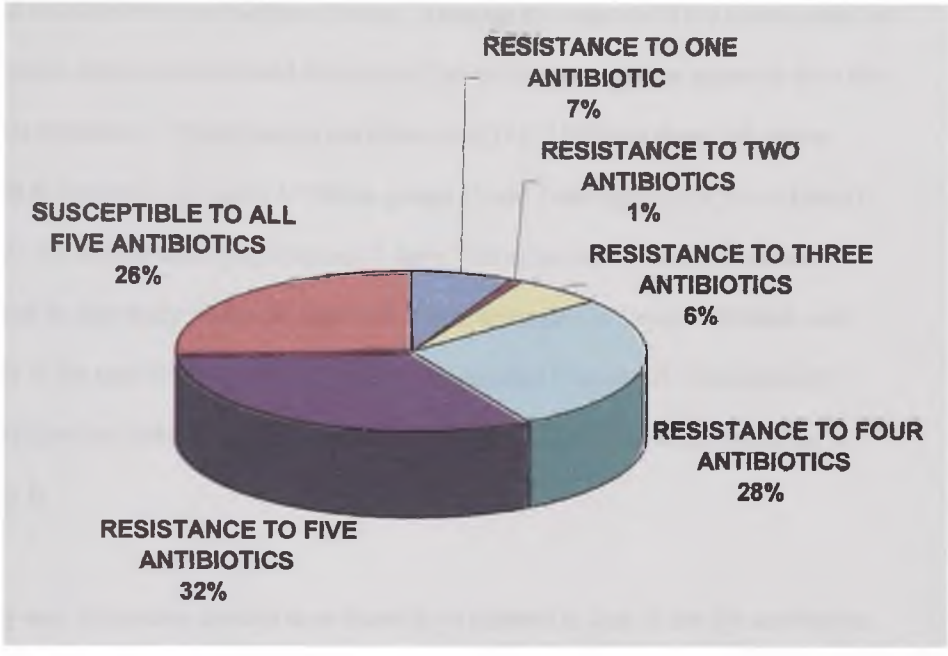

Figure 9. Susceptibility/resistance of the 115 isolated *Salmonella* strains to the different antibiotics

The level of resistance or susceptibility of all the 115 *Salmonella* strains was determined using disks impregnated with ampicillin (10µg), chloramphenicol (30µg), tetracycline (30µg), trimethoprim-sulphamethoxazole (1.25/23.75µg) and streptomycin (10µg) according to the guidelines set by the National Committee for Clinical Laboratory Standards (NCCLS).

The sources of this type of resistant strains are shown in Fig. 10. The figure compares the strains found to be resistant to all five antibiotics used (5-R) to the total number of strains isolated from each different source. Although the majority of the thirty-seven 5-R *Salmonella* strains were isolated from blood (78.38%), urine samples appear to have the highest percentage. Twenty-seven out of the total 37 (72.97%) of these 5-R strains belong to Group B, one each (2.70%) to groups C_1 and I and eight (21.62%) to group D. The 27 5-R strains belonging to Group B form 71% of the total Group B *Salmonella* isolated for this study whilst the eight 5-R strains belonging to Group D formed only 12.1% of the total Group D (8 out of 66) strains isolated (Figure 11). Thus majority (72.97%) of the *Salmonella* strains that were resistant to all five antibiotics belong to Group B.

Thirty-two *Salmonella* isolates were found to be resistant to four of the five antibiotics (4-R strains). Twenty-four of the thirty-two 4-R strains were susceptible to chloramphenicol but resistant to AmTeTsSt, seven were susceptible to tetracycline but resistant to AmCmTsSt whilst only one was susceptible to trimethoprim-sulphamethoxazole but resistant to AmCmTeSt. Thus, all the 4-R strains were resistant to ampicillin and streptomycin (Appendix A). Twenty-six (81.25%) of the 4-R strains were from blood and the distribution for the other sources for these 4-R strains is as found in Fig 12. The figure also compares the resistant strains to the total number of organisms from each of the sources, and indicates that the highest percentage was from blood.

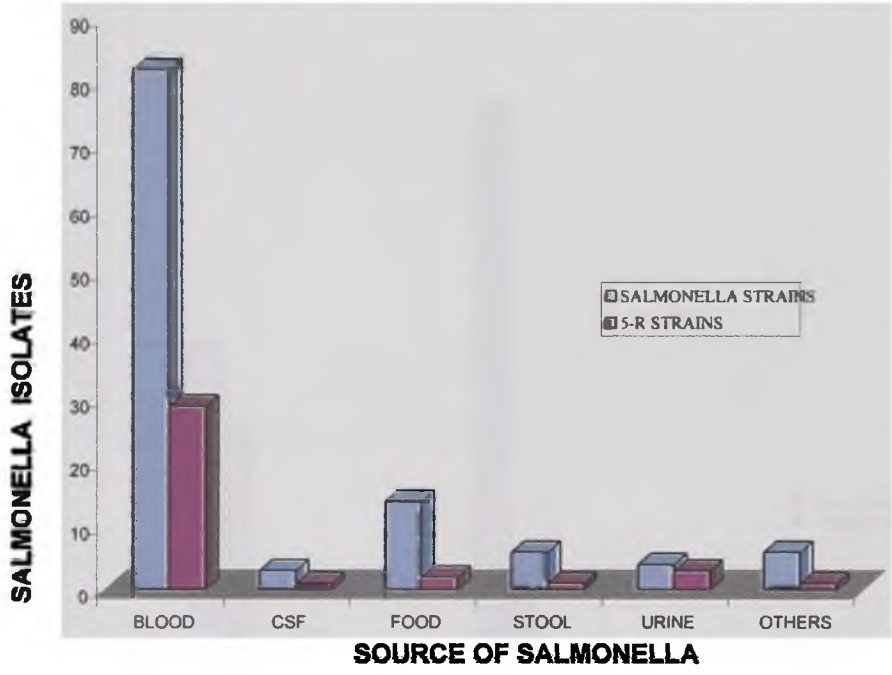

Figure 10. Sources of the thirty-seven *Salmonella* strains resistant to all five first line antibiotics.

The *Salmonella* strains were isolated from food sources and from patients suffering from typhoid fever. Resistance of *Salmonella* strains were determined using the standards described in the materials and methods.

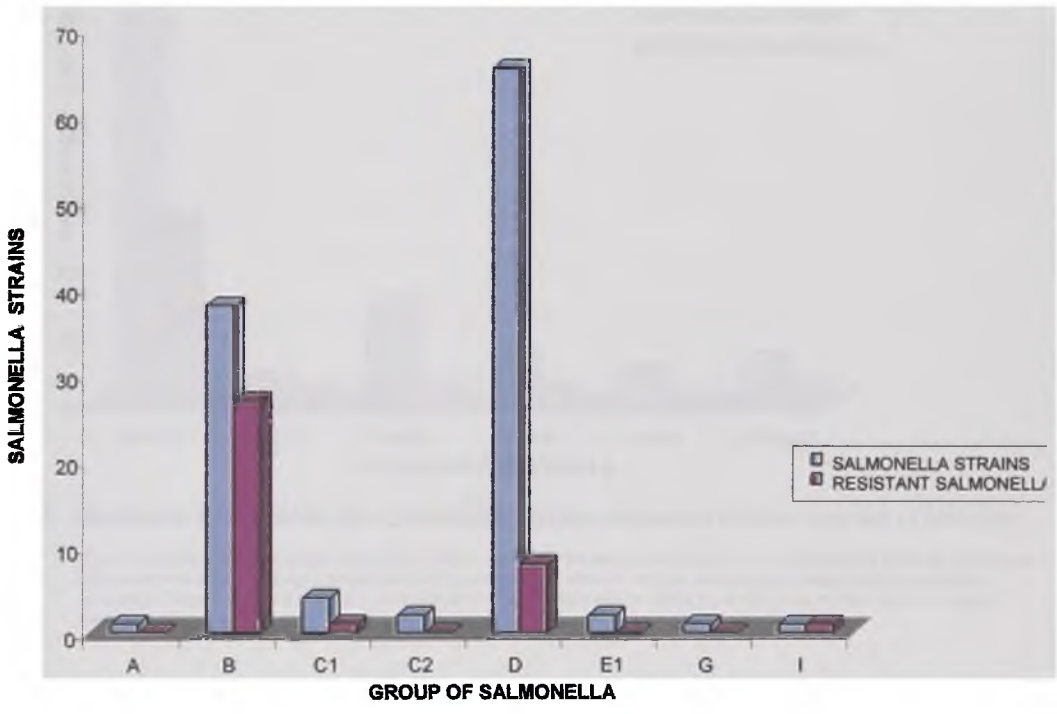

Figure 11. Groups of the thirty-seven *Salmonella* strains resistant to all five first line antiblotics.

Groups of the 37 *Salmonella* strains resistant to all five first line antibiotics were determined using *Salmonella* antisera kit from Denka Seiken Co., Ltd., Japan as described in the materials and methods.

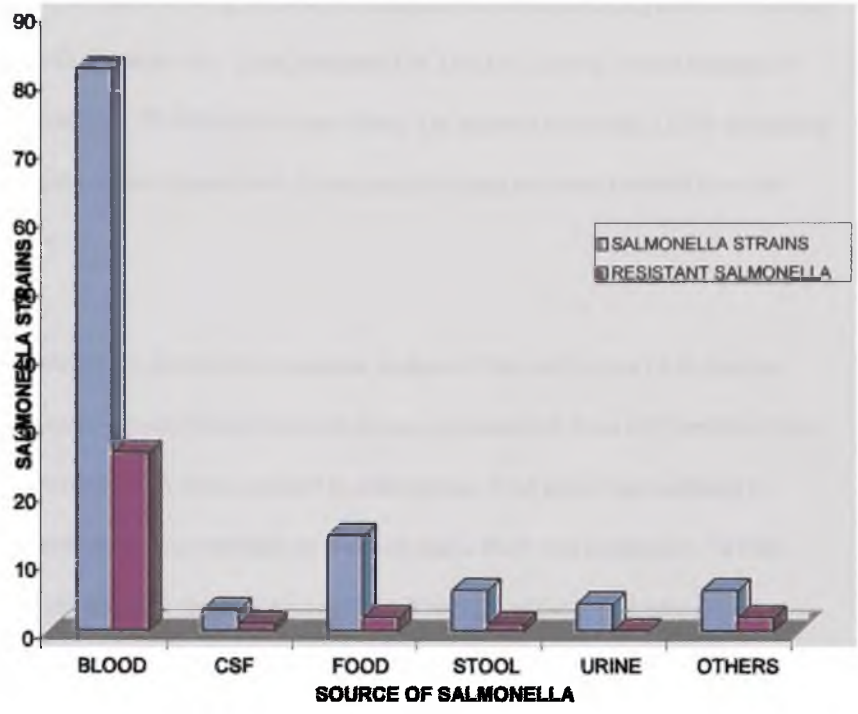

Figure 12. Resistance of the thirty-two *Salmonella* strains compared to their sources of isolation

The 32 resistant *Salmonella* strains were isolated from food sources and patients as described in the materials and methods. Briefly, the specimens were plated out using appropriate selective media such as MacConkey agar, blood agar and desoxycholate agar and/or *Salmonella/Shigella* agar, and colonies with the characteristics of *Salmonella* isolated and purified using standard microbiological methods

Four of the 32 4-R strains belong to Group B, one each to Groups C_1, C_2 and 25 to Group D whilst Group G also had one. Thus, majority (78.13%) of the 4-R strains belongs to Group D but form only 37.88% of the total Group Ds isolated with only 12.5% belonging to Group B. Figure 13 compares the 4-R strains to the total numbers isolated from the different groups.

Seven of the *Salmonella* strains were resistant to three of the antibiotics (3-R strains). Four (57.14%) were isolated from blood whilst one was isolated from CSF and two from stool. All the seven strains were resistant to tetracycline. One strain was resistant to AmCmTe, another strain was resistant to AmTeSt and a third was resistant to TeTsSt. The rest of the strains were resistant to AmTeTs. Thus, six of the 3-R strains were susceptible to chloramphenicol. One of the 3-R strains belong to Group B with six belonging to Group D (85.72%) The Group D, however, forms only 9.09% of the total Group Ds isolated.

Only one organism was resistant to two different antibiotics. This strain was isolated from blood and belongs to Group D.

Eight *Salmonella* strains were resistant to only one of the antibiotics (1-R strains). Four of these organisms were isolated from food, three from blood and one from urine. They were found to belong to Group B (62.50%), Group E_1 (12.50%) and Group D (25.00%). All eight organisms were susceptible to Cm and Ts.

Table 3. Summary of the resistance within the different groups to the different antibiotics

Group of organism	No of organisms	Level of resistance to the different antibiotics					Resistant strains
		5-R	4-R	3-R	2-R	1-R	
		37	32	7	1	8	85
A	1	0	0	0	0	0	0
B	38	27(71.05%)	4(10.53%)	1(2.63%)	0	5(13.16%)	37(97.37%)
C_1	4	1(25%)	1(25%)	0	0	0	2(50%)
C_2	2	0	1(50%)	0	0	0	1(50%)
D	66	8(12.12%)	25(37.88%)	6(9.09%)	1(1.52%)	2(3.03%)	42(63.64%)
E_1	2	0	0	0	0	1(50%)	1(50%)
G	1	0	1(100%)	0	0	0	1(100%)
I	1	1(100%)	0	0	0	0	1(100%)
TOTAL	115	37(32.17%)	32(27.83%)	7(6.09%)	1(0.87%)	8(6.96%)	85(73.91%)

The resistance levels were determined for the organisms from the different groups using antibiotic disks impregnated with antibiotics such as ampicillin (10µg), chloramphenicol (30µg), tetracycline (30µg), trimethoprim-sulphamethoxazole (1.25/23.75µg) and streptomycin (10µg). These antibiotics are the first line antibiotics used in the management of salmonellosis

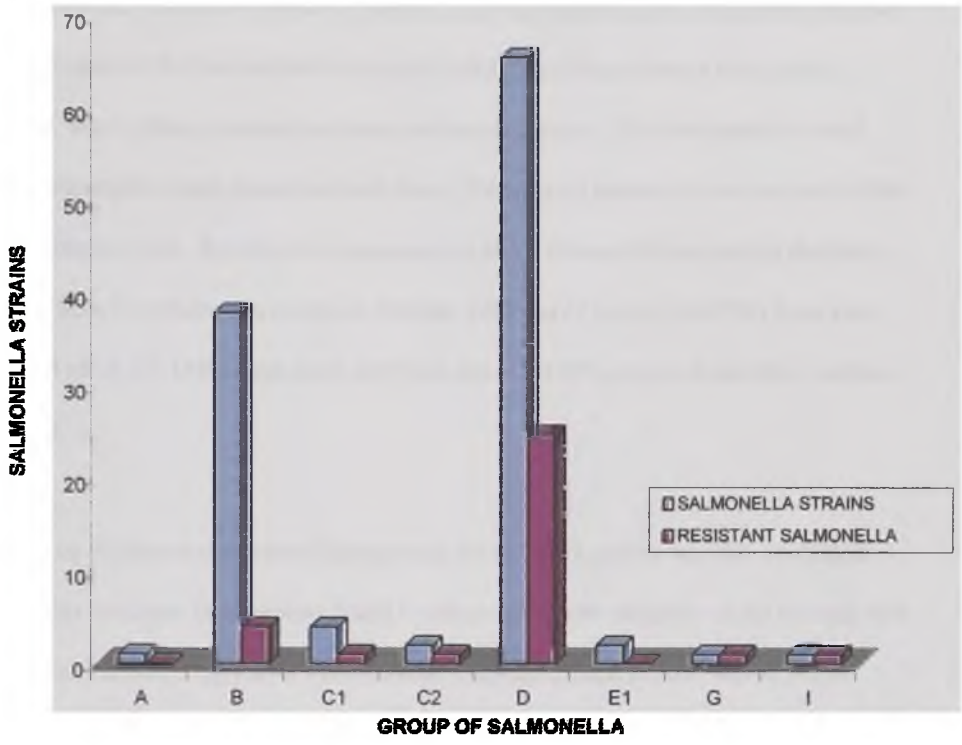

Figure 13. Groups of the thirty-two *Salmonella* strains resistant to four of the first line antibiotics

Salmonella antisera kit from Denka Seiken Co., Ltd., Japan was used to determine the groups of the 32 *Salmonella* strains resistant to four of the first line antibiotics as described in the materials and methods

The results also indicated that, resistance to the antibiotics tested varied with the sources of the samples. Sixty-three of the 82 strains (76.83%) isolated from blood were resistant to one or more of the five antibiotics with 60 (95.24%) of these strains being multi resistant, that is, being resistant to three or more antibiotics. The four strains isolated from urine and the three strains isolated from CSF were all resistant to one or more of the five antibiotics used. Resistance to one or more of the five antibiotics among the strains isolated from the other sources was as follows: Four out of the six (66.67%) from stool, eight out of 14 (57.14%) from food, and three out of 6 (50%) strains from other sources (Fig. 14).

Resistance of *Salmonella* strains belonging to the different groups was also evaluated. The results indicated that, Groups B and D which formed the majority of the isolates with 104 strains showed a high level of resistance. The two groups formed 90.4% of the isolates and the number resistant to one or more of the five antibiotics was 76%, (79 out of 104) or 68.70% of the total number isolated, that is, 79 out of the 115 strains. Resistance among Group B was 97.4% (37 out of 38) and 63.6% (42 out of 66) among Group D.

