Epidemiological, genetic, serological and antimicrobial susceptibility characterization of non-typhi *Salmonella* isolates from hospitalized children in Nairobi, Kenya

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DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any other university.

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DEDICATION

This work is dedicated first and foremost to the glory of God; my parents, Stephen Mburu and Serah Njeri; my husband, Jackim Kuria; and my children, Michael Githinji, Joyce Wangari and Allan Mburu.

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ABSTRACT

Non-typhi *Salmonella* (NTS) are a major cause of septicaemia and bacteraemia in humans worldwide. About 1.4 million cases are observed in the United States of America annually, out of which 600 are fatal. In sub-Saharan Africa, NTS are among the most common causes of invasive bacterial childhood disease. Multi-drug resistant NTS species have been isolated in Kenya and pose a major public health concern. This study was conducted to investigate the NTS that cause bacteraemia in children admitted in hospitals in Nairobi, Kenya. The objectives of the study were to investigate the characteristics of the households of NTS cases of children admitted in three major Nairobi hospitals; to determine the antigenic and genetic characteristics of the NTS isolates from the admitted children; and to determine the susceptibilities of the isolates to commonly used antimicrobial agents in Kenya.

The NTS isolates were obtained from children hospitalized in three hospitals, in Nairobi Kenya namely, Kenyatta National Hospital, the Aga Khan Hospital and Gertrudes' Garden Children's Hospital between June and November 2002. The identity of the isolates was confirmed by biochemical tests using the Analytical Profile Index (API) 20E system (API System Motalieu Vercieu, France) and serotyping using specific agglutinating antisera (Murex Diagnostics, Dartford, UK). Homes of the cases were visited in order to administer a questionnaire designed to capture the characteristics of the households that could favour the transmission and maintenance of the NTS infections. Informed consent to visit the homes was always obtained from the guardians before the visits. In addition, drinking water samples, and stool samples from the household mates of the cases were collected for culture and isolation of NTS.

Antimicrobial susceptibility tests to commonly available antimicrobials were performed for 83 isolates on Isosensitest agar using the disk diffusin technique. The antimicrobial agents included ampicillin 10μg, tetracycline 30μg, trimethoprim 5μg, chloramphenicol 30μg, streptomycin 10μg, gentamicin 10μg, ceftazidime 30μg, ciprofloxacin 5μg, cefuroxime 30μg, amoxicillin-clavulanic acid 30μg, sulphamethoxazole 100μg and nalidixic acid 10μg. Minimum inhibitory concentrations (MICs) for the commonly used antimicrobial agents were also determined for 50 isolates using the agar dilution technique. Plasmid DNA profiling as a method of strain differentiation was carried out. Plasmids were extracted using a commercial kit, Plasmid Mini Prep-Spin Kit (Qiagen, West Sussex, UK).

The majority (77.4%) of the NTS cases were from households in slum areas with very poor sanitary conditions. Over 75% of the NTS isolates were from children aged below 5 years. Seventy-four isolates from the hospital index cases were confirmed as NTS. Nine NTS isolates were recovered from the stool samples from household mates of the index cases. *S. enteritidis* was the most common (37.8%) serotype among the NTS bacteraemia cases in hospitalized children followed by *S. typhimurium* (14.9%). *Salmonella* gallinarum-pullorum was isolated from one case (1.4%). The other 45.9% of the case isolates could not be serotyped conclusively due to lack of specific antisera. All the *S. enteritidis* and *S. typhimurium* isolates were resistant to one or more of the commonly used antimicrobials. A high prevalence of multi-drug resistance (MDR) among the 74 NTS from children was recorded. Only 2 (2.7%) of these were susceptible to all the 12 antimicrobials tested; twenty-nine (39.2%) were resistant to five or more of the antimicrobials; and 7 (9.5%) were resistant to eight or more antimicrobials tested. Plasmids of sizes ranging from 2 - 105 Kb were extracted from 16 of the total 83 isolates. Presence of

plasmids was associated ($p<0.05$) with resistance to five or more antimicrobials. Plasmid 105Kb was significantly associated ($p<0.05$) with resistance to streptomycin while plasmid 8Kb was significantly associated ($p<0.05$) with resistance to both streptomycin and trimethoprim. Non-typhi *Salmonella* were not isolated from the 31 drinking water samples collected but were isolated from 9 of 123 (7.3%) stool samples from household mates of the NTS cases. Two of the stool sample isolates were different from those from index cases by their serotype and also by their antimicrobial resistance patterns while the rest were similar in both characteristics

This study showed that NTS affects mostly children aged less than 5 years and that antimicrobial resistance in the NTS is very common. The study also found resistance to antimicrobial agents to be associated but not restricted, to presence of plasmids. Presence or absence of plasmids was not related to virulence. In addition, poor sanitary conditions appeared to favour the maintenance of NTS. Improvement of sanitary conditions may therefore reduce the number of infections. Better studies, preferably case-control study types, are required to confirm the association of the potential risk factors of NTS in this high-risk age group of children.

Antimicrobial abuse, overuse and misuse in both human and veterinary medical practices may be the cause of the antimicrobial resistance observed among the NTS. Enforcement of the law (Cap. 244. Pharmacy and Poisons act) that regulates the use of the antimicrobial agents is therefore highly recommended as a way of dealing with the problem of antimicrobial resistance.

104 strain, which has been responsible for epidemics, particularly in Europe, USA and Canada, has reservoirs in cattle, and is transmitted mainly through consumption of contaminated meat, milk and milk products (Glynn *et al.*, 1998; Fey *et al.*, 2000; Threlfall, 2000; Murphy *et al.*, 2001). Animal carriers are also an important source of infection (Wiktor and Van Oye, 1955; Plowright 1957; Stewart, 1957).

In the USA, about 95% of NTS infections are related to food-borne transmission including contaminated poultry and poultry products, meat, milk and other dairy products (Cody *et al.*, 1999; Mead *et al.*, 1999; Ahmed *et al.*, 2000; Threlfall, 2000). Between 1985 and 1991, 82% of *S. enteritidis* outbreaks in the USA that could be attributed to a specific food vehicle were associated with eggs (Mishu *et al.*, 1994). As the incidence of *S. enteritidis* infection in chicken has increased in that country, so has the incidence of *S. enteritidis* infection in humans (St. Louis *et al.*, 1988; Mishu *et al.*, 1994). In most industrialized countries and also some less industrialized countries, *S. enteritidis* is transmitted through consumption of foods containing raw or incompletely cooked eggs and home cooked products containing eggs (Ahmed *et al.*, 2000; Palmer *et al.*, 2000; Indar-Harrinauth *et al.*, 2001; Liebena *et al.*, 2001).

Laboratory studies have confirmed that tomatoes and other fresh fruits and vegetables can support the growth of *salmonella* (Asplund and Nurmi, 1991) and are therefore an important source of infection.

Animal feeds have long been recognized as an important source of new *Salmonella* infections in livestock farms (Muller, 1957; Shapcott, 1984). *Salmonella* serotypes not previously detected in flocks of a broiler company in Australia were detected first in raw feed ingredients, and later appeared in live birds and processed carcasses (Mackenzie and Bains,

1976). Cox *et al.* (1983) collected poultry feed from commercial mills in the USA and found *Salmonella* in 92% of meat and bone meal samples and 58% of finished feed (mash) samples, but none in pelleted feed.

A recent study in Nairobi, Kenya, conducted to investigate the likely sources of NTS in humans did not show animals as the reservoirs (Kariuki *et al.*, 2002). Multi-drug resistant NTS have been isolated in Kenya, where resistance to two or more antibiotics including ampicillin, co-trimoxazole, streptomycin, tetracycline and chloramphenicol has been reported (Kariuki *et al.*, 2000; Oundo *et al.*, 2000). Sources of NTS that cause bacteraemia in children in Kenya have not been investigated.

This study was carried out to investigate NTS occurring in children admitted in major hospitals in Nairobi, Kenya. The general objective of the study was to determine the risk factors for invasive *Salmonella* infections in children and to determine the antigenic and genetic characteristics of the NTS involved. The specific objectives were:

1.To investigate the characteristics of households of NTS cases of children admitted in three major Nairobi hospitals, that may be risk factors;

2. To determine the antigenic and genetic characteristics of isolates of NTS from cases of children admitted to hospitals in Nairobi, Kenya;

3.To determine the susceptibilities of the isolates from 2) to commonly used antimicrobial agents in Kenya.