Analysis was made for multi-drug resistance (MDR), that is, resistance to three or more of the antibiotics. Multi-drug resistance among the two groups, that is, Groups B and D was high; 71 out of 76, or 93.4% with 42% among Group B and 51% among Group D. Of the 37 resistant organisms belonging to Group B, 32 or 86% showed MDR the majority (27 or 84%) of which had resistance to all 5 antibiotics used (5-R). Of the 42

resistant strains among Group D, 39 or 93% showed MDR and for most of these MDR strains, 25 or 60% had resistance to 4 of the antibiotics used (4-R). When the 37 5-R strains are considered, organisms in Group B constituted 73% whilst those in Group D formed 21.6%. Of the 32 4-R strains, only 12.5% belong to Group B and 78% to Group D. There were only seven 3-R strains and six of these, 85.7% belonged to Group D and only one to Group B. As Figure 15 shows, the rest of the strains showed various levels of resistance but the numbers are too small to be meaningful.

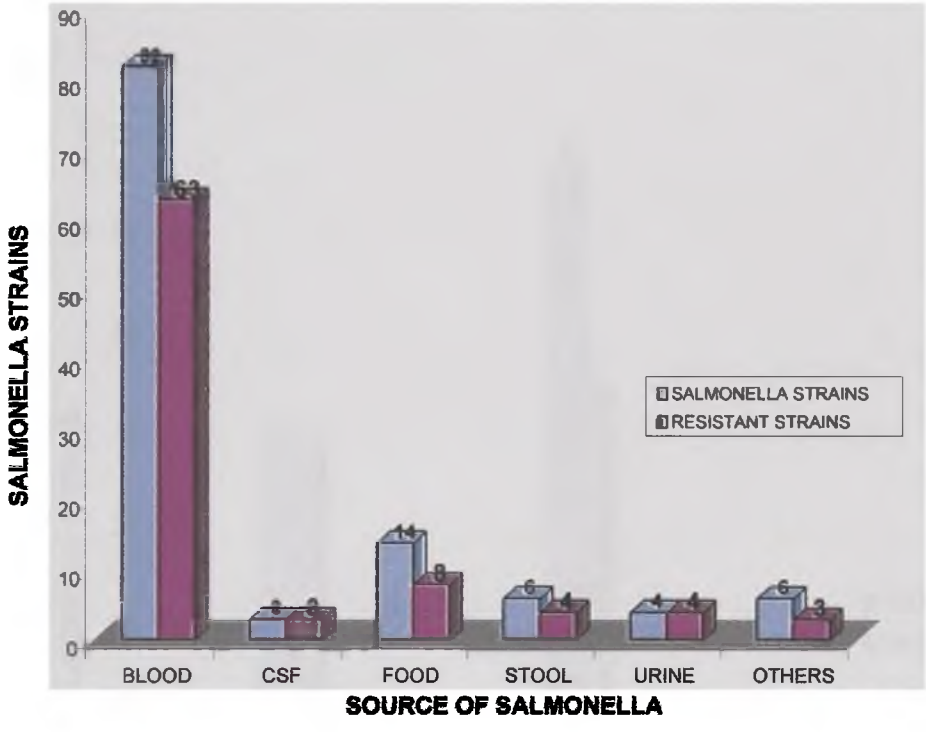

Figure 14. Level of resistance of *Salmonella* isolates compared to their sources of isolation

The resistance within each source of specimen was investigated using antibiotic disks impregnated with ampicillin (10µg), chloramphenicol (30µg), tetracycline (30µg), trimethoprim-sulphamethoxazole (1.25/23.75µg) and streptomycin (10µg) according to the guidelines set by the National Committee for Clinical Laboratory Standards. The organisms were isolated from different sources as described in the bacterial culture, isolation and identification under the materials and methods section.

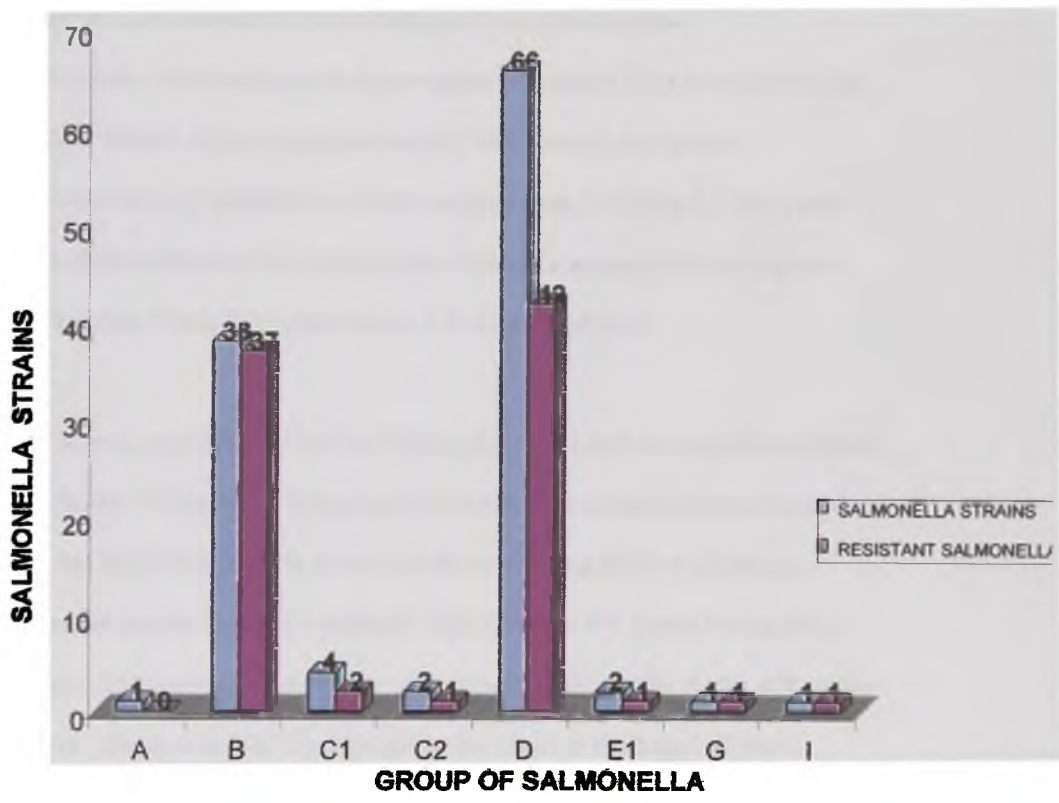

Figure 15. Level of resistance among the *Salmonella* strains within the different *Salmonella* groups to the five different antibiotics

Each group of *Salmonella* was determined using *Salmonella* antisera kit with details described in the materials and methods. The resistance, on the other hand, was determined using antibiotic disks prepared from ampicillin (10µg), chloramphenicol (30µg), tetracycline (30µg), trimethoprim-sulphamethoxazole (1.25/23.75µg) and streptomycin (10µg) and analysed using the guidelines set by the National Committee for Clinical Laboratory Standards

3.1.3 Minimum inhibitory concentration (MIC) of the antibiotics:

Four of the five antibiotics were used for the MIC determinations and the results are shown in Tables 4-8.

The 5-R strains that were resistant to all the five antibiotics used for the susceptibility test had very high MICs for ampicillin, chloramphenicol and trimethoprim-sulphamethoxazole. For example, with the exception of strains 67 and 89 which had the lowest MICs of 80mg/L all the organisms had MIC of 1280mg/L and above. Tetracycline was the only antibiotic with MICs ranging from 20-320mg/L. Thus, even though these strains were resistant to tetracycline during the susceptibility testing one needs not more than 320mg/L to eliminate the 5-R strains (Table 4).

For the 4-R strains, ampicillin had MIC of 1280mg/L or more with the exception of strain 38, which had MIC of 640mg/L. In the case of trimethoprim-sulphamethoxazole only four strains had MICs of 80mg/L or below with the rest having MICs of 1280mg/L. Chloramphenicol had the best MICs with only eight of the 32 4-R strains having MICs above 20mg/L. This compares with results of the susceptibility testing for the 4-R strains (Appendix A). The median MIC for tetracycline was found to be 80mg/L (Table 5).

The 3-R strains had MICs of 1280mg/L or above for ampicillin whereas chloramphenicol had MICs of 20mg/L except strain 7 that had MIC of 80mg/L. In the case of tetracycline 5 of the 7 strains had MICs of 80mg/L, and the other two had values of 40 and 160mg/L whilst values for that of trimethoprim-sulphamethoxazole were 20, 80 and 1280mg/L

(Table 6). These figures correlate with the results obtained for the susceptibility testing for the 3-R strains (Appendix A).

Table 4. MICs of four first line antibiotics using the *Salmonella* isolates which were found to be resistant to all the five antibiotics (5-R strains) in mg/l.

STRAIN	SOURCE	GROUP	Am	Cm	Te	Ts
8	URINE	D	>1280	1280	320	1280
13	BLOOD	D	>1280	1280	160	1280
33	MISC	D	>1280	1280	320	1280
39	BLOOD	C1	>1280	1280	20	1280
48	BLOOD	B	>1280	1280	320	1280
49	BLOOD	B	>1280	1280	320	1280
51	BLOOD	B	>1280	1280	20	1280
52	BLOOD	B	>1280	1280	320	1280
53	STOOL	D	>1280	1280	20	1280
55	BLOOD	B	>1280	1280	320	1280
66	FOOD	D	>1280	1280	320	1280
67	FOOD	I	80	80	160	80
72	BLOOD	B	>1280	1280	320	1280
73	BLOOD	B	>1280	1280	320	1280
74	BLOOD	B	>1280	>1280	320	1280
75	BLOOD	D	>1280	1280	320	1280
76	CSF	B	>1280	1280	320	1280
77	URINE	D	>1280	640	320	1280
78	URINE	B	>1280	1280	320	1280
83	BLOOD	B	>1280	1280	160	1280
89	BLOOD	D	80	80	80	80
90	BLOOD	B	>1280	1280	160	1280
95	BLOOD	B	>1280	1280	160	1280
97	BLOOD	B	>1280	1280	160	1280
98	BLOOD	B	>1280	1280	160	1280
99	BLOOD	B	>1280	1280	160	1280
101	BLOOD	B	>1280	1280	160	1280
102	BLOOD	B	>1280	1280	160	1280
104	BLOOD	B	>1280	1280	160	1280
105	BLOOD	B	>1280	1280	160	1280
106	BLOOD	B	>1280	1280	160	1280
108	BLOOD	B	>1280	>1280	20	>1280
109	BLOOD	B	>1280	1280	160	1280
110	BLOOD	B	>1280	1280	160	1280
111	BLOOD	B	>1280	1280	160	1280
112	BLOOD	B	>1280	1280	160	1280
115	BLOOD	B	>1280	1280	80	1280

The minimum inhibitory concentrations for four antibiotics against the 37 resistant *Salmonella* isolates were determined using the agar dilution technique according to the guidelines set by the National Committee for Clinical Laboratory Standards

Am=ampicillin
Cm=chloramphenicol
Te=tetracycline
Ts=co-trimoxazole.

Table 5. MICs of four first line antibiotics using the *Salmonella* isolates which were found to be resistant to four of the antibiotics (4-R strains) in mg/l.

STRAIN	SOURCE	GROUP	Am	Cm	Te	Ts
1	MISC	G	>1280	20	80	1280
3	BLOOD	D	>1280	20	80	1280
4	CSF	D	>1280	20	160	1280
6	BLOOD	D	>1280	20	80	1280
17	BLOOD	D	1280	80	20	80
21	STOOL	B	1280	20	80	80
22	BLOOD	D	>1280	20	160	1280
23	MISC	C2	>1280	320	80	20
27	BLOOD	D	>1280	20	80	1280
35	BLOOD	D	>1280	20	80	1280
38	BLOOD	D	640	20	80	1280
40	BLOOD	D	>1280	20	80	1280
46	BLOOD	D	>1280	20	160	1280
47	BLOOD	D	>1280	1280	20	1280
56	BLOOD	D	>1280	20	160	1280
63	FOOD	D	>1280	1280	20	1280
68	FOOD	B	>1280	1280	20	1280
71	BLOOD	B	>1280	1280	20	1280
79	BLOOD	D	1280	640	20	1280
81	BLOOD	D	>1280	20	80	1280
82	BLOOD	D	>1280	20	80	1280
84	BLOOD	D	1280	20	80	80
88	BLOOD	D	>1280	20	80	1280
91	BLOOD	D	1280	20	80	1280
92	BLOOD	D	>1280	20	80	1280
93	BLOOD	D	>1280	20	80	1280
94	BLOOD	C1	>1280	20	80	1280
96	BLOOD	D	>1280	20	80	1280
100	BLOOD	D	>1280	20	80	1280
103	BLOOD	D	>1280	20	80	1280
107	BLOOD	D	>1280	20	80	1280
114	BLOOD	B	>1280	1280	20	1280

The minimum inhibitory concentrations for the antibiotics against the 32 *Salmonella* isolates resistant to four of the antibiotics used were determined using the agar dilution technique according to the guidelines set by the National Committee for Clinical Laboratory Standards

Am=ampicillin
Cm=chloramphenicol
Te=tetracycline
Ts=co-trimoxazole

Table 6. MICs of four first line antibiotics using the *Salmonella* isolates which were found to be resistant to three of the antibiotics (3-R strains) in mg/l.

STRAIN	SOURCE	GROUP	Am	Cm	Te	Ts
2	BLOOD	D	>1280	20	80	1280
7	BLOOD	D	1280	80	80	20
14	BLOOD	D	1280	20	160	1280
24	CSF	D	>1280	20	80	20
28	BLOOD	D	>1280	20	80	1280
30	BLOOD	D	>1280	20	80	80
113	BLOOD	B	1280	20	40	80

Am=ampicillin Cm=chloramphenicol Te=tetracycline Ts=co-trimoxazole

The minimum inhibitory concentrations for the antibiotics using the 7 *Salmonella* isolates that were resistant to three of the antibiotics were determined using the agar dilution technique according to the guidelines set by the National Committee for Clinical Laboratory Standards

The only 2-R strain investigated had MIC of 20mg/L for chloramphenicol, tetracycline and trimethoprim-sulphamethoxazole and over 1280mg/L for ampicillin (Table 7). Compared to the susceptibility test (Appendix A), it is found that this strain was susceptible to chloramphenicol, tetracycline and trimethoprim-sulphamethoxazole.

The 1-R strains had, on the average low MICs of 20mg/L for chloramphenicol, tetracycline and trimethoprim-sulphamethoxazole. However, for ampicillin, the MIC was over 1280mg/L for four of the strains isolated from blood and urine, and MIC values of 20mg/L for the strains isolated from food sources (Table 8).

Table 7. MICs of four first line antibiotics using the *Salmonella* isolates which were found to be resistant to two of the antibiotics (2-R strains) in mg/l.

STRAIN	SOURCE	GROUP	Am	Cm	Te	Ts
87	BLOOD	D	>1280	20	20	20

The minimum inhibitory concentrations for the antibiotics against one *Salmonella* isolate which was found to be resistant to two of the antibiotics were determined using the agar dilution technique according to the guidelines set by the National Committee for Clinical Laboratory Standards

Am=ampicillin
Cm=chloramphenicol
Te=tetracycline
Ts=co-trimoxazole

Table 8. MICs of four first line antibiotics using the *Salmonella* isolates which were found to be resistant to only one of the antibiotics (1-R strains) in mg/l.

STRAIN	SOURCE	GROUP	Am	Cm	Te	Ts
26	URINE	B	>1280	20	20	20
29	BLOOD	B	>1280	20	20	20
36	BLOOD	D	>1280	20	20	20
58	FOOD	B	20	20	40	20
59	FOOD	B	20	20	40	20
61	FOOD	E1	20	20	20	20
65	FOOD	B	20	20	20	40
80	BLOOD	D	>1280	20	20	20

The minimum inhibitory concentrations for the antibiotics against the 8 *Salmonella* isolates resistant to only one of the antibiotics were determined using the agar dilution technique according to the guidelines set by the National Committee for Clinical Laboratory Standards

Am=ampicillin
Cm=chloramphenicol
Te=tetracycline
Ts=co-trimoxazole

3.2 MOLECULAR CHARACTERIZATION OF *SALMONELLA* STRAINS

3.2.1 Plasmid isolation and incompatibility testing

Purified plasmid preparations were used as template along with incompatibility group HI1A-specific primers in order to amplify a 365-bp region indicative of the RepHI1A replicon. Plasmid preparations from the *Salmonella* strains that were able to transfer antibiotic resistance genes via conjugation and their corresponding transconjugates yielded positive amplicons (Figure 16). The plasmid preparations derived from the transformants were negative. These data provide evidence that the conjugable plasmids in the *Salmonella* isolates examined belong to incompatibility group *Inc*HI.

3.2.2. Transfer of antibiotic resistance via conjugation:

The ability of the resistant *Salmonella* strains to transfer resistance phenotype via conjugation to *E. coli* HMS174 was investigated in order to determine if the antibiotic resistance associated with the *Salmonella* isolates was plasmid-encoded. In the investigation, the *Salmonella* strains with resistance to one or more of the antibiotics were mated with the recipient *E. coli* and the trans-conjugates selected on MacConkey media containing ampicillin, chloramphenicol or trimethoprim-sulphamethoxazole. The susceptibility for the trans-conjugates was carried out using the five antibiotics previously tested on the wild-type *Salmonella*. As presented in Table 9, 80 out of the total of 85 (94.12%) resistant strains possessed conjugable plasmids with 73 of the 80 (91.25%) strains conferring multi-drug resistance.

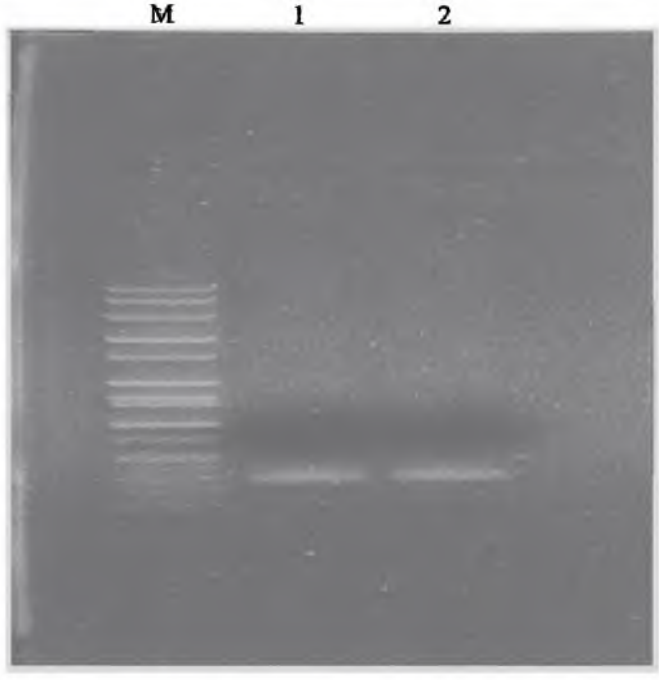

Figure 16. Representative agarose gel with 365-bp *Inc* amplicon.

The Rep HI1A replicon, present in *Inc* HI plasmids, was amplified via the polymerase chain reaction (PCR) using the primers 5'GGTCCAACCCATTGCTTTAC3' and 5'CACGGAAAGAAATCACAAC3' on a model PT 150 MiniCycler. Lane M, molecular mass markers; lane 1, amplicon from *Salmonella* strain 8; lane 2, amplicon from the corresponding *Salmonella* strain 8 transconjugate.

Table 9. Comparison of resistance pattern of wild-type *Salmonella* to the trans-conjugates

STRAIN	SOURCE	GROUP	RESISTANCE PATTERN (wild-type *Salmonella*)	TRANSCONGUGATES (recipient *E. coli*)
1	MISC	G	AmTsTeSt	AmTsTeSt
2	BLOOD	D	AmTsTe	AmTsTeSt
3	BLOOD	D	AmTsTeSt	AmTsTeSt
4	CSF	D	AmTsTeSt	AmTsTeSt
6	BLOOD	D	AmTsTeSt	AmTsTeSt
7	BLOOD	D	AmCmTe	-
8	URINE	D	AmCmTeTsSt	AmCmTeTsSt
13	BLOOD	D	AmCmTeTsSt	AmCmTeTsSt
14	STOOL	D	AmTeTs	AmTeTs
17	BLOOD	D	AmCmTsSt	AmCmTsSt
21	STOOL	B	AmTsTeSt	AmTsTeSt
22	BLOOD	D	AmTeTsSt	AmTsTeSt
23	MISC	C2	AmCmTeSt	AmCmTeSt
24	CSF	D	AmTeSt	Am
26	URINE	B	Am	Am
27	BLOOD	D	AmTeTsSt	AmTeTsSt
28	STOOL	D	AmTeTs	Am
29	BLOOD	B	Am	Am
30	BLOOD	D	AmTsTe	AmTsTe
33	MISC	D	AmCmTeTsSt	AmCmTeTsSt
35	BLOOD	D	AmTsTeSt	TsTeSt
36	BLOOD	D	Am	Am
38	BLOOD	D	AmTeTsSt	AmTeTsSt
39	BLOOD	C1	AmCmTeTsSt	AmTeTsSt
40	BLOOD	D	AmTeTsSt	AmTeTsSt
46	BLOOD	D	AmTeTsSt	AmTeTsSt
47	BLOOD	D	AmCmTsSt	AmCmTsSt
48	BLOOD	B	AmCmTeTsSt	AmCmTsSt
49	BLOOD	B	AmCmTeTsSt	AmCmTsSt
51	BLOOD	B	AmCmTeTsSt	AmCmTsSt
52	BLOOD	B	AmCmTeTsSt	AmCmTsSt
53	STOOL	D	AmCmTeTsSt	AmCmTsSt
55	BLOOD	B	AmCmTeTsSt	AmCmTsSt
56	BLOOD	D	AmTeTsSt	AmTeTsSt
58	FOOD	B	Te	-
59	FOOD	B	Te	-
61	FOOD	E1	St	-
63	FOOD	D	AmCmTsSt	AmCmTsSt
65	FOOD	B	St	-

Table 9 continued

STRAIN	SOURCE	GROUP	RESISTANCE PATTERN (wild-type *Salmonella*)	TRANSCONJUGATES (recipient *E. coli*)
67	FOOD	I	AmCmTeTsSt	AmCmTeTsSt
68	FOOD	B	AmCmTsSt	AmCmTsSt
71	BLOOD	B	AmCmTsSt	AmCmTsSt
72	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
73	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
74	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
75	BLOOD	D	AmCmTeTsSt	AmCmTeTsSt
76	CSF	B	AmCmTeTsSt	AmCmTsSt
77	URINE	D	AmCmTeTsSt	AmTeTsSt
78	URINE	B	AmCmTeTsSt	AmCmTsSt
79	BLOOD	D	AmCmTsSt	AmCmTsSt
80	BLOOD	D	Am	Am
81	BLOOD	D	AmTeTsSt	AmTeTsSt
82	BLOOD	D	AmTeTsSt	AmTeTsSt
83	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
84	BLOOD	D	AmTeTsSt	AmTeTsSt
87	BLOOD	D	AmSt	AmSt
88	BLOOD	D	AmTeTsSt	AmCmTsSt
89	BLOOD	D	AmCmTeTsSt	AmCmTeTsSt
90	BLOOD	B	AmCmTeTsSt	AmCmTsTeSt
91	BLOOD	D	AmTeTsSt	AmTeTsSt
92	BLOOD	D	AmTeTsSt	AmTeTsSt
93	BLOOD	D	AmTeTsSt	AmTeTsSt
94	BLOOD	C1	AmTeTsSt	AmTeTsSt
95	BLOOD	B	AmCmTeTsSt	AmCmTsSt
96	BLOOD	D	AmTeTsSt	AmTeTsSt
97	BLOOD	B	AmCmTeTsSt	AmCmTsSt
98	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
99	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
100	BLOOD	D	AmTeTsSt	AmTeTsSt
101	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
102	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
103	BLOOD	D	AmTeTsSt	AmTeTsSt
104	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
105	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
106	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
107	BLOOD	D	AmTeTsSt	AmTeTsSt
108	BLOOD	B	AmCmTeTsSt	AmCmTsSt
109	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt

Table 9 continued

STRAIN	SOURCE	GROUP	RESISTANCE PATTERN (wild-type *Salmonella*)	TRANSCONGUGATES (recipient *E. coli*)
112	BLOOD	B	AmCmTeTsSt	AmCmTeTsSt
113	BLOOD	B	TsTeSt	TsTeSt
114	BLOOD	B	AmCmTsSt	AmCmTsSt
115	BLOOD	D	AmCmTeTsSt	AmCmTsSt

Conjugation was carried out using the resistant *Salmonella* isolates as the donors and *E. coli* HMS 174 as the recipients. These organisms were grown separately overnight at 37°C with shaking in 3ml tryptic soy broth (TSB). Into fresh 3ml TSB was added 0.3ml overnight cultures and the mixture incubated at 37°C with shaking for about 1 hour or until 0.5 McFarland turbidity standard was obtained. Five hundred microlitres of each suspension of *Salmonella* strain was mixed with the same quantity of *E. coli* HMS 174 in a sterile tube and the suspension incubated at 37°C without shaking for 90 minutes. At 30-minute intervals, the cultures were mixed by gentle inversion. These conjugation mixtures were then plated on MacConkey agar plates containing 100μg/ml rifampicin and either ampicillin (32μg/ml), chloramphenicol (32μg/ml), or trimethoprim-sulphamethoxazole (4μg/ml; 16μg/ml) and incubated overnight at 37°C. Lactose fermenting colonies growing on the plates indicated conjugational transfer of antibiotic resistance to *E. coli* HMS 174.

Am=ampicillin
Cm=chloramphenicol
Te=tetracycline
Ts=co-trimoxazole St=streptomycin

3.2.3. Transfer of antibiotic resistance via transformation:

To determine if the antibiotic resistance associated with each *Salmonella* was located on a transformable plasmid, recipient *E. coli* were transformed with plasmid preparation obtained from each of the *Salmonella* strains resistant to one or more of the antibiotics used. These were then selected on media containing either ampicillin, chloramphenicol, or trimethoprim-sulphamethoxazole. The susceptibility of the transformants was done using the five antibiotics previously tested on the wild-type *Salmonella*. The results showed that out of the 85 wild-type resistant *Salmonella* only 15 (17.65%) of the recipient *E. coli* strains were transformed with all the transformants being resistant to ampicillin (Table 10).

Table 10. Comparison of resistance pattern of wild-type *Salmonella* to the transformants.

STRAIN	SOURCE	GROUP	RESISTANCE PATTERN (wild-type *Salmonella*)	TRANSFORMANTS (recipient *E.coli*)
1	MISC	G	AmTsTeSt	-
2	BLOOD	D	AmTsTe	-
3	BLOOD	D	AmTsTeSt	-
4	CSF	D	AmTsTeSt	AmTsTeSt
6	BLOOD	D	AmTsTeSt	AmTsTeSt
7	BLOOD	D	AmCmTe	-
8	URINE	D	AmCmTeTsSt	AmCmTeTsSt
13	BLOOD	D	AmCmTeTsSt	AmCmTeTsSt
14	STOOL	D	AmTeTs	-
17	BLOOD	D	AmCmTsSt	-
21	STOOL	B	AmTsTeSt	-
22	BLOOD	D	AmTeTsSt	AmTsTeSt
23	MISC	C2	AmCmTeSt	-
24	CSF	D	AmTeSt	Am
26	URINE	B	Am	-
27	BLOOD	D	AmTeTsSt	AmTeTsSt
28	STOOL	D	AmTeTs	-
29	BLOOD	B	Am	-
30	BLOOD	D	AmTsTe	-
33	MISC	D	AmCmTeTsSt	-
35	BLOOD	D	AmTsTe	-
36	BLOOD	D	Am	Am
38	BLOOD	D	AmTeTsSt	-
39	BLOOD	C1	AmCmTeTsSt	-
40	BLOOD	D	AmTeTsSt	-
46	BLOOD	D	AmTeTsSt	-
47	BLOOD	D	AmCmTsSt	-
48	BLOOD	B	AmCmTeTsSt	-
49	BLOOD	B	AmCmTeTsSt	-
51	BLOOD	B	AmCmTeTsSt	-
52	BLOOD	B	AmCmTeTsSt	-
53	STOOL	D	AmCmTeTsSt	-
55	BLOOD	B	AmCmTeTsSt	-
56	BLOOD	D	AmTeTsSt	Am
58	FOOD	B	Te	-
59	FOOD	B	Te	-
61	FOOD	E1	St	-
63	FOOD	D	AmCmTsSt	-

Table 10 continued

STRAIN	SOURCE	GROUP	RESISTANCE PATTERN (wild-type *Salmonella*)	TRANSFORMANTS (recipient *E. coli*)
66	FOOD	D	AmCmTeTsSt	-
67	FOOD	I	AmCmTeTsSt	-
68	FOOD	B	AmCmTsSt	-
71	BLOOD	B	AmCmTsSt	-
72	BLOOD	B	AmCmTeTsSt	-
73	BLOOD	B	AmCmTeTsSt	-
74	BLOOD	B	AmCmTeTsSt	-
75	BLOOD	D	AmCmTeTsSt	Am
76	CSF	B	AmCmTeTsSt	-
77	URINE	D	AmCmTeTsSt	Am
78	URINE	B	AmCmTeTsSt	-
79	BLOOD	D	AmCmTsSt	-
80	BLOOD	D	Am	-
81	BLOOD	D	AmTeTsSt	-
82	BLOOD	D	AmTeTsSt	-
83	BLOOD	B	AmCmTeTsSt	-
84	BLOOD	D	AmTeTsSt	Am
87	BLOOD	D	AmSt	-
88	BLOOD	D	AmTeTsSt	-
89	BLOOD	D	AmCmTeTsSt	Am
90	BLOOD	B	AmCmTeTsSt	-
91	BLOOD	D	AmTeTsSt	-
92	BLOOD	D	AmTeTsSt	Am
93	BLOOD	D	AmTeTsSt	Am
94	BLOOD	C1	AmTeTsSt	-
95	BLOOD	B	AmCmTeTsSt	-
96	BLOOD	D	AmTeTsSt	-
97	BLOOD	B	AmCmTeTsSt	-
98	BLOOD	B	AmCmTeTsSt	-
99	BLOOD	B	AmCmTeTsSt	-
100	BLOOD	D	AmTeTsSt	
101	BLOOD	B	AmCmTeTsSt	-
102	BLOOD	B	AmCmTeTsSt	-
103	BLOOD	D	AmTeTsSt	-
104	BLOOD	B	AmCmTeTsSt	-
105	BLOOD	B	AmCmTeTsSt	-
106	BLOOD	B	AmCmTeTsSt	-
107	BLOOD	D	AmTeTsSt	-
108	BLOOD	B	AmCmTeTsSt	-

Table 10 continued

STRAIN	SOURCE	GROUP	RESISTANCE PATTERN (wild-type *Salmonella*)	TRANSFORMANTS (recipient *E.coli*)
110	BLOOD	B	AmCmTeTsSt	-
111	BLOOD	B	AmCmTeTsSt	-
112	BLOOD	B	AmCmTeTsSt	-
113	BLOOD	B	TsTeSt	-
114	BLOOD	B	AmCmTsSt	-
115	BLOOD	D	AmCmTeTsSt	-

Competent cells were prepared by inoculating Luria-Bertani (LB) broth with a single colony of *E. coli* DH5α. Plasmid DNA was used to transform the *E. coli* DH5α according to standard procedures described by the NCCLS. Briefly, 2µl of the plasmid preparation was added to 100µl of the competent cell suspension and incubated on ice for 15-30min. The DNA-competent cell suspension was placed in a 42°C water bath for 90sec and then chilled on ice for 1min. Nine hundred microlitre volume of LB was added immediately and the cells incubated at 37°C for 1 hour for expression of antibiotic resistance. Selection of transformants was made on trypton soy agar (TSA) plates containing either ampicillin (32µg/ml), chloramphenicol (32µg/ml), or trimethoprim-sulphamethoxazole (4µg/ml:16µg/ml). Previous testing had proved that the *E. coli* DH5α was sensitive to all antibiotics investigated in this study.

3.2.4. DNA fingerprinting:

Three different genotypic procedures were used to investigate each *Salmonella* isolate to determine their clonal relatedness. Isolated plasmids yielded 5 distinct amplification patterns (Fig. 17), with the majority of the isolates belonging to pattern 2. Interestingly, patterns 2-4 were comprised of overlapping bands and all isolates belonging to either of these patterns demonstrated MDR. Patterns 1and 2 comprised mostly of the 3-R and 4-R strains whilst patterns 3 and 4 comprised mostly of the 5-R strains. Pattern 5 comprised all strains with 2-R and 1-R (Table 11).

Genotyping procedures involving ERIC-PCR (Fig. 18) resulted in 2 discrete amplification patterns. Strains not exhibiting MDR were unable to be discriminated from MDR isolates using this PCR-based approach (Table 11).

Genotyping procedures involving REP-PCR (Fig. 19) resulted in 3 discrete amplification patterns. However, this PCR-based procedure was also unable to discriminate MDR isolates from the non-MDR isolates (Table11).

Table 11. Distribution of *Salmonella* isolates into discrete genotypic patterns.

Genotypic pattern		Strains with indicated genotypic pattern
Plasmid Profile		
	1	24,107
	2	1-4,6,7,14,17,22,23,27,28,30,35,38,40,46,47,56,63, 68,71,79,81,82,84,88,92-94,96,100,103
	3	73,74,76,78,83,90,95,97,98,99,101,102,104-106,108, 109-111,114
	4	8,13,33,39,48,49,51-53,55,66,72,77,89,112,115
	5	21,26,29,36,58,59,61,65,67,75,80,87,91,113
REP	1	1-6,12,14,21-30,32,33-36,38-40,45,51,61, 67,76,80,90,93,95,96,99,103,105,108,111
	2	7-10,13,31,42,43,46,50,52,53,56,57,64-66,68-70,71,77, 81,83-88,92,94,97,98,100-102,104,106,107,109
	3	11,15-20,37,41,44,47-49,54,55,59,60,63,78,79,82 89,91,110,112-115
ERIC	1	2,3,5,6,7,8,9,12,14,16,18,19,22,23,25,26,27,29,31,34, 35, 37-50,52,55,57,58,60,67,84,87,88,96,99,107-109, 111-113
	2	1,4,10,11,13,15,17,20,21,24,28,30,32,33,36,51,53, 54,56,59,61-66,68-83,85,86,89-95,97,98,100-106, 110,114,115

Agarose gel electrophoresis was used to separate the plasmid and PCR products. Preparation of agarose gel size of 60 wells was done by dissolving 3g of agarose powder in 200ml of TAE after which 6µl ethidium bromide was added and the mixture poured into the appropriate electrophoretic trays. Each electrophoresis was run at 120V for 1½h. The electrophoretic chambers included a Minicell EC370M and Maxicell EC360M powered by a Bio-RAD model 250/2.5 power supply. Agarose gels were visualized using a UV Intensity Transilluminator and documented with Panasonic CCD Ultra Lum camera and scion image software. The TAE was prepared from a 50X stock (242g Tris, 57.1ml acetic acid, 4ml 0.5 EDTA/1L).