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of Salmonellosis

2.1.1 Aetiology

Salmonella species are gram-negative bacteria that are rod-shaped, facultatively anaerobic, and non-spore formers. They are motile, although non-motile serotypes also occur. They belong to the family Enterobacteriaceae and are pathogens of both man and animals. *Salmonella* are mainly non-lactose fermenters, although lactose fermenters have also been isolated (Easterling *et al.*, 1969). The *Salmonella* bacteria are very stable and can remain viable in the environment for a long time. They have been reported to survive for 87 days in tap water, 115 days in pond water, 120 days in pasture and soil, 280 days in garden soil, over 30 months in dried bovine manure, 28 months in naturally infected avian faeces, and 47 days in manure slurry (Erskine and Margu, 1974). *Salmonella* possesses group specific somatic O-antigens that are used to classify them into specific sero-groups A to Z, and species-specific flagella H-antigens that are used classify them into specific serotypes (Kauffmann, 1954).

For convenience, *Salmonella* are grouped into typhi *Salmonella* (causing typhoid and paratyphoid fever in man) and non-typhi *Salmonella* (NTS) species. Among the NTS, *S. typhimurium* is more widely distributed than any other serotype and causes severe outbreaks of salmonellosis in all kinds of animals (Baumler *et al.*, 1998).

2.1.2 Classification and Nomenclature of *Salmonella*

Early bacteriologists named *Salmonella* according to their importance in disease causation, e.g., *Bacterium typhi* (the agent of typhoid fever in man), or according to their association with particular animal hosts, e.g., *Bacillus gallinarum* (the agent of fowl typhoid in hens).

This nomenclature was discarded when it emerged that *Salmonella* species were not host-specific and that others did not cause the diseases that first inspired their names, e.g., *Bacillus choleraesuis,* the supposed agent of hog cholera (Le Minor, 1984). The Kauffmann-White Scheme (Kauffmann, 1954), in addition to names that were given species status, used the possession of the somatic-O, the flagella-H, and the capsular-V antigens to distinguish between each antigenically distinguishable type of *salmonella*, e.g., *S. enteritidis* (1, 9, 12: g, m: –), *S. typhimurium* (1, 4, [5], 12:i: 1, 2) and S. *typhi* (9, 12: [vi]: d: –). By 1925, only 20 species of *Salmonella* were known (Kauffmann, 1954). However, in addition to the commonly occurring types of *Salmonella* recognized then, there occurred others of local or temporary importance and a larger, perhaps very large number, of rare types, which come to light from time to time. This led to the replacement of the descriptive naming of *Salmonella* by a practice whereby, newly identified serotypes were named after the place of their first isolation, e.g., *S. adelaide*, *S. banana*, *S. Entebbe,* and others (Le Minor, 1984; Le Minor and Popoff, 1988). This nomenclature emphasized geographical rather than diagnostic or taxonomical importance.

Application of newer methods to the taxonomy of *Salmonella* indicates that all serotypes of *Salmonella* and those of former genus *Arizona* belong to one species comprising seven subspecies that are distinguishable by biochemical tests (Crosa *et al.*, 1973; Le Minor *et al.*, 1982). Although by strict application of nomenclature the single species should be named *S. choleraesuis*, a historic name formerly used, unfortunately, to designate a serotype, the alternative species name *S. enterica* was proposed (Le Minor and Popoff, 1987). The suggested subspecies ranking and names are subspecies I (*enterica*), II (*salamae*), IIIa (*arizonae*), IIIb (*diarizonae*), IV (*houtenae*), V (*bongori*), VI (*indica*). The formal presentation of any named *Salmonella* serotype, e.g., *S. enterica* ssp. Enterica serotype

Typhimurium is cumbersome for everyday communication and should be restricted to specialist papers (Le Minor and Popoff, 1987). It is suggested that serotypes in subspecies I be named artificially as if they were species, e.g., *S. typhimurium*, or, more correctly, they could be noted as Agona, Choleraesuis, Enteritidis, etc (Farmer *et al*, 1984; Le Minor and Poppov, 1987; Le Minor and Popoff, 1988; Old, 1990). Thus, simple designations acceptable for routine reporting of serotypes would include *Salmonella* serotype (ser) *Typhimurium*, *Salmonella Typhimurium* or even *Typhimurium*. If these proposals were accepted, naming a serotype would indicate that it is a member of subspecies I.

The Judicial Commission of the International Committee on the Systematics of Prokaryotes (2005) has issued an Opinion (Opinion 80), which results in the interpretation that the genus *Salmonella* includes only two species (*Salmonella enterica* and *Salmonella bongori)* and that the type *Salmonella enterica* should be divided into six subspecies (*enterica*, *arizonae*, *diarizonae*, *houtennae*, *indica* and *salamae*). This is the nomenclature currently in use by WHO and other organizations and it is hoped that it will find widespread usage.

2.1.3 Occurrence

Salmonella infects a wide variety of hosts including wild animals, domestic animals and humans. *Salmonella* species are recognized as some of the most common pathogens causing enteritis in humans worldwide (Akalin, 1993). The NTS infections in humans are the primary cause of food-borne disease in developed countries, resulting in considerable morbidity and occasionally death, especially in immuno-compromised patients (Levine *et al.*, 1991; Angulo and Swerdlow, 1995). In the USA, it was estimated that over 95% of food-borne infections are due to NTS (Mead *et al.*, 1999) and that only 1 to 5% of infections with *Salmonella* are reported (Chalker and Blaser, 1988). The incidence of salmonellosis in the USA has steadily

increased since World War II (CDC, 1991). In Africa, *Salmonella* species account for most paediatric bacteraemias and NTS are seen predominantly in children less than 5 years of age (Graham *et al.*, 2000). In Kenya, NTS, including *S. typhimurium* and *S. enteritidis*, are the main causes of bacteraemia in children below the age of 3 years (Oundo *et al.*, 2000). Among the NTS, *S. typhimurium* is more widely distributed than any other serotype and causes severe outbreaks of salmonellosis in all kinds of animals (Baumler *et al.*, 1998).

2.1.4 Transmission of *Salmonella*

The main reservoir of *Salmonella* is the intestinal tract of man and animals and cycles of transmission occur between animals, man, and the environment through foods, feed, and insects (WHO, 1976). In the developed countries, most *Salmonella* infections have been traced to reservoirs in food animals such as chicken, cattle and pigs. In the USA, as the incidence of *S. enteritidis* infection in chicken has increased, so has the incidence of *S. enteritidis* infection in humans (St. Louis *et al.*, 1988; Mishu *et al.*, 1994). Contamination during processing of foods of animal origin is a major source of *Salmonella* infection to humans (Fone and Barker, 1994; Pegues and Miller, 1994; Threlfall *et al.*, 1994a; Wray *et al.*, 1998).

In the USA, about 95% of NTS infections are related to food-borne transmission including contaminated poultry and poultry products, meat, milk and other dairy products (Cody *et al.*, 1999; Mead *et al.*, 1999; Ahmed *et al.*, 2000). In most industrialized countries and also in some less industrialized countries, *S. enteritidis* is transmitted through consumption of foods containing raw or incompletely cooked eggs and home-cooked products containing eggs (Ahmed *et al.*, 2000; Palmer *et al.*, 2000; Indar-Harrinauth *et al.*, 2001; Liebena *et al.*, 2001). Between 1985 and 1991, 82% of *S. enteritidis* outbreaks in the USA that could be attributed

to a specific food vehicle were associated with eggs (Mishu *et al.*, 1994). Animal feeds have been recognized as an important source of new *Salmonella* infections in livestock farms (Muller, 1957; Shapcott, 1984). *Salmonella* serotypes not previously detected in flocks of a broiler company in Australia were detected initially in raw feed ingredients, and later appeared in live birds and processed carcasses (Mackenzie and Bains, 1976).

Horizontal transmission of *Salmonella* within and between animals does occur. Anderson *et al.* (1961) found an increase in *Salmonella* infection in calves corresponding to an increase in holding time in lairages. In poultry flocks, horizontal transmission can be mediated by direct bird-to-bird contact, ingestion of contaminated faeces and litter, contaminated water (Gordon and Tucker, 1965; Nakamura *et al.*, 1994), personnel and equipment (Zecha *et al.*, 1977). Contaminated animal and poultry house environments are often implicated as among the principal sources of *Salmonella* (Williams and Newell, 1968; Kumar *et al.*, 1971). As chicks emerge through eggshells, *Salmonella* are released into the air and circulated around hatching cabinets on contaminated fluff and other hatching debris. Bailey *et al.* (1994) reported that 17% of eggshell samples and 215 of chick rinse samples obtained from commercial broiler hatcheries in the USA were positive for *Salmonella*. Cox *et al.* (1990) isolated *Salmonella* from more than 75% of samples of egg fragments, belting material, and paper pads from three broiler hatcheries. Cason *et al.* (1994) observed that 44% of chicks from uncontaminated eggs, hatched along with eggs dipped, before incubation, in a solution containing *S. typhimurium*, were found to carry *S. typhimurium* in their intestinal tracts upon removal from the hatchery.

Vertical transmission of *Salmonella* to the progeny of infected breeder flocks can result from the production of eggs contaminated by *Salmonella* in the egg contents or on the surface

(Snoeyenbos *et al.*, 1969). Experimentally-infected hens were reported to transmit *S. menstoni* to their offspring (Gordon and Tucker, 1965). The same *Salmonella* serotypes responsible for mortality in naturally infected chicks and poults have often been isolated from their parent flocks (Morris *et al.*, 1969; Kumar *et al.*, 1971). Naturally contaminated eggs have generally been found to contain very small numbers of *S. enteritidis* (Humphrey *et al.*, 1989; 1991), but the *S. enteritidis* population in eggs can expand to more dangerous levels if eggs are held at growth supporting temperatures (Humphrey *et al.*, 1991; Gast and Beard, 1992). Eggs become contaminated with *S. enteritidis* through cracks in the shell after contact with chicken faeces or by trans-ovarian infection (Snoeyenbos *et al.*, 1969). Lahellec *et al.* (1986) concluded that the greatest contribution to the eventual distribution of *Salmonella* serotypes in broiler houses came from the chicks themselves and not from their environment.

Biological vectors including rodents (Baumler *et al.*, 2000), cockroaches (Kopanic *et al.*, 1994) and the lesser mealworm (McAllister *et al.*, 1994) can both disseminate and amplify *Salmonella.* Mice have been found to be particularly important vectors for *S. enteritidis* in laying flocks. Henzler and Opitz (1992) detected *S. enteritidis* in 24% of the mice from environmentally contaminated laying farms but none of the mice from farms with environments free of *S. enteritidis*. They noted that a single faecal pellet could contain as many as 10^5 *S. enteritidis* cells. Infection in animals is maintained by recycling slaughterhouse waste in animal feed (Chambers, 1977; Sogaard and Nielson, 1979; Cox *et al.*, 1990), faecal-oral spread (Gordon and Tucker, 1965; Lee *et al.*, 1972; Nakumara *et al.*, 1994), and fecal contamination of hatching eggs (Lahellec *et al.*, 1986).

In developing countries, there is scanty data on the likely sources of NTS that cause human infections, and reservoirs of NTS are not clearly understood. Studies conducted in Kenya by

Kayihura (1982) to investigate *Salmonella* reservoirs in animals as sources of human infection, estimated the carrier rates in various animal species at 7.3% in rodents, 1.9% in pigs and 1.1% in cattle. Recent studies done in Nairobi, Kenya, did not reveal any significant sources of *Salmonella*, either in food animals or in the environment (Kariuki *et al.*, 2002). Kariuki (2002) recommended further studies to investigate the possible human-to-human transmission. Transmission of NTS from human carriers and patients to humans may occur through contaminated foods. Laboratory studies have confirmed that tomatoes and other fresh fruits and vegetables can support the growth of *salmonella* (Asplund and Nurmi, 1991) and are therefore an important source of infection.

2.1.5 Pathogenesis

Salmonella typically enter the host via food or water-borne contaminants and therefore must survive the acidic environment of the stomach and the degradative action of bile salts in order to reach their primary site of colonization, the small intestine. *Salmonella* serotypes initiate infection by adhering to the intestinal mucosa of the host (Baumler *et al.*, 1998). Adherence does not require metabolically active S*almonella,* but subsequent bacterial invasion of host cells requires protein synthesis by live *Salmonella* (Macbeth and Lee, 1993). Upon penetration of the intestinal mucosa of mammals, further spread of *Salmonella* is stopped by an effective barrier of macrophages that line the lymphatic sinuses of regional lymph nodes. In mammals, this host defense mechanism can successfully limit bacterial expansion to the intestines, the gut associated lymphoid tissue, and the mesenteric lymph nodes (Cotter and Dirita, 2000).

Humans infected with NTS serotypes usually develop an acute gastroenteritis, but in only 1 to 7% of clinical cases do bacteria manage to pass through the mesenteric lymph nodes and

cause bacteraemia (Blaser and Feldman, 1981). The replication of *Salmonella* within the host cells has also been found to be necessary for the full expression of pathogenicity (Leung and Finlay, 1991). The factors that cause infections to remain localized in the intestine and mesenteric lymph nodes, such as the gastroenteritis caused by most *S. enterica* subspecies I serotypes in humans (Baumler *et al.*, 1998) are not known. *Salmonella enterica* subspecies I serotypes frequently colonize internal organs of warm-blooded animals and the ability to survive and multiply in cells of the reticulo-endothelial system correlates with their capability to cause systemic disease in these hosts (Fields *et al.*, 1986; Barrow *et al.*, 1994). The ability of *S. enterica* serotypes to cause systemic disease is directly related to the capability to withstand an assault by the macrophages of a given host. The human-adapted *S. typhi* is able to survive *in-vitro* in human macrophages but not in murine macrophages, whereas *S. typhimurium*, which causes a systemic disease in mice, survives well *in vitro* in murine macrophages but not in human macrophages (Vladoianu *et al.*, 1990). Thus, it appears that mononuclear phagocytes are an important barrier that restricts the host range of *Salmonella* serotypes (Baumler *et al.*, 1998).

The overall virulence of *Salmonella* depends heavily on the initial degree of mucosal invasiveness (Amin *et al.*, 1994). Strong host-adapted serotypes of *S. enterica* subspecies I tend to cause higher mortality rates than those with a broad host range (Baumler *et al.*, 1998). Pathogenicity differences in chicks have been noted within single *Salmonella* serotypes, sometimes even among strains of the same phage type (Smith and Tucker, 1980; Barrow *et al.*, 1987). The bacterial characteristics responsible for the observed pathogenicity differences between *Salmonella* strains are not completely understood. However, Barrow *et al.* (1988) concluded that flagella and somatic antigens, mannose-sensitive haemagglutinins, and the serotype-specific plasmid of *S. typhimurium* were essential for the intestinal colonization.

Petter (1993) associated invasive properties of *S. enteritidis* variants with quantitative and qualitative differences in Lipopolysaccharide (LPS) expression. Three general categories of toxins (endotoxin, enterotoxin and cytotoxin) have been reported to play roles in the pathogenicity of *Salmonella*. Endotoxin is associated with the lipid-A portion of *Salmonella* cell wall LPS, and if released into the blood stream, following bacterial cell wall lysis, can produce fever in addition to causing liver and spleen lesions (Turnbull and Snoeyenbos, 1974). The LPS has also been associated with the resistance of bacterial cell wall to attack and digestion by host phagocytes. Loss of the ability to synthesize LPS has been associated with loss of virulence for *S. enteritidis* in mice (Chart *et al.,* 1989) and an impaired ability to colonize the caeca and invade the spleen in broiler chicks (Craven, 1974). *Salmonella* enterotoxin activity induces a secretory response by epithelial cells that results in fluid accumulation in the intestinal lumen (Koupal and Deibel, 1975). A heat labile enterotoxin was detected in 44% of 123 *S. typhimurium* strains from animal sources (McDonough *et al.*, 1989). The heat stable cytotoxin of *Salmonella* causes structural damage to intestinal epithelial cells, perhaps by inhibiting protein synthesis (Koo *et al.*, 1984). Differences in virulence and host range displayed by the various *Salmonella* subspecies have been proposed by Baumler *et al.* (1997; 1998) to reflect the phases of *Salmonella* evolution. *S. typhi* exhibits host specificity for humans in whom it causes typhoid fever, while *S. typhimurium and S. enteritidis* are zoonotic (Miller *et al.*, 1995).