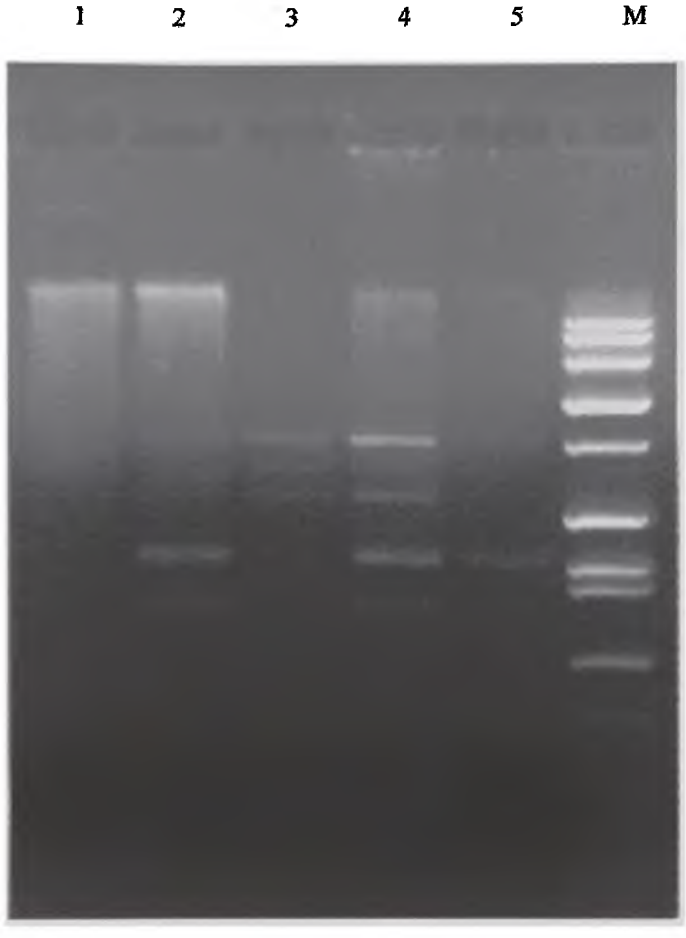

Figure 17. Agarose gel analysis of plasmids from wild-type *Salmonella*

Plasmids from the 115 strains were isolated using the QIAprep spin miniprep kit. A modification of the alkaline lysis method of Birnboim and Doly was used. Lane M, molecular mass marker; lanes 1, 2, 3, 4 and 5, represent the various wild-type *Salmonella* strains as summarized and presented in Table 11.

Figure 18. Agarose gel analysis of genotypic patterns obtained with ERIC-PCR.

Agarose gel electrophoresis was used to separate the PCR products. Chromosomal DNA for PCR was isolated from each *Salmonella* strain using the Wizard Genomic DNA Purification Kit. The primer used for ERIC was $^{5'}$GTGAATCCCCAGCAGCTTACAT$^{3'}$ and the amplification conditions were 94°C for 1min, 52°C for 1min and 72°C for 1min, all representing one cycle. PCR conditions included an initial 94°C for 5min and 72°C for 5min. Lane M, represents molecular mass marker; lanes 1 and 2 represent the various *Salmonella* strains as summarized and presented in Table 11.

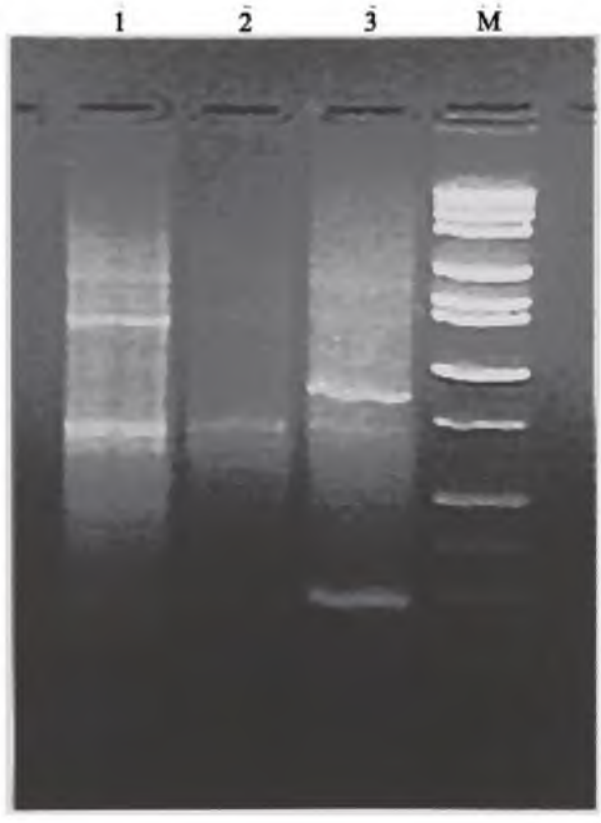

Figure 19. Agarose gel analysis of genotypic patterns obtained with REP-PCR.

The PCR products were separated by agarose gel electrophoresis. Chromosomal DNA was isolated from each *Salmonella* strain using the Wizard Genomic DNA Purification Kit. The primer used for REP were "REP1R-De 5'NCGNCGNCATCNGGC" and "RE 2D:5'RCGYCTTATCMGGCCTAC" (N=A,C,G, or T; M=A or C; R=A or G; Y=C or T). The amplification conditions were 94°C for 1min, 52°C for 1min and 72°C for 1min, representing one cycle. PCR conditions included an initial 94°C for 5min and final 72°C for 5min. Lane M, molecular mass marker; lanes 1, 2, and 3 represent the various *Salmonella* strains as summarized and presented in Table 11..

3.3 SUSCEPTIBILITY OF *SALMONELLA* STRAINS TO THE HERBAL PREPARATIONS

3.3.1. Potency of the herbal preparation:

Mueller-Hinton agar was used as the medium to test the efficacy/potency of the herbal preparations against standard *Salmonella* and *Staphylococcus* strains which previously showed susceptibility to all the five antibiotics used. In all, 12 plant preparations were analysed as well as the total extract ("Mist Enterica"). The results revealed that, seven of the preparations namely; *Paulina pinnata, Persea americana, Bidens pilosa, Veronia amydalina, Jatropha cucvas, Lantana camera* and *Citrus aurantifolia,* had no anti-bacterial properties against any of the standard strains. Preparations made from *Hoslundia opposita, Cnestis ferruginea,* and *Psidium guajava* were found to be very active against all three standard strains with zones of inhibition ranging from 9-25mm (Table 12). Preparations made from *Spondias monbin* and *Morinda lucida* also showed some activity against these standard strains but not as effective as the three above. The zones of inhibition were equal or less than 7mm.

Table 12. Antimicrobial activity of the herbal decoctions/concoction against selected standard bacterial strains.

HERBAL EXTRACT	*Salmonella typhi* (ATCC 33458)	*Salmonella paratyphi* B (ATCC 10719)	*Staphylococcus aureus* (12981)
Paulinia pinnata	-	-	-
Hoslundia opposita	+	+	+
Persea Americana	-	-	-
Bidens pilosa	-	-	-
Psidium guajava	+	+	+
Veronia amydalina	-	-	-
Jatropha cucvas	-	-	-
Lantana camera	-	-	-
Cnestis ferruginea	+	+	+
Spondias monbin	+	+	+
Morinda lucida	+	+	+
Citrus aurantifolia	-	-	-
"Mist Enterica"	+	+	+

The methods employed were as described in the British Pharmacopoeia (A147 Appendix XIVA, 1988, Vol. II). Each petri plate was flooded with a particular *Salmonella* strain and the plate allowed to dry at room temperature for an hour on a level surface. A sterilized borer of an internal diameter of about 6mm was used to bore holes in the medium and into these holes were added 100µl of the different herbal preparations. The plates were kept in the refrigerator overnight, for complete absorption of the extracts or antibiotic, and then incubated at 37°C for approximately 18 hours. The zones of inhibition produced by the various preparations were measured using a metric ruler

Positive (+) = Activity
Negative (-) = no activity

Figure 20 shows zones of inhibition for decoctions prepared from three of the 12 plants namely, *C. ferruginea, H. opposita* and *P. guajava* against standard *S. typhi.* The zones of inhibition were between 9-20mm for the *S. typhi.* The decoction prepared from *P. guajava* against the standard *S. aureus* gave an even bigger zone of inhibition of 29mm (Fig. 21). Figure 22 shows the zones of inhibition of the preparations from the 12 plants as well as a combination of *C. ferruginea, H. opposita* and *P. guajava* using standard *S. aureus.* The efficacy of "Mist Enterica" against the wild-type *Salmonella* strain 8 was tested using the ditch method. The result is as shown in Figure 23. Also disks impregnated with preparations from "Mist Enterica", *C. ferruginea, H. opposita, P. guajava* and chloramphenicol were tested for efficacy against the wild-type *Salmonella* strain 8 (Fig. 24).

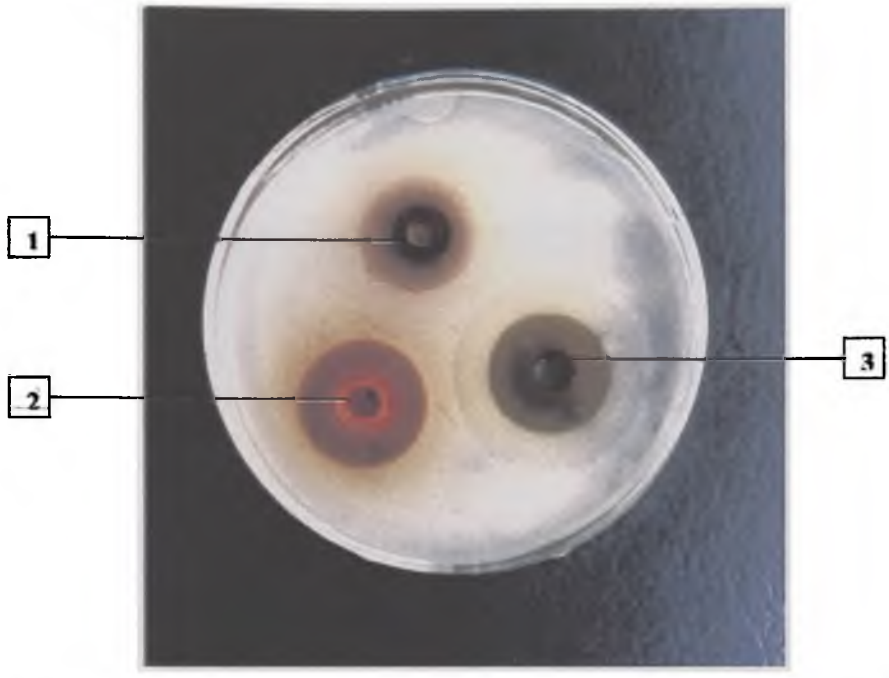

Figure 20. Inhibition of the growth of standard *Salmonella typhi* by three herbal preparations using the agar diffusion method

The plate was flooded with the *Salmonella typhi* and allowed to dry at room temperature for an hour on a level surface. A sterilized borer of an internal diameter of about 6mm was used to bore holes in the medium and into these holes were added 100µl of the different herbal preparations. The plate was kept in the refrigerator overnight, for complete absorption of the extracts and then incubated at 37°C for approximately 18 hours. The zones of inhibition produced by the various preparations were measured in millilitres. The three herbal preparations were represented by 1, *Cnestis ferruginea*, 2, *Hoslundia opposita* and 3, *Psidium guajava*

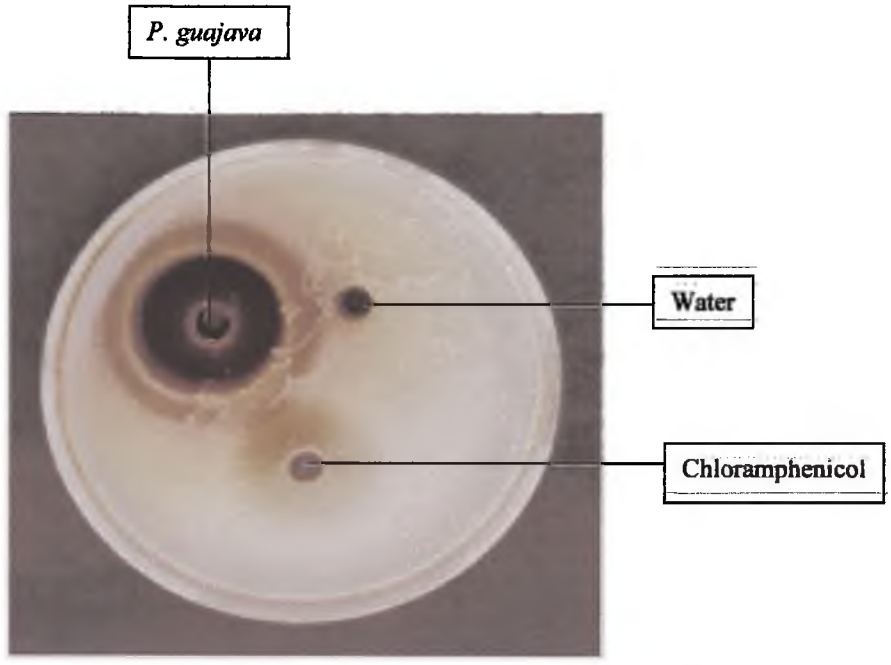

Figure 21. Inhibition of the growth of standard *Staphylococcus aureus* using decoction prepared from *Psidium guajava*.

The plate was flooded with the standard *S. aureus* and allowed to dry at room temperature for an hour on a level surface. A sterilized borer of an internal diameter of about 6mm was used to bore holes in the medium and into these holes were added 100µl of the different preparations. The plate was kept in the refrigerator overnight, for complete absorption of the extract and then incubated at 37°C for approximately 18 hours. The zones of inhibition produced by the various preparations were measured in millilitres

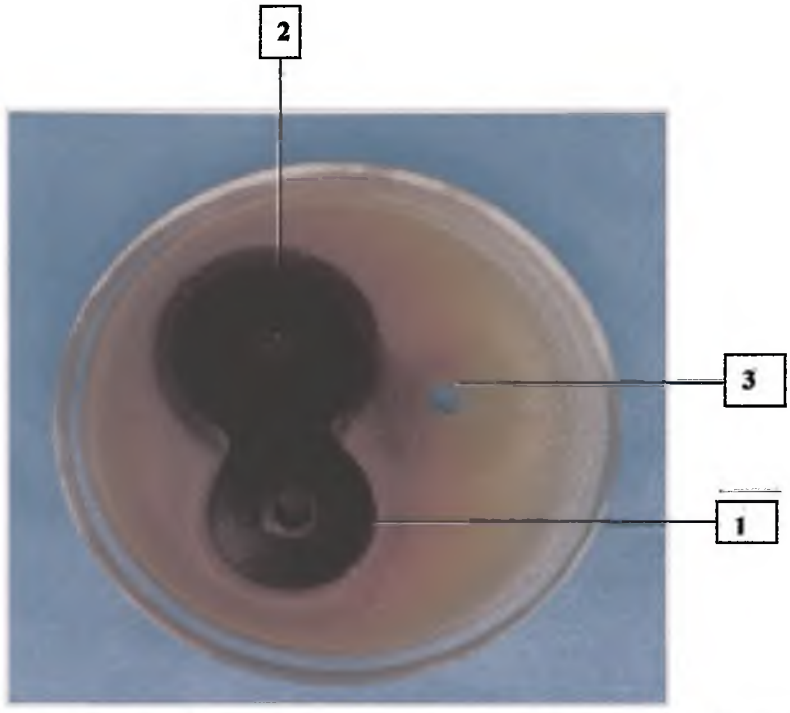

Figure 22. Inhibition of standard *Staphylococcus aureus by* 1-"Mist Enterica" and 2-combination of *P. guajava, C. ferruginea* and *H. opposita,* and 3-chloramphenicol

The plate was flooded with the standard *S. aureus* and allowed to dry at room temperature for an hour on a level surface. A sterilized borer of an internal diameter of about 6mm was used to bore holes in the medium and into these holes were added 100µl of the different preparations made from 1-"Mist Enterica" and 2-combination of *Psidium guajava, Cnestis ferruginea* and *Hoslundia opposita,* and 3-chloramphenicol. The plate was kept in the refrigerator overnight, for complete absorption of the extracts and then incubated at 37°C for approximately 18 hours. The zones of inhibition produced were measured in millilitres

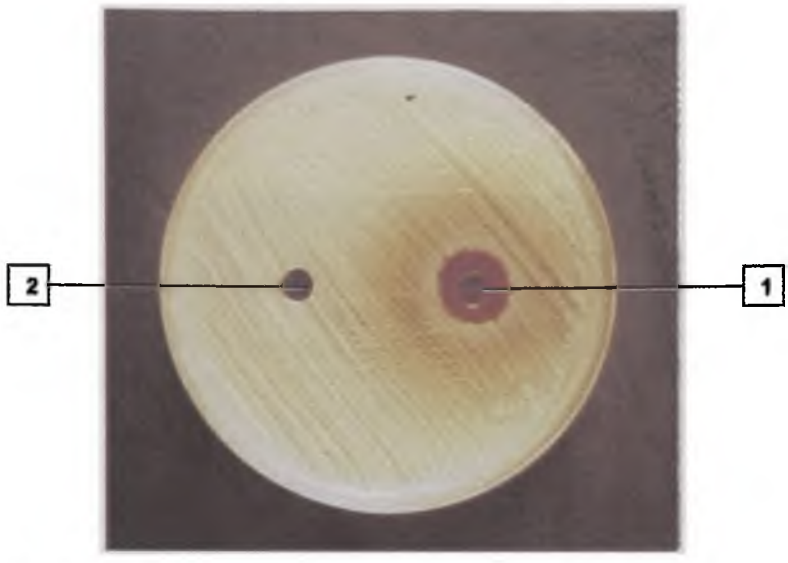

Figure 23. Inhibition of growth of wild-type *Salmonella* strain 8 using "Mist Enterica".