2.1.6 Clinical signs

Non-typhi *Salmonella* are capable of causing a variety of disease symptoms including enterocolitis, bacteraemia and focal infections (Darwin and Miller, 1999). Bacteraemia and focal infection usually follow or accompany enterocolitis (Darwin and Miller, 1999). Although there are over 2000 serotypes that have been associated with enterocolitis, only *S.*

enteritidis, S. typhimurium, and *S. heidelberg* account for the majority of infections (CDC, 1994; Tauxe, 1996). The incubation period ranges between 6-48 hours and is followed by headache, diarrhoea, abdominal pains and vomiting. Symptoms usually take about a week but *Salmonella* can be shed in faeces for up to 20 weeks in children under 5 years of age and for up to 8 weeks in adults (Gomez and Cleary, 1998).

2.2 Diagnosis of salmonellosis

Diagnosis of non-typhi salmonellosis is based on culture and isolation of the organisms using a variety of techniques which may include pre-enrichment, e.g., buffered peptone water to resuscitate sub-lethally damaged *Salmonella*; enrichment media, e.g., Selenite F broth and Rappaport-Vassiliadis that contain inhibitory substances to suppress non-*Salmonella* organisms; and selective plating agars, e.g., Brilliant green agar and deoxycholate-citrate agar to differentiate salmonellae from other *Enterobacteriaceae*. Various biochemical and serological tests are applied to the pure culture to provide a definitive confirmation of an isolated strain. Specific typing sera are used to determine the antigenic formulae of the serovars using the Kauffman-White Scheme. The number of *Salmonella* in faeces from asymptomatic animals, environment, animal feed and food is usually low and it is necessary to use pre-enrichment media (OIE, 2000).

2.3 Characterization of NTS using plasmid DNA profiling

The method of plasmid profile analysis (PPA) has been found useful by several investigators for strain differentiation (Hampton *et al.*, 1995; Threlfall *et al.*, 1994b; Threlfal *et al.*, 1996). The pulsed field gel electrophoresis (PFGE) of XbaI-digested DNA combined with PPA was found useful by Lindqvist *et al.* (1999) in discriminating between an endemic and feed-borne

S. infantis in cattle. The feed-related *S. infantis* was clonally related to the endemic infection by the ribotype, IS200-type and XbaI-profile but had a distinctive plasmid that appeared in pulsed-field gel electrophoresis as a 60kb band when cleaved with XbaI or linearized by SI-nuclease. Chiu *et al.* (2000) reported that all those serovars preferentially causing primary bacteraemia such as *S. enteritidis*, harboured a virulence plasmid. Plasmids are, however, regarded as fairly unstable genetic markers (Chrichton *et al.*, 1996). Olsen *et al.* (1994) reported that the same plasmid profile might be present in strains that are clearly different.

2.4 Control of non-typhi salmonellosis

In many countries, control of avian *Salmonella* is by strict enforcement of hygiene and management measures together with coordinated serological testing and slaughter of birds policy (Barrow, 1993). The management procedures include: obtaining chicks from sources free from fowl typhoid; avoiding mixing fowl typhoid-free stock with other chicken not known to be free of the disease; placing chicks in cleaned, sanitized and *Salmonella*-free environment; supplying feeds free from *Salmonella* contamination; and applying sound biosecurity systems (Shivaprasad, 1997). Steam-pelleting treatment of poultry feed under precisely defined conditions has been reported to eliminate both inoculated and naturally occurring *Salmonella* from chicken feed (Shackelford *et al.*, 1987; McCapes *et al.*, 1989). No salmonellae were isolated from pelleted feed by Cox *et al.* (1983) while *Salmonella* were isolated from 58% of finished feed (mash).

Most *Salmonella* strains appear to be susceptible to the lethal effects of irradiation (Thayer *et al.*, 1990). Combined heat and radiation treatments have been shown to be more effective in eliminating *Salmonella* than each used singly (Schaffer *et al.*, 1989; Thayer *et al.*, 1991). Acid treatment of feed has also been shown to reduce flock infection in chickens (Shapcot, 1984). The biosecurity system, according to Wray *et al.* (1996), has to ensure that the chicken

house is bird-proof, rodent-proof, and insect-proof. Drinking water should be potable and dead birds need to be properly disposed. In addition, precautions need to be taken with regard to mechanical carriers like footwear, human clothing, chicken equipment, processing trucks and poultry crates. Elimination of infected flocks must be combined with high flock management standards and hatchery discipline (Wray *et al.*, 1996). The control of transmission by mode of internally infected eggs is difficult because the egg-laying hens are usually asymptomatic (St. Louis *et al.*, 1988). Ozone and formaldehyde fumigation have been reported to be effective poultry hatchery disinfectants and can therefore be used to reduce the level of *Salmonella* on hatching eggs (Whistler and Sheldon, 1989). In addition to these measures, vaccination has been reported to be protective when properly done (Smith, 1956; Barrow, 1993; Wray *et al.*, 1996; Shivaprasad, 1997).

Other control measures of animal salmonellosis include: proper abattoir effluent disposal (Chambers, 1977; Sogaard and Nielson, 1979); good slaughter practices where holding time prior to slaughter is minimal (Anderson *et al.*, 1961); avoiding stress to animals as this may convert non-shedding carriers to contaminators of the environment (Kampelmacher *et al*, 1963; Williams and Newell, 1970).

Control of human salmonellosis is mainly by proper handling of foods of animal origin (Cody *et al.*, 1999; Ahmed *et al.*, 2000; Threlfall, 2000), by maintaining high standards of hygiene during food processing, strict segregation of cooked and uncooked foods, and proper heat treatment of foods (Glynn *et al.*, 1998; Fey *et al.*, 2000; Threlfall, 2000; Ahmed *et al.*, 2000; Palmer *et al.*, 2000; Indar-Harrinauth *et al.*, 2001; Murphy *et al.*, 2001; Liebena *et al.*, 2001). Undercooking contaminated foods is a major source of human infections.

Improvement of sanitary conditions, proper sewage disposal, proper washing of vegetables and fruits would also greatly control human infections. Proper washing of hands after every visit to the toilet as well as ensuring that sick persons are treated completely to ensure that they do not become carriers would reduce human- to- human transmissions through the fecal-oral route.

2.5 Treatment of non-typhi salmonellosis

In humans, management of *Salmonella* infections is usually supportive and consists of oral or parenteral rehydration, as required. Treatment of salmonellosis with antibiotics is not usually undertaken because it does not shorten the duration of diarrhoea, and also tends to induce the carrier-state (Barrow, 1993). Treatment with antibiotics is only indicated when there is accompanying septicaemia, fever lasting more than 48 hours, or when there is metastatic infection (Threlfall *et al.*, 1992). Oliesuk *et al.* (1973) found that five antimicrobial agents had only very limited value for preventing or eliminating experimental *S. typhimurium* infection in chickens. The administration of some antibiotics has been reported to increase the susceptibility of poultry to *Salmonella* infections, perhaps by suppressing the growth of other micro flora capable of exerting inhibitory activity against *Salmonella* (Manning *et al.*, 1992; 1994). Both therapeutic and sub-therapeutic antibiotic administration has been shown to select for drug-resistant strains of *Salmonella*, thereby potentially compromising the effectiveness of those drugs in both humans and animals (Kobland *et al.*, 1987; Gast and Stephens, 1988; Gast *et al*, 1988). Multi-drug resistant *Salmonella,* insensitive to the effects of several antimicrobial agents, have become increasingly prevalent among poultry isolates in both the United Kingdom and North America (D'Aoust *et al.*, 1992; Threlfall *et al.*, 1993). Multi-drug resistant *Salmonella* species have been isolated in some African children (Graham *et al.*, 2000) including Kenya, where resistance to two or more antibiotics including

ampicillin, co-trimoxazole, streptomycin, tetracycline and chloramphenicol, has been reported (Kariuki *et al.*, 2000; Oundo *et al.*, 2000). Antimicrobial use on farm premises leads to local amplification and perhaps persistence of resistant strains (Williams *et al.*, 1978). A worldwide consensus is emerging in support of reducing the total amount of antimicrobials used, eliminating inappropriate and non-essential antimicrobial uses in both human and animal medicine, and reserving specific antimicrobials for critical human applications (Angulo *et al.*, 1998).