The plate was flooded with the wild-type *Salmonella strain* 8 and allowed to dry at room temperature for an hour on a level surface. A sterilized borer of an internal diameter of about 6mm was used to bore holes in the medium and into these holes were added 100µl of 1-"Mist Enterica" and 2-chloramphenicol. The plate was kept in the refrigerator overnight, for complete absorption of the extract and then incubated at 37°C for approximately 18 hours. The zones of inhibition produced were measured in millilitres

Figure 24. Inhibition of growth of wild-type *Salmonella* strain 8 using disk impregnated with 1-P. *guajava*, 2-*C. ferruginea*, 3-*H. opposita*, 4-"*Mist* Enterica" and 5- Chloramphenicol.

The plate was flooded with the wild-type *Salmonella strain* 8 and allowed to dry at room temperature for an hour on a level surface. The susceptibility of the wild-type *Salmonella* strain 8 to the different disks was investigated using the Kirby-Bauer disks diffusion method

Antimicrobial activity of the herbal decoctions against other wild-type pathogenic bacteria was also investigated. The three decoctions prepared from *C. ferruginea, H. opposita, P. guajava* and a mixture of these three were tested for their antibacterial and antifungal activity using *C. albicans* from high vaginal swab (*Candida*-HVS), *S. saprophyticus,* isolated from a female patient with vaginitis, *S. aureus*, haemolytic *E. coli* both isolated from a patient with urethral discharge, and *N. gonorrhoea* from a male having urinary tract infection (UTI). The results are presented in Table 13.

Each of the three plant extracts showed complete bactericidal activity against the Gram positive bacteria, *S. aureus* and *S. saprophyticus* at a concentration of 320mg/ml. However, the bactericidal activity varied depending on the medicinal plant used in the case of the Gram negative bacteria. Of the Gram negative organisms, *N. gonorrhoea* appeared to be the most susceptible to the medicinal plant extracts at a concentration of 320mg/ml whilst haemolytic *E. coli* was not susceptible to *C. ferruginea* and *H. opposita* and only slightly susceptible to *P. guajava.* All four herbal preparations showed antifungal activity against *Candida*-HVS at a concentration of 320mg/ml.

Table 13. Effects of different herbal decoctions against wild-type pathogenic bacteria.

Organisms	*C. ferruginea*	*H. opposita*	*P. guajava*	3 in 1
S. aureus	+++	+++	+++	+++
S. saprophyticus	+++	+++	+++	+++
Haemolytic *E. coli*	NS	NS	+	NS
Candida (HVS)	+++	++	+	+++
N. gonorrhoea	+++	++	++	+++

Each petri plate was flooded with a particular pathogen and the plate allowed to dry at room temperature for an hour on a level surface. A sterilized borer of an internal diameter of about 6mm was used to bore holes in the medium and into these holes were added 100µl of the different herbal preparations. Detailed method is described in the materials and methods

NS = organism is not susceptible
+ = zone of inhibition ≤ 9mm
++ = zone of inhibition ranging from10-15mm
+++ = zone of inhibition ≥16mm

The effect of heat on "Mist Enterica" was investigated by autoclaving the plant concoction at 121°C for 15min. Using the ditch method, 100µl each of autoclaved and non-autoclaved "Mist Enterica" concoctions were pipetted into wells made in Mueller-Hinton agar plates inoculated with the standard *S. typhi*. Figure 25 shows no significant difference between the two zones of inhibition. This investigation was extended to the decoctions made from *C. ferruginea, H. opposita* and *P. guajava.* Similar results were obtained for all three of them using the standard *S. typhi*.

In an effort to find out whether the most active anti-bacterial agents from the aqueous extracts were more hydrophobic or more hydrophilic, a solvent-solvent extraction was performed. Four organic solvents, petroleum ether, chloroform, ethylacetate and n-butanol with different degrees of polarity were used. Only the n-butanol and ethylacetate fractions, the two most polar solvents, inhibited bacterial growth of wild-type *Salmonella* and the standard strains of *Salmonella*, with zones of inhibition less than 9mm (Table 14). Overall results show that none of the plant fractions tested was as effective as the crude extract.

Figure 25. Effect of heat on the potency of the "Mist Enterica".

The effect of heat on the potency of "Mist Enterica" was investigated by autoclaving some of the extract at 121°C for 15mins before being used. Briefly, the plate was flooded with the standard *Salmonella typhi* and allowed to dry at room temperature for an hour on a level surface . A sterilized borer of an internal diameter of about 6mm was used to bore two holes in the medium and into one of these holes was added 100µl of unautoclaved "Mist Enterica" (1) whilst the other hole was added autoclaved "Mist Enterica" (2). The plate was kept in the refrigerator overnight, for complete absorption of the extract and then incubated at 37°C for approximately 18 hours. The zones of inhibition produced were measured in millilitres

Table 14. Zones of inhibition for the organic fractions with antimicrobial activity against the standard strains *S. aureus*, *S. typhi* and *S. paratyphi*

Organisms	Butanol	chloroform	Ethylacetate	Petroleum ether
S. aureus	+	NS	+	NS
S. typhi	+	NS	+	NS
S. paratyphi	+	NS	+	NS

Each petri plate was flooded with a particular standard strain and the plate allowed to dry at room temperature for an hour on a level surface. A sterilized borer of an internal diameter of about 6mm was used to bore holes in the medium and into these holes were added 100µl of the different organic fractions. The plate was kept in the refrigerator overnight, for complete absorption of the extract and then incubated at 37°C for approximately 18 hours. The zones of inhibition produced were measured in millilitres

NS = organism is not susceptible
+ = zone of inhibition ≤ 9mm

3.3.2. MICs of the herbal preparations

Table 15 shows the MICs of the herbal preparations used against the standard strains. *H. opposita* produced the lowest MIC with "Mist Enterica" having the highest at 32mg/ml for the standard strains used. *C. ferruginea* had MIC of 6.4 mg/ml, *H. opposita* had 1.6mg/ml, whilst *P. guajava* was 1.6-3.2mg/ml depending on the type of bacteria involved. In the case of the wild-type *Salmonella* strains, *C. ferruginea* had lower MICs of 1.6mg/ml as against 3.2mg/ml for *H. opposita* and *P. guajava* (Table 16).

Minimum inhibitory concentrations were determined for other pathogenic organisms using decoctions from *C. ferruginea, H. opposita* and *P. guajava*. As shown in Table 17 *C. ferruginea* gave the lowest MIC using *Candida* but had similar or same MICs as those of *H. opposita* and *P. guajava*.

Figure 26 illustrates the appearance of 115 *Salmonella* strains on typical test and control plates.

Table 15. MICs (mg/ml) of the decoctions/concoction with antimicrobial activity against the standard strains.

Organisms	*H. opposita*	*P. guajava*	*C. ferruginea*	Mist Enterica
S. aureus	1.6	1.6	6.4	32
S. typhi	1.6	3.2	6.4	32
S. paratyphi	1.6	3.2	6.4	32

The MIC for the herbal preparation was determined using the agar dilution method

Table 16. MICs (mg/ml) of the decoctions with antimicrobial activity against representative wild-type *Salmonella* strains.

Organisms	*H. opposita*	*C. ferruginea*	*P. guajava*
Salmonella strain 1	3.2	3.2	3.2
Salmonella strain 2	3.2	3.2	3.2
Salmonella strain 3	3.2	1.6	3.2
Salmonella strain 4	3.2	1.6	3.2

The agar dilution method was used to determine the MICs of the herbal preparations

Table 17. MICs (mg/ml) of the decoctions with antimicrobial activity against other pathogenic organisms.

Organisms	*H. opposita*	*C. ferruginea*	*P. guajava*
E. coli	3.2	3.2	6.4
Candida	12.8	6.4	12.8
S. aureus	1.6	1.6	1.6
S. saprophyticus	1.6	1.6	1.6

The MIC for the herbal preparation was determined using the agar dilution method

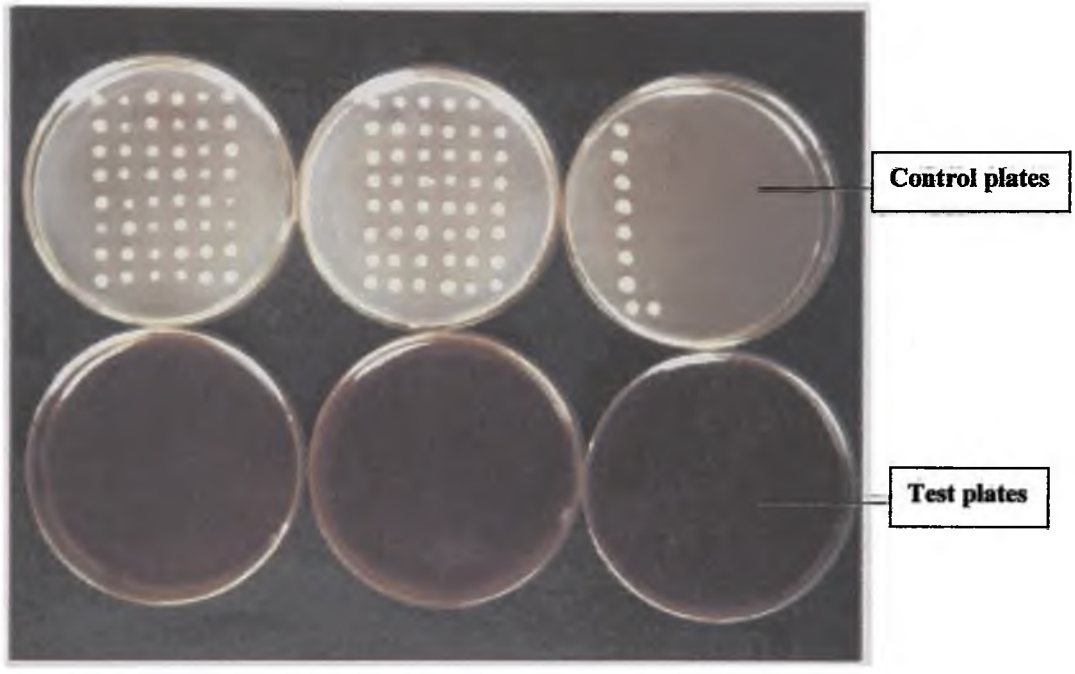

Figure 26. Mueller-Hinton agar plates showing the inhibition of *Salmonella* strains on test plates compared to the control plates

The MICs for the herbal preparations were determined using the agar dilution method

CHAPTER FOUR

DISCUSSION AND CONCLUSION

4.1 DISCUSSION

The recent increases in antibiotic resistant bacteria of all genera have prompted the scientific community to perform routine surveillance of microbial populations to determine the extent of the resistance (Mourad *et al.*, 1993; Wallace and Yousif, 1993). This study therefore focused on the isolation and identification of the groups/types of *Salmonella* found in Ghana as well as their resistance pattern and prevalence. Also studied was the efficacy of "Mist Enterica" and its component twelve plants as anti-typhoid agents using standard as well as the isolated or wild-type *Salmonella* strains.

A total of 115 *Salmonella* strains were isolated from different sources, identified according to the Group they belong to and investigated with respect to their susceptibility to the first-line anti-typhoid antibiotics namely ampicillin, chloramphenicol, tetracycline and trimethoprim-sulphamethoxazole as well as streptomycin. MICs for these antibiotics with the exception of streptomycin were determined. The study showed that 74% of the isolates (85 out of 115) were resistant to one or more of the five antibiotics used, with 66% classified as having multi-drug resistance (MDR) based on their resistance to three or more of the five antibiotics tested. Thirty-seven of the 85 resistant *Salmonella* strains were resistant to all five antibiotics. The prevalence rate of 43% compares with findings from India which reported a prevalence rate of 45% (Panigrahi *et. al.*, 1996). India is known to have extremely high levels of multi-drug resistant *Salmonella*. These findings from Ghana have grave implications for antibiotic therapy.

The emergence of resistant strains has been attributed to improper use of antibiotics (WHO, 1993). In Ghana, antibiotics are easily obtained as over-the-counter drug, in spite of laws and regulations to the contrary which specify their sale only on prescription. Due to poverty and ignorance most Ghanaians continue to abuse antibiotics, through wrong dosage, non-compliance and misapplication of the knowledge of the colour codes for the various antibiotics. The abuse may lead to the selection of resistant strains. The best strategy to minimize the development of resistant strains must be to intensify education, not only for people who patronize the antibiotics, but also those who sell them.

Ten to fifteen years ago, typhoid fever and other salmonellosis were treated successfully with three inexpensive drugs; ampicillin, chloramphenicol and trimethoprim-sulphamethoxazole, but many *Salmonella,* especially *S. typhi* strains today are showing increasing resistance to these antibiotics (Mourad *et. al.*, 1993; Wallace and Yousif, 1993). Due to the misinterpretation or inaccuracy of the "Widal test" leading to the misapplication of chloramphenicol and other first line antibiotics, there is now a stable R factor coding for plasmid multi-drug resistance to these antibiotics. Although current treatment of typhoid fever is multi-drug therapy, multi-drug resistance is now common among many pathogenic microorganisms such as *S. typhi* (MDRST) which show *in vivo* as well as *in vitro* resistance to all three first line anti-typhoid antibiotics (Smith *et al.*, 1984; Panigrahi, 1996; Stormon *et al.*, 1997).

The first major outbreak of typhoid fever caused by multi-drug resistant strains occurred in 1972 in Mexico City (Olarte and Galindo, 1973) and in South India (Paniker and

Vimla, 1972). In both outbreaks, drug resistance was shown to be plasmid mediated. Since then the occurrence of MDR strains of *S. typhi* has been reported from Britain (Ward *et al.*, 1982), India (Threlfall *et al.*, 1992), South Africa (Coovidia *et al.*, 1992), Pakistan (Bhutta *et al.*, 1994), Egypt (Farid, 1992), Bangladesh (Ahasan *et al.*, 1993) and many other places. Strains resistant to these "first line" antibiotics are not well documented in most African countries including Ghana even though these countries also have some of the same socioeconomic problems encountered in other areas of the developing world where antibiotic resistance seems to be on the increase (Olarte and Galindo, 1973; Anderson and Smith, 1972; Awotedu *et. al.*, 1992; Ibe and Wariso, 1996). Thus, this work was designed to characterize the *Salmonella* found in Ghana, by looking at the level of resistance, presence of an R-factor plasmid as well as the clonal relatedness, in addition to evaluating the efficacy of an herbal preparation used to treat typhoid fever.

The study identified eight groups of *Salmonella* in Accra, Ghana. The most common isolates were from groups B (33.0%) and D (57.4%) a total of about 90%, with the least found in groups A, G and I (Table 3). The high incidence of organisms in groups D and B indicates the prevalence of typhoid and paratyphoid fevers, respectively, in Accra. *S. typhi* the causative agent of typhoid fever belongs to Group D whilst *S. paratyphi* B which causes paratyphoid fever belongs to Group B. The Group B had as much as 73% of the strains being resistant to all 5 antibiotics and this could be due to the fact that paratyphoid fever is milder compared to typhoid fever, and therefore most patients

normally do not give the disease the needed attention, leading to sub-optimal doses of antibiotics and/or early curtailment of regimen.

No single strain of *Salmonella* was found to be resistant to chloramphenicol or trimethoprim-sulphamethoxazole alone. Thus any of the organisms found to be resistant to chloramphenicol and/or trimethoprim-sulphamethoxazole was also resistant to ampicillin and/or tetracycline. Similar results have been reported by other workers, who indicated no single strain of *Salmonella* being resistant to chloramphenicol alone (Panigrahi *et al*., 1996). This implies that chloramphenicol and/or trimethoprim-sulphamethoxazole could still be useful in the treatment of uncomplicated typhoid fevers and other salmonellosis. However, it is advisable to carry out antibiotic susceptibility tests on all strains to ensure that they are not multi-drug resistant.

The appearance of such antibiotic-resistant strains of *Salmonella* is not only linked to antibiotic use for the treatment of human infection but also usage in poultry farming, which provides selective pressure favouring resistant strains (WHO, 1978). It is therefore not surprising that the drugs most commonly affected by bacterial resistance in Ghana and many other developing countries are generally the inexpensive and popular broad-spectrum antibiotics such as the five used in this study. The spread of these resistant organisms may also be traced to socio-economic and behavioural antecedents contributing to the escalating resistance to antibiotics worldwide. Misuse of antibiotics by either the physicians in clinical practice, the unskilled practitioners, or by the public, sub-optimal dosage and poor quality of antibiotics, all bring about resistance (Okeke,

1999). The resistance may, however, appear rapidly or slowly, depending on the organism concerned, the volume and type of antibiotic used, and the method of application (WHO, 1978).

In this study, Groups B and D together constituted about 90% of the total *Salmonella* isolates (104 out of 115) (Table 3) which is not surprising since they are the most prevalent *Salmonella* strains in developing countries (Cheesbrough, 2003). For instance, *S. paratyphi* B and *S. typhi* that cause enteric fever belong to Groups B and D respectively whilst *S. typhimurium* and *S. enteritidis* belonging to Groups B and D respectively, are the main causative agents of diarrhoeal diseases in most developing countries including Ghana. Of the 104 *Salmonella* strains making up Groups B and D, 76% were resistant to one or more of the five antibiotics used (Table 3). The total number of organisms found to be resistant to one or more of the five antibiotics in this study were 85 out of 115 strains. Out of these 85 resistant strains, Groups B and D together formed about 93% (79 out of 85) and again about 93% (71 out of 76) of the multi-drug resistant (MDR) strains (Table 3). The Ghana Medical Association has highlighted the public health implications of the increasing incidence of typhoid fever and other salmonellosis in Ghana especially in urban slums and rural communities (National Symposium on Enteric fever/salmonellosis for the Southern sector, 1998). However, the level of typhoid resistance as well as its management has not been extensively investigated. There is therefore an urgent need for a country-wide study on the antimicrobial sensitivity patterns of the major bacterial pathogens especially *Salmonella*.

The high level of MDR found in this study must be a source of worry. Group B for instance, had almost all strains, that is, 97% (37 out of 38) of the strains being resistant to one or more of the five antibiotics with 84% of these being MDR. As much as 71% of these MDR strains were resistant to all five antibiotics (Table 3). This is a group that includes two important pathogenic *Salmonella*; *S. paratyphi* B and *S. typhimurium*, causative agents of enteric fever and *Salmonella* food-poisoning/diarrhoea. In the case of Group D, 64% were resistant to one or more of the five antibiotics used with 59% being MDR and of the 32 strains belonging to 4-R, 78% were from group D (Table 3).

The *Salmonella* strains were isolated from patients attending the Korle-Bu Teaching Hospital, one of the best specialist/referral hospitals in Ghana. Thus the high MDR strains might have evolved due to the sub-standard treatment of the disease elsewhere with the subsequent selection of the MDR *Salmonella*. Unfortunately, consumption of antibiotics appears to be rising on a worldwide scale, and in Ghana, since most people lack knowledge about emergence of resistant strains and the dangerous effects antibiotics pose to vital organs of the body, the situation becomes worse. Thus, an alternative medicament that has therapeutic effects on both sensitive and multi-drug *Salmonella* strains must be explored and developed to alleviate the sufferings of the people especially the rural folks who are mostly afflicted with the disease. Herbal preparations could provide such an alternative.

The use of antimicrobial drugs in animals for growth promotion may also lead to the selection of resistant strains of pathogens, which may be transmitted to humans through

food (Ryder *et al.*, 1980; Oosterom, 1991; Khachtourians, 1988; Mensah, 1999). A typical example involves the emergence and spread of drug-resistant salmonellae from ampicillin and tetracycline used in animals during the 1960s, with the subsequent transmission of these salmonellae to man resulting in many human infections (Anderson, 1968). A study conducted in Accra revealed that both imported and locally produced chicken are the potential source of multiple-antibiotic-resistant enteropathogenic bacteria (Sackey *et al.*, 2001). In their study, they isolated *Salmonella* from seven live birds from poultry farms and attributed this to contaminated water used to feed the poultry on the farm. For instance, at the time of their study the farm was using an alternative supply of water, which was stored in the open and was patronized also by flying birds. The same study also detected *Salmonella* from supermarkets, which display chicken under refrigeration.

Richmond (1972) reported that sewage and surface waters contribute to the distribution and circulation of resistant organisms. These sources represent a natural medium in which transfer of resistant plasmid in organisms can occur under certain physical, chemical or biological conditions, the plasmids being subsequently transferred to food and drinking water, leading to recycling to man and animals. In this study, as much as 57% of the *Salmonella* (8 out of 14) isolated from food, were resistant to one or more of the antibiotics used (Fig. 10). Four out of these 8 resistant strains were resistant to only one antibiotic, two to tetracycline and two to streptomycin, two very important antibiotics some time past. These 1-R strains constituted 50% of all the strains resistant to only one

antibiotic even though the food samples formed only 9% of the 85 resistant strains (Appendix A, Table 5).

Also these 4 1-R strains were the only 1-R strains that did not transfer their resistance to tetracycline or streptomycin onto recipient *E. coli* by conjugation, neither were their plasmids able to transform recipient *E. coli*. This implies that one does not need multiple antibiotics to eradicate them from the population (Tables 9 and 10).

Several studies have indicated that much of the multi-drug resistance associated with *S. typhi* and other *Salmonella* are plasmid mediated and that plasmids belonging to the incompatibility group *Inc*HI are frequently the source of resistance to ampicillin, chloramphenicol, trimethoprim-sulphamethoxazole, and tetracycline. These plasmids are quite large, ranging in size from 140 to180 kb (Shanahan *et al.*, 1998; Mirza and Hart, 1993; Threlfall *et al.*, 1992; Karmaker *et al.*, 1991; Goldstein *et al.*, 1986). Results from this study (Fig. 16), showed that plasmids that transferred resistance via conjugation belonged to the *Inc*HI incompatibility group, thus pointing to them as the source of the resistance.

Streptomycin is obtained from a group of microorganisms named *Streptomyces* and was added in this study because it has been used to treat typhoid fever and other bacterial infections such as tuberculosis in times past. Most of the transconjugates in this study acquired streptomycin resistance in addition to the other antibiotics mentioned. This

aminoglycoside may therefore be added to the list of common antibiotic resistance determinants associated with *Inc*HI plasmids.

As direct cell-to-cell contact appears to be a common mechanism for transfer of antibiotic resistance genes, the possibility that these organisms could harbour transformable plasmids containing antibiotic resistance genes was examined. The results indicate that *E. coli* containing plasmids with ampicillin resistance were the most frequently transformed (Table 10). This is not surprising because ampicillin is one of the antibiotics mostly prescribed by health workers and it is for a wide range of diseases including infections of the middle ear, urinary, respiratory, and gastrointestinal tracts. In Ghana, ampicillin is one of the antibiotics abused by many people for all kinds of diseases to the extent that they are used to treat open wounds.

Few isolates contained transformable plasmids with MDR genes, including two organisms, strains 8 and 13, isolated from urine and blood, respectively. Each of them contained transformable plasmids with resistance determinants to the five antibiotics examined (Table 10). Similar results have been obtained in studies on *S. typhi* isolates from Pakistan, which were shown to contain self-transmissible 98 Mda plasmids encoding resistance to chloramphenicol, ampicillin, tetracycline, streptomycin, and trimethoprim-sulphamethoxazole (Mirza and Hart, 1993).

To determine the mode of transmission of the observed antibiotic resistance in this study, mating experiments were performed between each *Salmonella* isolate and *E. coli*. That

an identical antibiotic resistance phenotype was conferred on the recipient *E. coli* in 94% (80 out of 85) of the cases where the *Salmonella* isolate was resistant shows that the resistance was encoded by conjugation plasmids (Table 9). Furthermore, transferring antibiotic resistance to the recipient *E. coli* via transformation was accomplished in only 18% (15 out of 85) strains. These data indicate that genetic determinants for antibiotic resistance are located on conjugative plasmids.

No differences were noted in transferable resistance patterns when matings were performed at either 28°C or 37°C. Most notably, transferable tetracycline resistance was in some cases only accomplished at 28°C, while in other instances it occurred at only 37°C; oftentimes the use of both conjugation temperatures resulted in tetracycline resistance (Data not presented). These data suggest that, multiple conjugative plasmids that are temperature sensitive with respect to transfer exist in the host cell and these plasmids may or may not contain tetracycline resistance determinants. Although temperature sensitive conjugative transfer has been documented in *S. typhi*, no difference in resistance phenotypes were noted in a previous study (Shanahan *et al.*, 1998). Temperature sensitive MDR in *Salmonella* and other enteric bacteria has been documented by other workers (Cohen *et al.*, 1993; Kunonga *et al.*, 2000).

Epidemiological investigations examining the clonal nature of disease outbreaks and for routine surveillance are commonplace in clinical settings. Such data give public health officials the necessary tool to determine if the causative organisms are part of the transient or resident populations. Many genetic fingerprinting techniques are available

for examining the clonal relatedness, each demonstrating variable degrees of utility. The clonal nature of the *Salmonella* isolated in this study was investigated using plasmid profiling and the PCR-based methodologies of ERIC-PCR and REP-PCR. Plasmid profiling was the simplest procedure, yet was the best in discriminating among the resistant types. Five distinct patterns were observed upon electrophoresis with majority of the isolates belonging to pattern 2 (Fig. 17). Interestingly, patterns 2-4 were comprised of overlapping bands and all isolates belonging to either of these patterns demonstrated MDR. Patterns 1 and 2 comprised mostly of the 3-R and 4-R strains whilst patterns 3 and 4 comprised mostly of the 5-R strains. Pattern 5 comprised all strains with 2-R and 1-R (Fig. 17). In contrast, little variation was observed among the 115 isolates examined in this study using the PCR-based methodologies although both REP and ERIC, composed of short extragenic repetitive sequences located throughout the chromosome, have been used to fingerprint many bacterial species (De Bruijn, 1992; Georghiou *et al.*, 1994; Rivera *et al.*, 1995; Rodriguez-Barradas *et al.*, 1995; Beyer *et al.*, 1998). The lack of discrimination may be that only two sampling sites in a relatively small geographic location were selected.

Further studies were conducted to evaluate the antibacterial properties of "Mist Enterica" used at the CSRPM clinic for the treatment of typhoid fever, as well as those of the component herbs making up "Mist Enterica". The results revealed that "Mist Enterica" had antibacterial activities against a wide range of organisms including Gram positive and Gram negative bacteria and fungi (Tables 12). Preparations made from three of the plants making up "Mist Enterica", *Hoslundia opposita*, *Cnestis ferruginea*, and *Psidium*

guajava were found to be very active against all three standard strains of bacteria used in this study, that is, *Salmonella typhi* (ATCC 33458), *Salmonella paratyphi* B (ATCC 10719) and *Staphylococcus aureus* (12981) (Table 12). Each of the three plant extracts also showed complete bactericidal activity against wild-type *S. aureus* and *S. saprophyticus* at a concentration of 320mg freeze dried material per milliliter of sterile distilled water. *N. gonorrhoea* was very susceptible to all the herbal components at a concentration of 320mg/ml whilst haemolytic *E. coli* was resistant to *C. ferruginea* and *H. opposita* and only slightly susceptible to *P. guajava*. All four herbal preparations from *C. ferruginea*, *H. opposita*, *P. guajava*, and the 3-in-1 respectively showed complete anti-fungal activity against *Candida*-HVS at a concentration of 320mg/ml (Table 13).

Herbal/plant preparations have been used for the management of a range of ailments including infectious diseases in Ghana over the years. These preparations are sold at most public places and patronized by people from almost all social classes but the knowledge about these medicinal substances and their pharmacological potentials have been passed on generation after generation only by oral tradition. Hence, Ghana and most African countries lack a comprehensive pharmacopoeia of potential medicinal herbs. For instance, even though most of these plants used for this study have been in existence and used by our herbalist since time immemorial, they have not been exhaustively researched scientifically. *P. guajava* is only documented to have anti-diarrhoeal and antimicrobial properties, *H. opposita* as being used to treat gonorrhoea whilst *C. ferruginea* is used to treat wounds (Mshana *et al.*, 2000; Ghana Herbal

Pharmacopoiea, 1992). This is the first study indicating that these three plant materials are effective against standard and wild type strains of *Salmonella* species.

This study has shown that the bacterial strains used, both standard and wild-type, were completely susceptible to the herbal preparations (Tables 12 and 13). This is of significance because a large proportion, 74%, of the wild-type *Salmonella* was found to be resistant to the commonly used anti-typhoid antibiotics such as ampicillin, chloramphenicol, tetracycline, and trimethoprim-sulphamethoxazole. Thus "Mist Enterica" used to treat fever may also serve as a potential effective treatment for other salmonellosis as well as treatment for other infectious diseases. This is the first study indicating *P. guajava, H. opposita* and *C. ferruginea* as potential treatment against typhoid fever. Further scientific investigations such as the safety assessment as well as clinical trials are needed in order to formulate and standardize an alternative medicament not only for salmonellosis but diseases caused by other microorganisms that may be susceptible to the plant preparations.

This study has shown that, preparations from individual plants or a combination of *C. ferruginea, H. opposita* and *P. guajava* can also be used as effective means for the treatment against Gram positive bacteria that are human pathogens. *S. aureus* infection is an ongoing problem in hospitals, especially for immune suppressed patients but the herbal preparations from the individual plants as well as a combination of the three were able to inhibit the growth of both standard and wild-type *Staphylococcus* (Table 13). Thus "Mist Enterica" and some of its componenets are not only anti-*Salmonella* but also

anti-*Staphylococcus*. In addition, the plant preparations showed activity against organisms associated with sexually transmitted infections (STI) such as *Candida* and *N. gonorrhoea*. The results warrant further studies into the possibility of using these extracts in the treatment of specific STIs.

With the estimation by WHO that 80% of the world's population presently use herbal medicine for some aspect of primary healthcare (WHO, 2003), one can only conclude that these three plants show much promise for the future.

The effect of heat on "Mist Enterica" and its three efficacious components was investigated by autoclaving them at 121°C for 15min but no significant difference was observed in the zones of inhibitions produced implying that they are heat resistant (Fig. 25). This is very advantageous since most of these herbal preparations are boiled to extract the active components.

Minimum inhibitory concentrations of "Mist Enterica" and its three components were analysed to find out the lowest concentrations of the preparations needed to inhibit the growth of the micro-organisms. "Mist Enterica" had the highest MIC at 32mg/ml for the standard strains, *C. ferruginea, H. opposita* and *P. guajava* had MICs of 6.4 mg/ml, 1.6mg/ml and 1.6-3.2mg/ml, respectively. For the wild-type *Salmonella* strains, *C. ferruginea* had MIC of 1.6mg/ml as against 3.2mg/ml for *H. opposita* and *P. guajava* (Table 16). The results indicate that these herbal preparations are efficacious and can be

used for the management of diseases caused by these strains of bacteria after toxicity tests are carried out on them to ensure their safety.

4.2 CONCLUSION AND RECOMMENDATIONS

This study has demonstrated that, most of the clinical *Salmonella* isolates from patients suspected of having enteric fever are resistant to the 'first line' antibiotics routinely used in treating patients, the majority showing multi-drug resistance and belong to Groups B and D. The genetic basis for this resistance appears to reside on conjugative plasmids. The organisms appear to be derived from a similar clonal lineage. Studies such as these are recommended to be made routine in clinical laboratories in order to aid our medical practitioners prescribe the right type of antibiotics. Having such knowledge can also help in the development of antibiotic combinations that will be effective in the treatment of MDR infections.

The study has also demonstrated that "Mist Enterica", *C. ferruginea, H. opposita* and *P. guajava* have bactericidal as well as fungicidal activities with zones of inhibition ranging from 9-29mm and MICs between 1.6-32mg/ml. The study further revealed that a combination of *C. ferruginea, H. opposita* and *P. guajava* could be a better formulation to replace "Mist Enterica" and perhaps, lead to the development and production of an effective standardized plant-derived pharmaceutical product for salmonellosis and other diseases of bacterial origin. It is therefore recommended that further investigations into the safety and efficacy be conducted *in vivo* in order to produce a very standardized herbal preparation safe for all to drink.

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APPENDIX A

TABLE 1. *Salmonella* isolates resistant to all the five antibiotics

AmCmTeTsSt	8,13,33,39,48,49,51-53,55,66,67,72-78,83, 89,90,95,97-99,101,102,104-106,108-112,115

TABLE 2 *Salmonella* isolates resistant to four of the antibiotics

AmCmTeSt	23
AmCmTsSt	17,47,63,68,71,79,114
AmTeTsSt	1,3,4,6,21,22,27,35,38,40,46,56,81,82,84,88,91-94,96, 100,103,107

TABLE 3. *Salmonella* isolates resistant to three of the antibiotics

AmCmTe	7
AmTeSt	24
AmTeTs	2,14,28,30
TeTsSt	113

TABLE 4. *Salmonella* isolates resistant to two of the antibiotics

AmSt	87

TABLE 5. *Salmonella* isolates resistant to one of the antibiotics

Am	26,29,36,80
Te	58,59
St	61,65

MULTIPLE RESISTANT SALMONELLA IN ACCRA, GHANA

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SUMMARY

A total of 115 Salmonella strains isolated during 1998-1999 in Accra, Ghana were examined for drug/multiple drug resistance, using first-line anti-typhoid antibiotics, namely, ampicillin (Am), chloramphenicol (Cm), Tetracycline (Te) and Trimethoprim/sulphamethoxazole (Ts). These organisms were isolated from urine, stool, food, cerebrospinal fluid, blood and other sources. The number of organisms isolated from blood alone was 82(71.3%). Eight serological groups were identified and the most common isolates were groups D (57.4%) and B (33%), with the least found in groups A, G and I.