2.6 Antimicrobial susceptibility tests

Antimicrobial susceptibility is defined as the capability of an organism to be acted upon by an antimicrobial agent (McAllister, 1979). Antimicrobial susceptibility tests help clinicians assess their choice of chemotherapy by providing them with a measure of the potential usefulness of various antimicrobial agents (McAllister, 1979). The concentration of the antimicrobial agent at the site of infection is a function of the site itself, the antimicrobial agent's ratio of diffusion from body fluids, and its inactivation or binding by body proteins (Balows, 1974). The absolute criteria for the evaluation of the antimicrobial agent for therapy should therefore be based on the clinical response of the patient in the course of treatment (Bailey and Scott, 1974). Dilution and diffusion are the two fundamental principles underlying techniques for the determination of susceptibility to antimicrobial agents (Bailey and Scott, 1974; Balows, 1974). Dilution techniques involve exposing a measured number of microorganisms to decreasing concentrations of the antimicrobial agent either in liquid or in solid media. After incubation at 37°C the lowest antimicrobial agent concentration that prevents visible growth is determined and is defined as the minimum inhibitory concentration (MIC). Organisms are regarded as susceptible to an agent if the attainable blood levels are three to five times as high as the *in vitro* MIC (Washington and Barry, 1974).

In diffusion techniques, the test organism is exposed to a concentration gradient of the antibiotic in an agar medium. An impregnated paper disc filled with the antimicrobial agent serves as the reservoir from which the antimicrobial agent diffuses out (McAllister, 1979). The diffusion technique is a dynamic process- the concentration gradient changes with the amount of antimicrobial per unit volume of agar dropping to lower levels further away from the reservoir, and the concentration peak near the disc becoming shallower (McAllister, 1979). The speed of diffusion is influenced by the amount of antimicrobial agent in the disc, the density of the agar gel, the agent's diffusibility in aqueous solution, the ionic strength and composition of the medium, the depth of the agar, the geometry of the disc placement and storage conditions of both agar and the antimicrobial agent (Bailey and Scott, 1974; Balows, 1974). When all variables are held constant for a given antimicrobial agent, the size of the inhibition zone is a function of the microorganisms' susceptibility to the antimicrobial agent (Balows, 1974). The Bauer-Kirby single high-potency disc diffusion method is universally recommended for susceptibility testing (Bailey and Scott, 1974; Balows, 1974). Obtaining results from this method has been speeded up by the introduction of zone-interpretive devices and by the reports that rapidly growing bacteria yield reliable zones in seven to eight hours of incubation at 37°C (McAllister, 1979). The Bauer-Kirby disc method is based on the fact that for a given antimicrobial agent, the size of the zone of inhibition is inversely related to the MIC (Balows, 1974). The size of the zone increases as the MIC decreases, demonstrating that zone size is a measure of MIC.

Pure cultures are obtained before the administration of the antimicrobial agent discs so that the true infecting organisms are isolated. An organism known to be sensitive to the antimicrobial agent(s) used is tested under the same conditions as the test organism in order

to obtain more standard and reliable results. The inhibition zones of the reference strain are measured to the nearest millimeter, and the results are compared to the expected results (Balows, 1974).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study area

The study was conducted in the city of Nairobi and its environs. Nairobi, with a human population estimated at 2,143,254 (Government of Kenya, 1999), is the capital city of Kenya and borders Kiambu District to the west, Thika District to the north, Kajiado to the south, and Machakos District to the east. More than half (50.24%) of the human population in Nairobi live below the poverty line and 56.7% do not have access to good sanitation (Ministry of Finance and planning, 2000a and 2000b). Kiambu and Thika districts are high potential areas whereas Kajiado and Machakos districts are semi-arid. Farming activities in Kiambu and Thika include smallholder dairy production as well as broiler and layer production. Much of the milk, eggs and chicken meat for Nairobi residents originate from Kiambu and Thika Districts.

3.2 Sampling for non-typhi *Salmonella*

For the purposes of this study, a NTS case was a child who had been hospitalized with signs of fever, aged less than 15 years and from whom NTS had been isolated from whole blood cultured on MacConkey, *Salmonella-Shigella* and Deoxycholate agars, and whose home was within Nairobi and its environs. The NTS isolates were submitted from cases who had been hospitalized in three major Nairobi hospitals namely, Kenyatta National Hospital, Gertrudes' Garden Children Hospital and the Aga Khan Hospital for the year 2002. The isolates had been preserved in Tryptic Soy broth with 15% glycerol at -70^0C. The history, patient data as well as contact address of the cases were also submitted.

The parents/guardians of the cases were contacted by phone in order to get an informed consent to participate in the study. Once the consent (Appendix 8.1) was obtained, details of the home location were obtained and a convenient date agreed on for the follow-up visit. During the visit, a questionnaire (Appendix 8.2) was administered via personal interviews in order to obtain the characteristics of the households. Information (Names, age, sex, occupation) on the household mates was also obtained and the consent form signed. Stool samples from all the household mates of the case as well as drinking water samples from the households. Stool samples of two index cases were also inadvertently collected during the household visits. Labelled and sterile stool and water bottles were used for the collection of the samples. A small amount of stool was collected using a spoon attached to the specimen bottle lid. Drinking water used in the households was collected directly into the sterile bottle. All specimens collected were transported to the laboratory in a cool box and processed within 24 hours.

3.3 Laboratory tests for non-typhi *Salmonella*

3.3.1 Serotyping

Isolates obtained from the cases were confirmed as *Salmonella* by biochemical tests using the Analytical Profile Index (API) 20E system (API System, Motalieu Vercieu, France) and serotyped using agglutinating antisera (Murex Diagnostics, Dartford, UK). The isolates were first tested with poly-O somatic *Salmonella* antisera. Only isolates showing agglutination with the poly-O somatic *Salmonella* antisera were tested with the specific somatic-O or flagellar antisera. Those NTS that showed agglutination with somatic-O antigen "9" and flagella-H phase-1 antigen "g", i.e. (O9: g: -), were classified as *S. enteritidis;* those agglutinating with somatic-O antigen "9" only i.e. (O9: -: -), as *S. pullorum-gallin*arum; and those that agglutinated with somatic-O antigen "4", flagella-H phase-1 antigen "i" and phase-

2 antigens "1" and "2" i.e. (O4: i: 1,2), were classified as *S. typhimurium*. A polyphase agglutinating antisera was also used and isolates were classified as positive when they showed agglutination with it. All NTS isolates that showed agglutination with the poly-O somatic *Salmonella* antisera but could not be identified using the available antisera were classified only as *Salmonella* species.

3.3.2 Culture and isolation

Culture and isolation of NTS from the stools of the cases' household mates and water samples was by standard laboratory methods requiring pre-enrichment, selective enrichment and indicative plating and serotyping (ISO, 1993). The stools were selectively enriched in selenite-F broth followed by selective plating onto xylose lysine deoxycholate (XLD), MacConkey, and Salmonella-Shigella (SS) agars (ISO, 1993). A small amount of stool was placed in a universal bottle containing about 10 ml of the Selenite-F broth and incubated at 37°C overnight. The following morning, a loopful of the Selenite-F broth culture was sub-cultured onto the selective Xylose Lysine Deoxycholate (XLD), MacConkey, and *Salmonella-Shigella* (SS) agar plates. The plates were incubated at 37°C overnight. The next day, the culture plates were examined for the presence of non-lactose fermenters. Non-lactose fermenters appear whitish or colourless on both MacConkey, and *Salmonella-Shigella* (SS) agars while in XLD they are of pinkish colour. A single colony of the non-lactose fermenting colonies was again sub-cultured onto MacConkey agar and incubated at 37°C overnight to obtain a pure culture, after which, a bacterial suspension of about 0.5 Mcfarland Standard turbidity was made with 10 ml sterile water. This bacterial suspension was used to inoculate an API 20E System strip described below. The strip was incubated for 18-24 hours during which bacterial metabolism occurred causing colour changes that were read immediately after incubation or after addition of specific reagents.