Forty-four (38.3%) isolates were found to be sensitive to all four antibiotics whilst 71 (61.7%) were resistant to one or more of the antibiotics used. Thirteen (11.3%) of the resistant strains were resistant to only one of the antibiotics, 6 (5.2%) were resistant to two of the antibiotics, 22 (19.1%) were resistant to three of the antibiotics, and 30 (26.1%) were resistant to all four of the antibiotics used. All the organisms were however, sensitive to Ciprofloxacin, and Ceftriaxone and Gentamicin.

These findings indicated that multiple resistant Salmonella are prevalent in Ghana and national surveillance to determine the level of resistance is needed for the nation.

Keywords: Antibiotic susceptibility, multiple resistance, Salmonella

INTRODUCTION

The number of officially recorded cases of human salmonellosis has significantly increased over the past two decades in many countries all over the world[1,2,3]. In Ghana, salmonellosis, especially typhoid fever is of public health concern in urban slums and rural communities where its prevalence is highest in children and young people[4]. This is because older people often seem to possess partial immunity, probably following exposure to frequent sub-clinical infective doses of typhoid bacillus[5]. A review conducted in rural areas of three African countries namely Ghana, Zambia and Kenya in 1994, revealed that incidence of typhoid was much higher in Ghana compared to the rest[3]. This was linked to poor water supply, inadequate sewage disposal and unhygienic conditions.

Treatment of typhoid fever is usually by antibiotics and the drug of choice has been chloramphenicol, a broad-spectrum antibiotic. This drug is inexpensive and has remarkably been effective in the treatment of typhoid fever in the past. However, it has recently been reported to increasingly becoming ineffective in treating typhoid cases[6]. This is very disturbing because it has been reported that about 12-16% of patients die within four weeks of the disease if not well managed[7]. This has led to the recommendation of the use of multi-drug therapy and/or third generation drugs. Multi-drug resistance is, however, now common among these pathogenic microorganisms, which show both *in vivo* as well as *in vitro* resistance to the four first-line antityphoid antibiotics namely, ampicillin, chloramphenicol, tetracycline and trimethoprim-sulphamethoxazole[8, 9, 10,11,11,12].

Quinolone derivatives such as Ciprofloxacin or Pefloxacin and third generation cephalosporins such as Ceftriaxone or Cefotaxin are very effective for treating diseases caused by multi-drug resistant *S. typhi* strains, particularly Ciprofloxacin given by oral route in a 7-day course of therapy[13]. However, these drugs are expensive and out of reach of the poor in the endemic areas, hence, chloramphenicol is still prescribed in many health facilities in Ghana and this has led to the general notion that typhoid is difficult to treat.

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The present study was carried out to evaluate the prevalence of multiple resistant Salmonella in Accra, Ghana during the period August 1998 to July 1999.

MATERIALS AND METHODS

The above study was carried out at the Bacteriology Unit, Noguchi Memorial Institute of Medical Research (NMIMR), University of Ghana, Legon. The isolates of Salmonella from stool, blood, urine, cerebrospinal fluid, food and chicken, were from the Microbiology Department, Korle Bu Teaching Hospital, and the Bacteriology Unit, NMIMR, Legon. The specimens were plated on the appropriate media (MacConkey agar, chocolate agar, blood agar or Dexycholate agar) from Oxoid, (Maryland, USA), depending on the source of the sample and colonies with the characteristics of Salmonella isolated using standard microbiologic methods[14] Identification of Salmonella from the various sources was done using the following biochemical tests; Triple Sugar Iron (TSI) agar test, Sulphur Indole Motility (SIM) agar test, and Urea agar test, all from Oxoid (Maryland, USA) and the presence of Samonella was confirmed and grouped using Salmonella antesera kit from Denka Seiken Co., Ltd., Japan.

In vitro antibiotic susceptibility against ampicillin (10μg), chloramphenicol (30μg), tetracycline (30μg), and trimethoprim/sulphamethoxazole (1.25/23.75μg) from Britania (Buenos Aires, Argentina) was tested according to the guidelines set by the National Committee for Clinical Laboratory Standards (NCCL)[15].

RESULTS

In all, one hundred and fifteen (115) Salmonella strains were isolated. Table 1 shows the sources and number of Salmonella strains isolated.

Table 1 Number of Salmonella strains isolated

Source of salmonella	Number of salmonella (%)
Blood	82 (71.3)
Food	14 (12.2)
Stool	6 (5.2)
Urine	4 (3.5)
Cerebrospinal fluid	3 (2.6)
Others	6 (5.2)
Total	115 (100)

The study serologically identified eight groups of Salmonella, Group D (57.4%). Group B (33%), Group C_1 (3.5%), Group C_2 (1.72%), Group E_1 (1.7%) and Groups A, G and I (0.9%) each.

The antibiotic susceptibility tests showed that, 44(38.3%) were susceptible to all four antibiotics and the remaining 71 (61.7%) of the 115 Salmonella strains were resistant to one or more of the first-line anti-typhoid antibiotics (Table 2).

Table 2 Susceptibility patterns of Salmonella isolated (n=71).

Resistance pattern	Total number of resistant strains (%)
AmCmTeTs	30 (42.3)
AmCmTe	1 (1.4)
AmTeTs	17 (23.9)
CmTeTs	4 (5.6)
AmTs	1 (1.4)
CmTe	1 (1.4)
TeTs	4 (5.6)
Am	4 (5.6)
Te	9 (12.7)
Total	71 (100)

Am=Ampicillin Cm=Chloramphenicol
Te= Tetracycline Ts = Trimethoprim/Sulphamethoxazole

Of these resistant isolates, 6(5.2%) were resistant to two antibiotics, 22 (19.1%) were resistant to three antibiotics whilst 30 (26.1%) were resistant to all four antibiotics. Interestingly, 4 (3.5%) were resistant to ampicillin alone, 9 (7.8%) were resistant to tetracycline alone but none was resistant to chloramphenicol or trimethoprim/sulphamethoxazole alone.

DISCUSSION

There is an increase in the prevalence of salmonellosis in most rural tropical areas and urban slums probably due to HIV, malnutrition, sickle cell anaemia, G6PD-deficiency[3,16], nematodiasis and schistosomiasis[16]. Most of these clinical conditions impair mononuclear cells, hence susceptibility to Salmonella bacteraemia. The high incidence of such infectious diseases has resulted in the acquired bacterial resistance in isolates of even healthy persons and from patients with community-acquired infections[17].

The present study confirms the presence of multi-drug resistant strains (MRS) of Salmonella in Ghana[12,26]. In the study, 60% of the 115 Salmonella species isolated, during a 12 month period, were resistant to one or more of the four first-line anti-typhoid antibiotics, with 70% of these being multi-drug resistant strains. Thus, the multi-drug resistant strains form 45.2% of the total number of organisms investigated. This is similar to the percentage reported from the Indian sub-continent[9], known for its extremely high levels of multi-drug resistant *Salmonella*. These findings have grave implications for antibiotic therapy in Ghana as the resistance is to the inexpensive first-line antibiotics.

No single strain of Salmonella was found to be resistant to chloramphenicol or trimethoprim/sulphamethoxazole alone with the rest being susceptible, and this corroborates investigations by other workers, who indicated no single strain of Salmonella being resistant to chloramphenicol alone[9]. The organisms that were found to be resistant to chloramphenicol and/or trimethoprim-sulphamethoxazole were always resistant to ampicillin and/or tetracycline but never vice versa. All the resistant Salmonella strains in this study were resistant to ampicillin and/or tetracycline, and therefore treatment with chloramphenicol and/or trimethoprim-sulphamethoxazole cannot be relied on to eliminate these pathogens. Fortunately, all the isolates were susceptible to ciprofloxacin, ceftriaxone and gentamicin.

The appearance of such antibiotic-resistant strains of Salmonella is closely linked to antibiotics use for the treatment of human infection and in poultry farming, which provides selective pressure favouring resistant strains[18,19]. It is therefore not surprising that, the drugs most commonly affected by bacterial resistance in Ghana and many other developing countries are generally the inexpensive and popular broad-spectrum antibiotics such as the four used in this study.

The spread of these resistant organisms may be traced to socio-economic and behavioural antecedents contributing to the escalating resistance to antibiotics worldwide. This may result from the misuse of antibiotics by either the physicians in clinical practice, the unskilled practitioners, or by the public, sub-optimal use and poor quality of antibiotics, all of which may bring about resistance[20]. The resistance may however appear rapidly or slowly, depending on the organism concerned, the volume and type of antibiotics used, and the method of application[18]. Unfortunately, precise data on antibiotic use in Ghana and many other countries are not available but consumption appears to be rising on a worldwide scale. This is not surprising since in Ghana antibiotics are easily obtained as over-the-counter drug, in spite of laws and regulations to the contrary which specify their sale only on prescription.

The use of antimicrobial drugs in animals for growth promotion has also led to selection of resistant strains of pathogens, which may be transmitted to humans through food[1,2,21,22]. A typical example involves the emergence and spread of drug-resistant salmonellae from antibiotics used in animals during the 1960s[23], with the subsequent transmission of these salmonellae to man resulting in many human infections. A study conducted in Accra revealed that, imported and locally produced chicken are a potential source of multiple-antibiotic-resistant enteropathogenic bacteria[24]. Richmond[25] reported in 1972 that, sewage and surface waters contribute to the distribution and circulation of resistant organisms. These sources represent a natural medium in which R-plasmid transfer can occur under certain physical, chemical or biological condition and transferred to food and drinking water, leading to recycling to man and animals. It is therefore not surprising that, as much as 11(79%) of the Salmonella isolated from food for this study, were resistant to one or more of the antibiotics used.

The results of this study would suggest that, control measures be tackled through multisectoral methods and not only be dependent on the health sector. Thus, the unnecessarily frequent use of antibiotics must be curbed whilst prompt diagnosis and antibiotic therapy for patients and proper management of asymptomatic carriers be practiced. The provision of potable drinking water and 21st century sewage disposal practices as well as health education must be intensified to help control the disease.

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Antibiotic resistance and genotyping of clinical group B *Salmonella* isolated in Accra, Ghana

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ABSTRACT

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Aims: The purpose of this study was to investigate the antibiotic resistance and clonal lineage of serogroup B *Salmonella* isolated from patients suspected of suffering from enteric fever in Accra, Ghana.

Methods and Results: Serogroup B *Salmonella* were isolated from blood ($n = 28$), cerebral spinal fluid (CSF) ($n = 1$), or urine ($n = 2$), and identified based on standard biochemical testing and agglutinating antisera. Isolates were examined for their susceptibility to ampicillin, chloramphenicol, tetracycline and trimethoprim–sulfamethoxazole. Most of the isolates could be classified as multiple-drug resistant. Furthermore, the genetic location of resistance genes was shown to be on conjugative plasmids. Genetic fingerprinting by plasmid profiling, enterobacterial repetitive intergenic consensus (ERIC)-PCR, and repetitive element (REP)-PCR were performed to determine the diversity among the isolates. Plasmid profiling discriminated five unique groupings, while ERIC-PCR and REP-PCR resulted in two and three groupings, respectively.

Conclusions: A high rate of antibiotic resistance was associated with the *Salmonella* isolates and the genes responsible for the resistance are located on conjugative plasmids. Also, there appears to be minimal diversity associated with the isolates.

Significance and Impact of the Study: As a result of the increasing antibiotic resistance among bacteria of all genera, surveys to monitor microbial populations are critical to determine the extent of the problem. The inability to treat many infectious diseases with current antibiotic regimens should prompt the medical community to be more prudent with its antibiotic use.

Keywords: antibiotic resistance, genotyping, Group B *Salmonella*.

INTRODUCTION

Typhoid fever poses a serious health problem to the developing world. The annual incidence of this disease is estimated to be 20 million cases, resulting in greater than 700 000 deaths (Thong *et al.* 1994). Most cases are the result of infection with *Salmonella typhi* (DuPont 1993), but colonization with *Salm. paratyphi* A, B or C, results in paratyphoid fever, a contagious condition similar to typhoid fever (Barrow 2000). Clinically, both typhoid and paratyphoid fever are grouped together under the category of enteric fever and patients exhibit headache, high fever, stomach and intestinal pain, and vomiting. Cases of enteric fever are self-limiting if the appropriate antimicrobial regimen is started promptly. Chloramphenicol has been the 'first line of defense' for many years, but the emergence of chloramphenicol-resistant strains prompted the use of ampicillin and trimethoprim/sulfamethoxazole, which are considered appropriate alternatives to chloramphenicol

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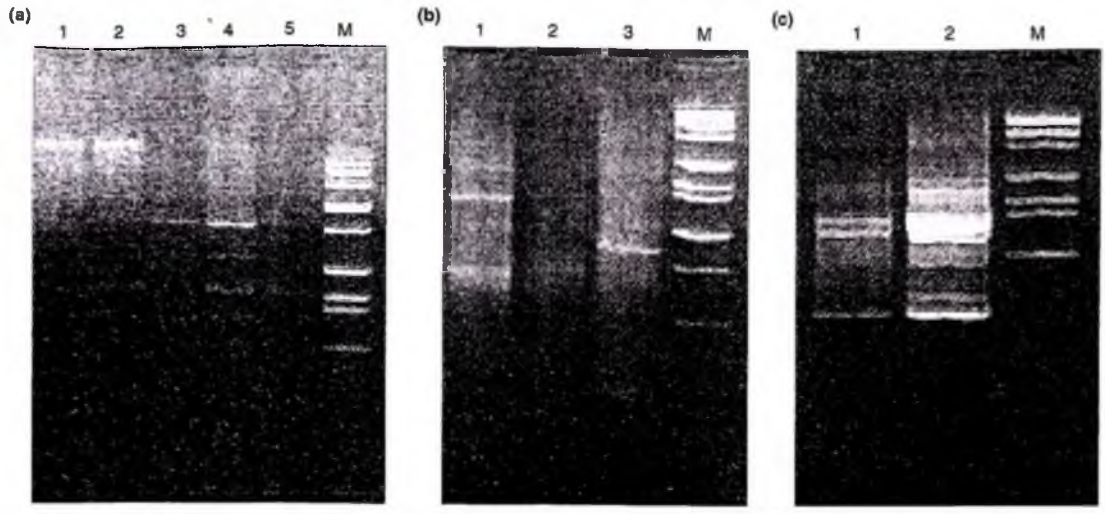

Fig. 1 Agarose gels of representative genotypic patterns obtained with (a) plasmid profiling, (b) REP-PCR, and (c) ERIC-PCR. The numbers above each gel refer to the different amplification pattern (refer to Table 2). M, markers

Table 2 Distribution of group B *Salmonella* isolates into discrete genotypic patterns

Genotypic pattern	Strains with indicated genotypic pattern
PP*	
1	1,2
2	3,4
3	5–7,9
4	8,10–29,31
5	30
REP	
1	1–7,26,27
2	8–14,30,31
3	15–25,28,29
ERIC	
1	1–7,16–19,25,26,28–30
2	8–15,20–24,27,31

*PP, plasmid profiling.

DISCUSSION

Enteric fever is predominately the result of infection by *Salm. typhi, Salm. paratyphi* A, B, or C. Ten to fifteen years ago, patients with enteric fever were successfully treated using either of the 'first line' antibiotics (ampicillin, chloramphenicol, or trimethroprim/sulfamethaxozole). However, as with the trend of many bacterial pathogens in today's world, classical antibiotic treatment regimens are no longer effective (Mourad *et al.* 1993; Wallace and Yousif 1993). We initiated this study to examine if this trend was occurring in patients suspected of suffering from enteric fever in Accra, Ghana. Thirty-one group B *Salmonella* isolates were examined for their susceptibility to five different antibiotics. An alarming rate of antibiotic resistance was noted, as 93% of the isolates could be classified as MDR. Although these 'first line' antibiotics had no effect, all isolates were sensitive to ceftriaxone and ciprofloxacin (data not shown). The cost of these antibiotics, however, will have to become less prohibitive if they are to find application in the developing world.

Previous studies have indicated antibiotic resistance associated with *Salm. typhi* is in large part the result of large conjugative plasmids (Goldstein *et al.* 1986; Karmaker *et al.* 1991; Mirza and Hart 1993). To determine if the observed antibiotic resistance in this study was encoded by conjugative plasmids, mating experiments between each *Salmonella* isolate and *E. coli* were carried out. An identical antibiotic resistance phenotype was conferred on the recipient *E. coli* in all cases where the *Salmonella* isolate was resistant (Table 1). Furthermore, transferring antibiotic resistance to the recipient *E. coli* via transformation was not accomplished. These data indicate the genetic determinants for antibiotic resistance are located on conjugative plasmids. We did note differences in transferable resistance patterns when matings were performed at either 28 or 37°C. Most notably, transferable tetracycline resistance was in some cases only accomplished at 28°C, while in other instances it occurred at only 37°C; oftentimes both conjugation temperatures resulted in tetracycline resistance. These data

suggest multiple conjugative plasmids that are temperature sensitive with respect to transfer exist in the host cell and these plasmids may or may not contain tetracycline resistance determinants. Although temperature sensitive conjugative transfer has been documented in *Salm. typhi*, no difference in resistance phenotypes were noted in a previous study (Shanahan *et al.* 1998). Furthermore, temperature sensitive MDR in *Salmonella* and other enteric bacteria has been documented (Cohen *et al.* 1993; Kunonga *et al.* 2000), and could possibly account for our observations. However, we currently have no data to support this hypothesis.