One milliliter of the water from the households was placed in sterile bottles containing 10mls of peptone water and incubated at 37°C for 18-24 hours. A loopful of the contents of the peptone water cultures were plated onto the selective agar plates and the process continued as for the stool specimen.

Non-lactose fermenters were identified as *Salmonella* species by biochemical tests using the API 20E system and by serotyping with specific agglutinating anti-sera as previously described.

3.3.2.1 API 20E system

The API 20 E system is a commercial test system used for the identification of Enterobacteriaceae and other gram-negative rods. The API 20E System strip consists of 20 microtubes containing dehydrated biochemical reagents (API Motalieu, Vercieu, France)(Appendix 8.3). The strip and suspension medium were removed from the refrigerator and allowed to equilibrate to room temperature on the bench. The incubation chamber was moistened with about 5 ml of tap water using a dispenser bottle. The laboratory reference number of the test sample was then recorded on the elongated tab of the tray. The strip was then removed from its packaging and placed in the chamber. The microtubes for citrate utilization, acetoin production and presence of the enzyme gelatinase tests, and marked CIT, VP, GEL respectively on the API test strip, were filled completely with the bacterial suspension using a pasteur pipette. The tubes of the other tests were half-filled with the bacterial suspension. The arginine dehydrolase, lysine decarboxylase, ornithine decarboxylase hydrogen sulphide and urease production tests, marked ADH, LDC, ODC, H_2S and URE on the API 20 E strip tests were overlaid with mineral oil to achieve anaerobiasis. The chamber plastic cover was placed and the strip incubated at 35 - 37°C for

18-24 hours. The next day the strip was removed and read by reference to the interpretation table. All spontaneous reactions were recorded on the report sheet. One drop of tryptophane deaminase reagent was added to tryptophane deaminase (TDA) test. A dark brown colour indicated a positive reaction. One drop of James reagent was added to indole production (IND) test. An immediate pink colour development in the whole cupule indicated a positive reaction. One drop each of sodium pyruvate reagents 1 and 2, VP1 and VP 2 were added to acetoin production (VP) test cupule and left for ten minutes. A bright pink/red colour indicated a positive reaction. On the report sheet, the tests were separated into groups of 3 and a number 1, 2, or 4 was indicated for each test. By adding the numbers corresponding to positive reactions within each group, a 7-digit profile number was obtained for the 20 tests of the API 20 E strip. The reactions were read according to the interpretation table (Apendix 8.3) and the patterns of reactions obtained were then coded into a numerical profile as indicated in the instructions manual. The, 7-digit profile number was then entered into the APILAB software identification system in the computer under API 20 E programme, and the results read.

3.3.3 Plasmid DNA analysis

Plasmid DNA from the NTS isolates was extracted using the commercial kit, Plasmid Mini Prep-Spin (Qiagen, West Sussex, UK) and according to the manufacturers' instructions. Briefly, the NTS isolates were first sub-cultured onto MacConkey agar, which was incubated at 37°C for 18 hours. A single colony from the fresh cultures was then sub-cultured into 5ml Luria-Bertani broth. The broth cultures were incubated at 37°C in a shaking incubator for 12-16 hours. One millilitre of the broth cultures was transferred into 1.5ml eppendorf tubes, capped and centrifuged at 13000rpm for 3 minutes at room temperature. The supernatant was discarded and more broth added and centrifuged, a little at a time, until all the 5ml was

centrifuged and a pellet obtained at the bottom of the tube. The pellet obtained was used in the DNA plasmid extraction. Briefly, the pellet obtained was resuspended in 250µl of resuspension buffer to which RNase had been added and gently mixed to ensure no visible clumps. Two hundred and fifty microlitres of cell lysis buffer was added and mixed gently by inverting the tube 4-6 times. Then 350µl of neutralizing buffer was added within 5 minutes of adding the cell lysis buffer. The mixture was centrifuged at 13000rpm for 5 minutes. The supernatant was carefully decanted into Qiagen spin columns and centrifuged for 5 minutes. The columns were then placed onto clean eppendorf tubes and 50µl of elution buffer placed in the middle of the spin columns, allowed to stand for one minute and then centrifuged at 13000 rpm at room temperature to elute the plasmids into the clean tubes. The spin columns were discarded. Twenty microlitres of each of the extracted plasmids were then loaded with 10µl of loading buffer incorporating a tracking dye (sucrose 25%, 8mM sodium acetate, 3.5 mM SDS, 0.7 mM bromophenol blue) and electrophoresed at 100V for 2 hours on horizontal 1% agarose gels stained with 0.05% ethidium bromide (Sigma, Poole, UK).

Plasmid bands were visualized using a UV transilluminator (UVP Inc., CA, USA) and photographed using a Polaroid MP-3 camera (Polaroid, MA, USA). The molecular weights of the extracted plasmids were determined by comparison with plasmids of known molecular weights (147, 63, 43.5, 6.9 kb) of *E. coli* 39R861 (NCTC 50192). Plasmid DNA from the NTS were recorded and analyzed based on their molecular weights.

3.3.4 Antimicrobial susceptibility tests

Antimicrobial susceptibility tests of the NTS isolates to commonly used antimicrobials were done on Isosensitest (Oxoid, Basingstoke, UK) agar by the disk diffusion technique (Bauer *et al.*, 1966), with a few modifications. The 12 antimicrobial agents used were ampicillin 10µg,

tetracycline 30μg, trimethoprim 5μg, chloramphenicol 30μg, streptomycin 10μg, gentamicin 10μg, ceftazidime 30μg, ciprofloxacin 5μg, tetracycline 30μg, amoxicillin-clavulanic acid 30μg, sulphamethoxazole 100μg and nalidixic acid 10μg. Fresh colonies of the isolates were suspended in sterile distilled water to conform to 0.5 MacFarland turbidity. A sterile cotton swab was then dipped into the bacterial suspension, squeezed on the side of the bottle to remove excess broth and the bacteria streaked uniformly onto the Isosensitest agar plate. The antimicrobial disks were then applied onto the plates. *Escherichia coli* ATCC 25922 of known zones of inhibition was always used as a control. The plates were incubated at 37°C for 18 hours. The diameters of the zones of growth inhibition of the control plate were measured in millimeters using a zone reader and recorded in millimeters. The recorded readings of the control plate were compared with those given in the National Committee for Clinical Laboratory and Standard (NCCLS) Guidelines 2002 to ensure that they were within the expected ranges; otherwise, the test was repeated. The test plate zones of growth inhibition were then read and recorded. The results were interpreted as sensitive, intermediate or resistant, according to the NCCLS 2002 Guidelines (Appendix 8.4).

Minimum inhibitory concentrations (MIC) for the commonly used antimicrobials were also determined by agar dilution technique. Antimicrobial tablets (adatabs) of known antimicrobial contents (Adatabs, Mast Laboratories, Liverpool, UK) were used to make various dilutions in Isosensitest agar (Oxoid). The adatabs were first diluted in sterile-distilled water to make double dilutions of the antimicrobial agents before making the final appropriate dilutions in the agar. The dilutions made were determined by both the initial concentration of the adatabs and the MIC of the antimicrobial agent for *E. coli* ATCC 25922, and were made in such a manner as to ensure that the latter was included in the range of the dilutions made. Two milliliters of each of the antimicrobial agent dilution were pipetted into

sterile labelled (antimicrobial agent and the dilution) plates and 18ml of Isonsesitest agar (previously autoclaved and cooled to about 50°C) added and mixed by gently swirling the plates on the bench. The plates were covered and the mixture allowed to set at room temperature before inoculating the bacterial isolates.

The bacterial suspension broth cultures were delivered using a 32-multipoint inoculator. A single colony of the NTS isolates grown on MacConkey agar was sub-cultered in 5ml Luria-Bertani broth and incubated overnight at 37°C. One milliliter of the broth cultures was put in the wells of the multipoint inoculator except the corner wells, which were omitted since they fall outside the plate circumference during inoculation. Distilled water and *E. coli* ATCC 25922 of known MIC, were included in each plate as negative and positive controls, respectively. The plates were marked to ensure easy identification of the isolates and controls on the plate.

The bacteria, including the distilled water and *E. coli* ATCC 25922 controls, were delivered onto the antimicrobial agent-agar plates. A table was drawn and the identities of the isolates and the controls entered to correspond with their positions in the multipoint inoculator wells. The inoculated plates were incubated at 37°C overnight. The plates were then examined to ensure that there was no growth in the positions corresponding to the areas where distilled water was inoculated and that the concentration of antibiotic that resulted in complete inhibition or growth of five or fewer discrete colonies of the *E. coli* ATCC 25922 was within the expected ranges in accordance with the NCCLS Guidelines (2002); otherwise, the test was repeated. The MIC was taken as the concentration of antibiotic that resulted in either complete growth inhibition or growth of five or fewer discrete colonies. The isolates were

classified as either susceptible or resistant to the antimicrobial agents, in accordance with the NCCLS Guidelines 2002 (Appendix 8.4).

3.4 Data handling and analysis

All data were entered and managed in Microsoft Excel and transported to Statistix (Statistix Version 4.0 Analytical Software, Tallahassee, Florida, USA) package for analysis. Descriptive statistics were generated and the odds ratio (OR) was used in the tests of association between drug resistance and the presence of plasmids.

CHAPTER 4

RESULTS

4.1 Characteristics of households of non-typhi *Salmonella* cases

On the basis of the responses obtained through the administration of the questionnaire (Appendix 8.2), the households of the NTS cases were classified into 4 broad categories, namely: those with and without domestic animals and animal feeds; those who ate or did not eat animal products; those who boiled or did not boil drinking water; and those where sanitation was good or poor. Poor sanitation was defined as the absence of running water in the household and/or presence of communal toilets. The households in the latter category were mainly those from slums areas. The household categories were not mutually exclusive and a household could belong to one or more categories.

A total of 31 households were visited and the questionnaire administered. Animal products were eaten in all the households visited and drinking water was reportedly boiled in all the households. Of the 31 households visited, 24 (77.4%) were in areas that could be categorized as slums. Sanitary conditions in these areas were poor with overcrowding; sometimes free-flowing sewage could be seen; and tap water was mainly lacking. Animals and animal feeds were found in only 2 (6.5%) of the households visited. A total of 40 NTS isolates were obtained from the household members; 31 from the hospitalized cases and 9 from household mates. Of the 40 NTS isolates, 33 (82.5%) were from slum areas; seven were from non-slum areas; and 4 were from households with animals and animal feeds. In 16 of the 31 households visited, the NTS cases had travelled less than three months prior to onset of illness. Animal products, mainly milk, eggs and beef, were eaten in all the households visited.

4.2 Serotypes of non-typhi *Salmonella* Isolates

A total of 74 NTS isolates (Table 4.1) were obtained from hospitalized children. The age distribution of 70 of the 74 NTS cases was: 53 (75.7%) were below 5 years of age; 14 (20%) were above 5 but less than 10 years of age; and 3 (4.3%) were above 10 years but less than 15 years old (Fig. 4.1). Of the 74 isolates, 28 (37.8%) were classified as *Salmonella enteritidis*, 11 (14.9%) as *Salmonella typhimurium* and 1(1.4%) as *Salmonella gallinarum-pullorum* (Table 4.1). The remaining 34 isolates could not be identified further because they did not agglutinate with the available typing antisera and were, thus, only classified as *Salmonella* species. A total of 123 stool samples were obtained from the household mates of 31 hospitalized children who could be followed-up to their homes. Out of the 123 stools samples, *Salmonella* was confirmed in only 9 (7.3%) of the samples. The *Salmonella* isolates were classified as *S. enteritidis* (2), *S. typhimurium* (1) and *Salmonella* species (6). Of the 6 NTS isolates from household mates classified as *Salmonella* species, 5 had the somatic antigens "O67" and the other one "O4". The household mates from whom the 9 NTS were isolated did not have any signs of disease. Seven of the 9 household mates from whom NTS were isolated were adults aged over 20 years and the remaining two were children aged 3 and 2 years. Non-typhi *Salmonella* were not isolated from any of the 31 water samples collected.

Six of the cases, all below one year of age, were fatal although they had been treated. *Salmonella* was still detectable in two of the cases that had already completed their treatment, but the clinical signs of the disease had subsided.

Table 4.1: Antigen agglutinating patterns of non-typhi *Salmonella* serotypes isolated from children hospitalized in Nairobi, Kenya, 2002.

Agglutinating antigens				Non-Typhi *Salmonella* serotype	Number of isolates
"O"	"H1"	"H2"	Polyphase "H1" and"H2"		
9	g,m	*	Negative	*S. enteritidis*	28
9	*	*	Negative	*S. pullorum- gallinarum*	1
4	i	1,2	Positive	*S. typhimurium*	11
4	i	*	Positive	*Salmonella* species	5
4	*	*	Positive	*Salmonella* species	9
4	*	*	Negative	*Salmonella* species	6
4	e	*	Positive	*Salmonella* species	1
4	r	*	Positive	*Salmonella* species	1
6,7	*	*	Positive	*Salmonella* species	10
6,8	*	*	Positive	*Salmonella* species	2
Total					74

* Could not be typed further with the available antisera

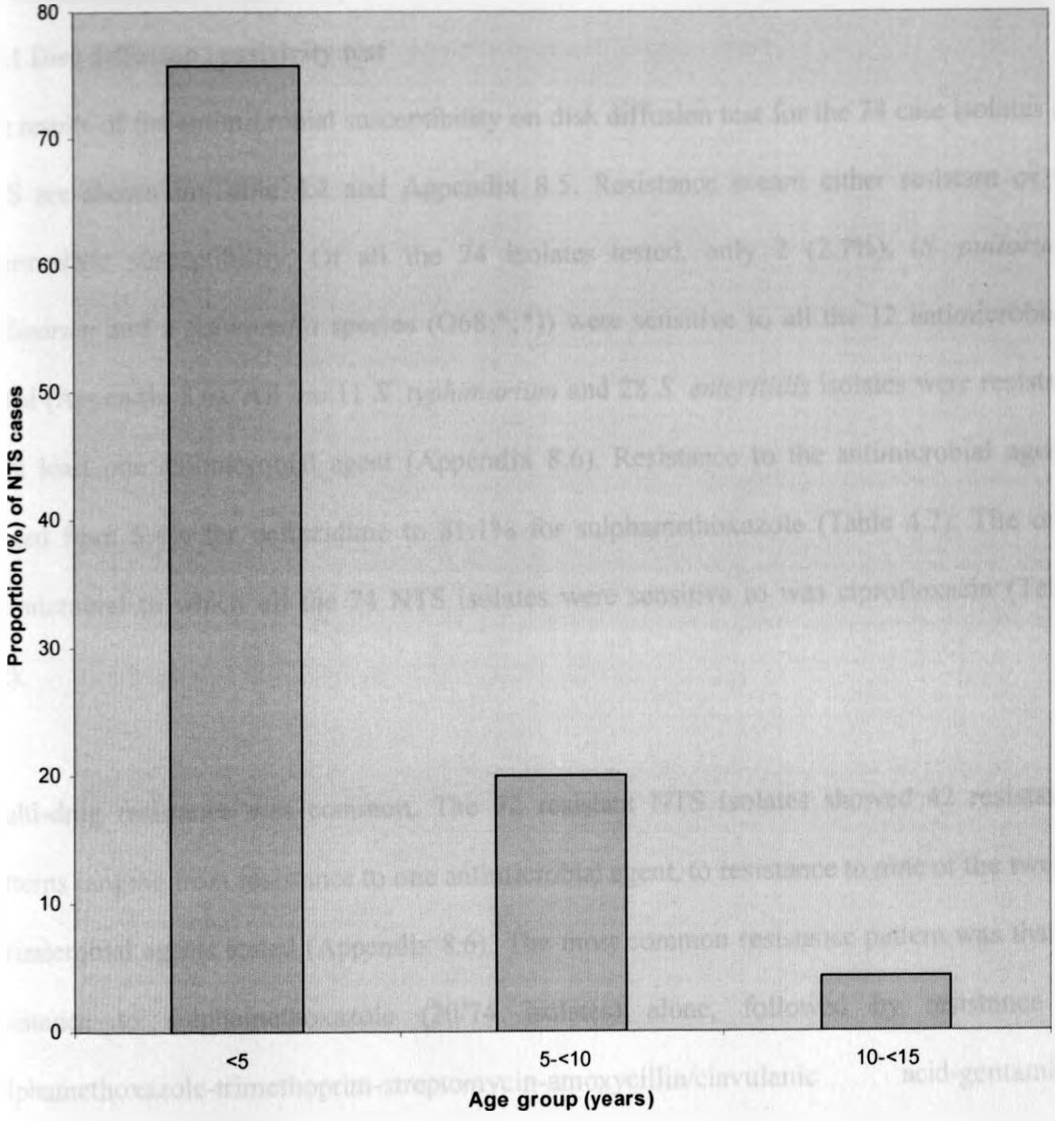

Figure 4.1: Age distribution of 70 non-typhi *Salmonella* cases hospitalized in Nairobi hospitals, Kenya, 2002.