Epidemiological investigations examining the clonal nature of disease outbreaks and for routine surveillance are commonplace in clinical settings. Such data allows public health officials the ability to determine if the causative organisms are part of the transient or resident populations. Many genetic fingerprinting techniques are available, each demonstrating variable degrees of utility. We investigated the clonal nature of the group B *Salmonella* isolated in this study using plasmid profiling and the PCR-based methodologies of ERIC- and REP-PCR. Plasmid profiling was the simplest procedure, but yet yielded the most genotypic groupings. Five distinct patterns were observed upon electrophoresis, and most importantly, this procedure grouped two strains that were sensitive to all of the antibiotics examined and one strain that was only resistant to ampicillin into two unique genotypic groupings (Table 2). In contrast, we observed little variation among the 31 isolates examined in this study using the PCR-based methodologies. Both REP and ERIC are composed of short extragenic repetitive sequences located throughout the chromosome and have been used to fingerprint many bacterial species (De Bruijn 1992; Georghiou *et al.* 1994; Rivera *et al.* 1995; Rodriguez-Barradas *et al.* 1995; Beyer *et al.* 1998). The lack of discrimination (Table 2) may be explained by only having two sampling sites in a relatively small geographic location.

In conclusion, we have demonstrated that clinical group B *Salmonella* isolated in Accra, Ghana, from patients suspected of having enteric fever are MDR to the 'first line' antibiotics routinely used in treating patients. The genetic basis for this resistance appears to reside on conjugative plasmids and the organisms as a whole appear to be derived from a similar clonal lineage. It is important that studies such as this are routine practice in clinical environments worldwide in order for the medical community to understand the molecular basis of MDR. Having such knowledge will aid in the development of antibiotic analogs that will be useful in the treatment of MDR organisms. Studies such as this, however, will be useless if the medical community does not alter its indiscriminate use of antibiotics.

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Molecular characterization of antibiotic resistance in clinical *Salmonella typhi* isolated in Ghana

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Abstract

Fifty-eight clinical *Salmonella typhi* strains isolated from patients suspected of suffering from typhoid fever were obtained at the Korle-Bu Teaching Hospital and the Noguchi Memorial Institute for Medical Research, both located in Ghana, Africa. Each isolate was examined for susceptibility to ampicillin, chloramphenicol, streptomycin, tetracycline, and trimethoprim/sulfamethoxazole by the disk diffusion assay. Five of the isolates were resistant to all five antibiotics while 10 isolates were resistant to ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole, which are considered 'first line' antibiotics in the treatment of typhoid fever. Thirty-four isolates were resistant to at least one of the antibiotics tested and 62% of these isolates possessed conjugable plasmids belonging to incompatibility group *Inc*HI. Ninety percent of the conjugable plasmids conferred a multiple drug-resistant phenotype on the strains harboring them. Additionally, 14 strains contained plasmids that were transformable and six of them encoded multiple drug resistance. Our findings indicate that multiple drug resistance to the 'first line' antibiotics in *S. typhi* may be more prevalent in Africa than previously thought.

Keywords: Multiple antibiotic resistance; *Inc*HI; Incompatibility group; *Salmonella typhi*

1. Introduction

Typhoid fever remains a serious health threat to developing countries. Globally, it is estimated there are over 20 million cases annually, resulting in greater than 700 000 deaths [1]. The incidence of this disease in the industrialized world is rare primarily due to proper sanitary facilities. The sporadic outbreaks that occur, however, are oftentimes due to transmission from an endemic region or country. In the majority of cases, infection with *Salmonella typhi*, the causative agent, is not lethal if effective antimicrobial therapy is administered in a timely fashion [2]. The antibiotic of choice for many years was chloramphenicol [3], but like many pathogens in today's world, chloramphenicol-resistant strains have emerged. Additionally, many strains have developed resistance to ampicillin and trimethoprim/sulfamethoxazole, which are considered appropriate alternatives to chloramphenicol [3]. Indeed, resistance to each of these 'first line' antibiotics is often plasmid encoded [4,5] and strains harboring a plasmid encoding resistance to all three antibiotics have been isolated in Southeast Asia [6]. In many cases, plasmids responsible for the resistance belong to the incompatibility complex group *Inc*HI [7]. Patients harboring these multidrug-resistant strains have been successfully treated with fluoroquinolones [8,9], but drug resistance to fluoroquinolones has emerged [10–12]. Although multiple drug resistance (MDR) in *S. typhi* is increasing worldwide, these organisms are documented minimally in Africa where some of the first reports of chloramphenicol-resistant *S. typhi* were isolated [5,13–15]. In lieu of these observations, we investigated the drug resistance associated with *S. typhi* isolated from patients suspected of suffering from typhoid fever in Ghana, Africa.

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2. Materials and methods

2.1. Bacterial isolation

Fecal, blood, urine, or cerebrospinal fluid samples were obtained from patients exhibiting the classical symptoms of typhoid fever (sudden fever, severe headache, loss of appetite, and nausea) at the Korle-Bu Teaching Hospital and the Noguchi Memorial Institute for Medical Research, both located in Accra, Ghana. Samples were processed and tested biochemically according to standard conditions for *Salmonella* isolation [16]. Confirmation of 58 *Salmonella enterica* serovar Typhi isolates was accomplished using agglutinating antisera (Denka Seiken Co., Japan).

2.2. Antimicrobial susceptibility testing

S. typhi were tested for susceptibility to various antibiotics using the disk diffusion method according to guidelines set by the National Committee for Clinical Laboratory Standards (NCCLS) [17–20]. Briefly, organisms were grown overnight at 37°C in 3 ml Mueller–Hinton (MH) broth. Ten microliters of the overnight culture were used to inoculate a fresh 3 ml MH broth followed by incubation at 37°C with shaking until a 0.5 McFarland turbidity standard was obtained. A sterile swab was dipped into this culture and used to inoculate the surface of a fresh MH agar plate. Antibiotic disks impregnated with ampicillin (10 μg), chloramphenicol (30 μg), streptomycin (300 μg), tetracycline (30 μg) and trimethoprim/sulfamethoxazole (1.25 μg/23.75 μg) were placed on the surface of the inoculated agar plate. After incubation at 37°C for 24 h, zones of inhibition around each antibiotic disk were measured. Using NCCLS guidelines, each organism was classified as either resistant or susceptible to the antibiotics. Specifically, organisms were considered resistant if the diameter of the zone of inhibition was equal to or less than 13 mm for ampicillin, 12 mm for chloramphenicol, 6 mm for streptomycin, 14 mm for tetracycline, and 10 mm for trimethoprim/sulfamethoxazole [18]. Antibiotic disks were purchased from Becton Dickinson Microbiology Systems (Cockeysville, MD, USA).

2.3. Transfer of antibiotic resistance via conjugation and transformation

Conjugation experiments were carried out using each *S. typhi* isolate as the donor and *Escherichia coli* HMS174 obtained from the *E. coli* Genetic Stock Center (Yale University, New Haven, CT, USA). Previous examination of *E. coli* HMS174 indicated it was sensitive to all antibiotics examined in this study. Briefly, the *S. typhi* isolates, which are sensitive to 50 μg ml^{-1} rifampicin and *E. coli* HMS174, which is resistant to 50 μg ml^{-1} rifampicin, were grown overnight at 37°C with shaking in tryptic soy broth (TSB). The overnight cultures were used to inoculate fresh TSB and incubated at 37°C with shaking until a 0.5 McFarland turbidity standard was obtained. Five hundred microliters of each *S. typhi* culture were mixed with 0.5 ml of *E. coli* HMS174 in a sterile tube and the suspension incubated at 37°C without shaking for 90 min. At 30-min intervals, the cultures were mixed by gentle inversion. Conjugation mixtures were plated on MacConkey agar plates containing 50 μg ml^{-1} rifampicin and either ampicillin (32 μg ml^{-1}), chloramphenicol (32 μg ml^{-1}), or trimethoprim/sulfamethoxazole (4 μg ml^{-1}; 16 μg ml^{-1}) and incubated overnight at 37°C. Lactose fermenting colonies growing on the plates indicated conjugational transfer of antibiotic resistance to *E. coli*.

Plasmid DNA was isolated from each *S. typhi* strain using a QIAprep spin miniprep kit according to the manufacturer's guidelines (Qiagen, Valencia, CA, USA). Transformation of *E. coli* DH5α was performed according to standard procedures [21]. Previous testing had determined that *E. coli* DH5α was sensitive to all antibiotics investigated in this study. Selection of transformants was made on TSA plates containing either trimethoprim/sulfamethoxazole (4 μg ml^{-1}; 16 μg ml^{-1}), ampicillin (32 μg ml^{-1}), or chloramphenicol (32 μg ml^{-1}).

2.4. Plasmid incompatibility testing

Plasmid DNA was isolated from each *S. typhi* strain, transformants and transconjugates to determine if they belonged to the incompatibility group *Inc*HI. The RepHI1A replicon, present in *Inc*HI plasmids, was amplified via the polymerase chain reaction using the primers 5′-GGTCCAACCCATTGCTTTAC-3′ and 5′-CACGGAAAGAAATCACAAC-3′ as previously reported [22] on a model PT150 MiniCycler (MJ Research, Watertown, MA, USA). Reaction conditions consisted of 50 ng plasmid DNA and 50 nM of each primer in a buffer composed of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM dNTP mixture, and 1 U of *Taq* polymerase in a final volume of 100 μl. Amplification conditions were 30 cycles of 94°C/30 s, 55°C/30 s, and 72°C/30 s, with a final extension step of 72°C for 10 min. Amplicons of 365 bp were considered positive for the RepHI1A replicon as previously reported [23].

3. Results

3.1. Antimicrobial susceptibility testing of S. typhi isolates

Fifty-eight confirmed *S. typhi* obtained from either blood ($n=50$), stool ($n=4$), urine ($n=2$), or cerebrospinal fluid ($n=2$) of infected patients were examined for their antibiotic susceptibility. The antibiotics chosen for testing were based on current treatment regimens for *S. typhi* infections [2,3] as well as NCCLS standards [17–20].

Table 1
Antibiotic resistance patterns for *S. typhi* clinical isolates and corresponding *E. coli* transconjugates and transformants

Strain #	Resistance pattern[a]		
	Clinical *S. typhi*	*E. coli* transconjugates	*E. coli* transformants
8, 13	AmCmTsTeSt	AmCmTsTeSt	AmCmTsTeSt
77	AmCmTsTeSt	AmTsTeSt	Am
89	AmCmTsTeSt	–	Am
47	AmCmTsTeSt	AmTsTeSt	–
53, 79, 115	AmCmTsSt	AmCmTsSt	–
17	AmCmTsSt	–	–
14	AmCmTsTe	–	–
2, 46, 81, 88	AmTsTeSt	AmTsTeSt	–
4, 6, 22	AmTsTeSt	AmTsTeSt	AmTsTeSt
27	AmTsTeSt	–	AmTsTeSt
56, 84, 92, 93	AmTsTeSt	AmTsTeSt	Am
24	AmTeSt	–	Am
96	AmTsTe	Am	–
30	AmTsTe	AmTsTe	–
28, 38, 40, 100	AmTsTe	–	–
7	AmTe	–	–
36	Am	Am	Am
80	Am	–	–
87, 107	Am	–	–
See below[b]		–	–

[a]Am, ampicillin; Cm, chloramphenicol; St, streptomycin; Te, tetracycline; Ts, trimethoprim/sulfamethoxazole
[b]Strain Nos. 3, 9, 11, 12, 15, 16, 18, 19, 20, 31, 35, 37, 41–45, 50, 75, 82, 85, 86, 91, and 103 were sensitive to all antibiotics tested.

Table 1 demonstrates that 30 of the 58 isolates (52%) exhibited MDR and 10 strains were resistant to all three 'first line' antibiotics. We defined MDR as resistance to any three or more antibiotics according to NCCLS guidelines.

3.2. Conjugational and transformable plasmid transfer of antibiotic resistance

To determine if the antibiotic resistance associated with the *S. typhi* isolates was plasmid encoded, we investigated the ability to transfer resistance phenotypes via conjugation and transformation to *E. coli*. In conjugation experiments, we mated each *S. typhi* strain with recipient *E. coli* and selected for transconjugates on MacConkey media containing either trimethoprim/sulfamethoxazole, ampicillin, or chloramphenicol. Ampicillin resistance was conferred on *E. coli* in all cases where conjugational transfer occurred (Table 1). The transconjugates were examined for resistance to the five antibiotics previously tested on the wild-type *S. typhi*. Out of 34 wild-type *S. typhi* exhibiting antibiotic resistance to at least one antibiotic, 21 of them (62%) possessed conjugable plasmids. Interestingly, 19 of the 21 (90%) conjugable plasmids conferred MDR.

We also questioned if antibiotic resistance associated with each *S. typhi* strain was located on a transformable plasmid. We transformed *E. coli* with a plasmid preparation obtained from each *S. typhi* strain and selected for transformants on media containing either ampicillin, chloramphenicol, or trimethoprim/sulfamethoxazole. Similar to the conjugation experiments, ampicillin resistance was associated with all successful transformations (Table 1). Examination of the 14 transformed *E. coli* strains indicated that six of them (43%) had a MDR phenotype.

Fig. 1. Representative agarose gel with 365-bp *Inc*HI amplicon. Lane M, molecular mass markers; lane 1, amplicon from *S. typhi* strain 8; lane 2, amplicon from corresponding *S. typhi* strain 8 transconjugate. All *S. typhi* strains possessing conjugable plasmids gave a similar amplification pattern.

3.3. Incompatibility testing

Purified plasmid preparations were used as template along with incompatibility group HI1A-specific primers in order to amplify a 365-bp region indicative of the RepHI1A replicon. Only plasmid preparations from *S. typhi* strains that were able to transfer antibiotic resistance genes via conjugation and their corresponding transconjugates resulted in positive amplicons (Fig. 1). Plasmid preparations derived from transformants were negative for the 365-bp amplicon (data not shown). These data provide ample evidence that the conjugable plasmids in the *S. typhi* isolates examined belong to incompatibility group *Inc*HI.

4. Discussion

Recent increases in antibiotic-resistant bacteria of all genera have prompted the scientific community to perform routine surveillance of microbial populations to determine the extent of the resistance. In this study, 58 clinical *S. typhi* isolates were examined for their susceptibility to various antibiotics used in the treatment of typhoid fever. We classified 30 of the 58 isolates as MDR based on their resistance to three out of the five antibiotics tested. Ten to 15 years ago typhoid fever was treated successfully with three inexpensive drugs (ampicillin, chloramphenicol, trimethoprim/sulfamethoxazole), but many *S. typhi* strains today are showing increasing resistance to these antibiotics [24,25]. Interestingly, strains resistant to these 'first line' antibiotics are not well documented in Africa [5,13–15]. This is surprising because Africa suffers from some of the same socioeconomic problems encountered in other areas of the developing world where antibiotic resistance seems to be on the increase. We isolated 10 organisms resistant to all three of these drugs, indicating resistance to these 'first line' antibiotics may be more problematic than once thought. Other antibiotics, such as expanded-spectrum cephalosporins and fluoroquinones have shown utility in treating *S. typhi* [26,27]. Unfortunately, the expense of these antimicrobials precludes their use in developing nations. All isolates in this study were sensitive to ceftriaxone (data not shown), which may show future application in treatment of typhoid fever if the cost becomes less prohibitive.

We also examined the genetic locus of the antibiotic resistance. Several studies have indicated that much of the resistance associated with *S. typhi* was plasmid mediated [23,28–31]. Specifically, plasmids belonging to the incompatibility group *Inc*HI are frequently the source of resistance to ampicillin, chloramphenicol, trimethoprim/sulfamethaxone, and tetracycline. These plasmids are quite large, ranging in size from 140 to 180 kb [23,29]. Our data correlate with these findings as all plasmids transferred via conjugation belonged to the *Inc*HI incompatibility group. No attempt was made to determine their size. Furthermore, most of the transconjugates in this study acquired streptomycin resistance in addition to the above mentioned antibiotics, thus adding this aminoglycoside to the list of common antibiotic resistance determinants associated with *Inc*HI plasmids.

As direct cell-to-cell contact appears to be a common mechanism of antibiotic resistance gene transfer, we also examined the possibility that these organisms could harbor transformable plasmids containing antibiotic resistance genes. Plasmids with ampicillin resistance were the most frequently transformed plasmids; however, a few isolates contained transformable plasmids with MDR genes. Indeed, two organisms, strains 8 and 13, isolated from urine and blood, respectively, each contained transformable plasmids containing resistance determinants to the five antibiotics examined. This is supported by *S. typhi* isolates from Pakistan, which were shown to contain self-transmissible 98 Mda plasmids encoding resistance to chloramphenicol, ampicillin, tetracycline, streptomycin, and sulfamethoxazole/trimethoprim [28].

Data obtained in this study suggest MDR *S. typhi* may be more prevalent in this locale within Africa than previously thought. These findings are also consistent with current global trends indicating MDR phenotypes are on the rise [25]. Societal misuse of antibiotics, in both the developed and developing world, is fueling the emergence of antibiotic-resistant organisms. For example, in developing countries, the misuse of antibiotics is compounded by financial constraints. Patients begin a treatment regimen, but in many cases once they are feeling better they discontinue their antibiotic use due to high cost in order to save the remaining tablets for future illness. Antibiotics effective 10 years ago in combating routine infections are now largely ineffective, and as a result, these infections are becoming increasingly fatal. Indeed, the World Health Organization (WHO) estimates that antibiotic-resistant bacteria are responsible for approximately 60% of nosocomial infections worldwide [32]. These, and other factors, have fueled the global spread of antibiotic resistance.

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