4.3 Antimicrobial susceptibility of non-typhi *Salmonella* isolates

4.3.1 Disc diffusion sensitivity test

The results of the antimicrobial susceptibility on disk diffusion test for the 74 case isolates of NTS are shown in Table 4.2 and Appendix 8.5. Resistance meant either resistant or of intermediate susceptibility. Of all the 74 isolates tested, only 2 (2.7%), (*S. pullorum-gallinarum* and a *Salmonella* species (O68;*;*)) were sensitive to all the 12 antimicrobials tested (Appendix 8.6). All the 11 *S. typhimurium* and 28 *S. enteritidis* isolates were resistant to at least one antimicrobial agent (Appendix 8.6). Resistance to the antimicrobial agents ranged from 5.4% for ceftazidime to 81.1% for sulphamethoxazole (Table 4.2). The only antimicrobial to which all the 74 NTS isolates were sensitive to was ciprofloxacin (Table 4.2).

Multi-drug resistance was common. The 72 resistant NTS isolates showed 42 resistance patterns ranging from resistance to one antimicrobial agent, to resistance to nine of the twelve antimicrobial agents tested (Appendix 8.6). The most common resistance pattern was that of resistance to sulphamethoxazole (20/74 isolates) alone, followed by resistance to sulphamethoxazole-trimethoprim-streptomycin-amoxycillin/clavulanic acid-gentamicin combination (5/74 isolates).

Table 4.2: Antimicrobial disc susceptibility test results of 74 non-typhi *Salmonella* isolates from children admitted in Nairobi hospitals, Kenya, 2002.

Antimicrobial agent	Susceptibility			% Resistant
	Sensitive	Intermediate	Resistant	
Sulphamethoxazole	14	10	50	81.1
Trimethoprim	43	1	30	41.9
Streptomycin	38	8	28	48.6
Ampicillin	47	1	26	36.5
Amoxycillin-clavulanic acid	47	8	19	36.5
Gentamicin	55	3	16	25.7
Tetracycline	51	9	14	31.1
Chloramphenicol	59	3	12	20.3
Nalidixic acid	67	3	4	9.5
Cefuroxime	63	7	4	14.9
Ceftazidime	70	1	3	5.4
Ciprofloxacin	74	0	0	0.0

4.3.2 Minimum inhibitory concentration tests

The MIC tests were done on 50 of the 74 NTS case isolates. There were not enough antimicrobial tablets to test all the 74 isolates. All the 50 NTS isolates tested were sensitive to ceftazidime (Table 4.3). The resistance ranged from a low of 2% to a high of 60%. For similar antimicrobials, the results of the disc sensitivity tests and those of minimum inhibitory concentration tests for the 50 isolates were not significantly different, especially when isolates were categorized into either, fully sensitive or resistant.

Table 4.3: The minimum inhibitory test results of 50 non-typhi *Salmonella* isolates from children admitted in hospitals in Nairobi, Kenya, 2002.

Antimicrobial agent	µg/ml (Number of isolates sensitive)	% Isolates Resistant
Cotrimoxazole	2(12) 4(19) 16(2) >128(17)	34
Ampicillin	1(1) 2 (26) 4(6) 8(1) 32(1) 128(1) >128(14)	32
Co-amoxiclav	0.5(3) 1(31) 2(1) 8(1) 16(3) 32(11)	28
Gentamicin	0.25(10) 0.5(7) 1(1) 2(4) 4(17) 8(2) 16(2) >32(7)	22
Cefuroxime	8(20) 16(21) 32(6) 64(2) >128(1)	60
Tetracycline	1(1) 2(7) 4(35) 8(1) 32(3) >64(3)	14
Nalidixic acid	2(5) 4(20) 8(19) 16(4) >64(2)	12
Ciprofloxacin	0.01325(46) 0.25(1) 1(2) 4(1)	2
Cefotaxime	0.25(29) 0.5(9) 1(3) 2(1) 4(7) 16(1)	2
Ceftazidime	0.5(24) 1(25) 4(1)	0

4.3.3 Comparison of antimicrobial susceptibility of hospital-based non-typhi *Salmonella* isolates with those isolated from household mates

A total of 9 NTS were isolated from family members of 5 hospital cases (Table 4.4). Two of the isolates (a *Salmonella* species and a *S. enteritidis*) were of different serotypes and also exhibited different antimicrobial resistance patterns from the isolates of the hospital cases. The remaining 7 isolates were of the same serotype as the hospital cases and also showed fairly similar antimicrobial susceptibility patterns (Table 4.4). The isolates are grouped in households.

Table 4.4: Comparison of antimicrobial resistance patterns of non-typhi *Salmonella* isolates on disc diffusion method by source of isolates, in Nairobi, Kenya, 2002.

Source of NTS isolate (Hospital/ Household)	Antimicrobial susceptibility											
	CXM	RL	TMP	CHL	STR	NAL	CIP	AMP	AMC	CAZ	CN	TCY
S. enteritidis / *S. enteritidis*	S/S	R/R	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S
Salmonella species / *Salmonella* species	S/S	R/R	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/I
Salmonella species / *Salmonella* species	S/S	R/R	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/S	S/I
Salmonella species / *Salmonella* species	S/S	R/R	S/S	S/S	S/S	I/S	S/S	S/S	S/S	S/S	S/S	S/S
S. enteritidis / *S. enteritidis*	S/S	I/I	S/S	S/S	R/I	I/S	S/S	S/S	S/S	S/S	I/S	I/S
S. enteritidis / *S. enteritidis*	S/R	I/S	S/S	S/S	R/I	I/S	S/S	S/S	S/S	S/S	I/S	I/I
S. enteritidis / *S. enteritidis*	S/S	R/R	R/R	R/R	R/I	S/S	S/S	R/R	R/R	S/S	S/S	R/R
S. enteritidis / *S. typhimurium*	S/S	R/S	R/S	R/S	R/S	S/S	S/S	R/S	R/S	S/S	S/S	R/I
S. enteritidis / *Salmonella* species	I/S	R/S	R/S	R/R	R/S	S/I	S/S	R/S	I/S	S/S	R/S	R/I

Key: CXM - Cefuroxime
RL- Sulphamethoxazole
TMP- Trimethoprim
CHL- Chloramphenicol
STR- Streptomycin
NAL- Nalidixic acid
CIP- Ciprofloxacin
AMP- Ampicillin
AMC- Amoxycillin-clavulanic acid
CAZ- Ceftazidime
CN- Gentamicin
TCY- Tetracycline

4.4 Plasmid profiles of non-typhi *Salmonella* isolates

Extraction of plasmids was attempted on all the 74 isolates from the hospitalized children and all the 9 isolates from household members. Out of the 83 NTS isolates only 16 (19.3%) yielded plasmids (Plate 4.1) ranging in size from approximately 2Kb to 105Kb: 5 *S. typhimurium*; 2 *S. enteritidis*; 9 *Salmonella* species. Most of the plasmids (13/16 (81.3%)) were in isolates that had the "O4" somatic antigen. Five out of 11 (45.5%) *S. typhimurium* and two out of 28 (7.1%) *S. enteritidis* isolates had plasmids. The various plasmid sizes as well as profiles were not serotype-specific. The plasmid DNA profiles of the 16 isolates are shown in (Table 4.5). Table 4.6 shows the frequency of the various plasmid sizes. Thirteen of the isolates had at least 2 plasmids. Nine different plasmid sizes were identified ranging in size from approximately 2Kb to 105Kb (Table 4.6). Eight of the nine different plasmid sizes were less than 20Kb and one plasmid size was 105Kb. The 8Kb and 105Kb plasmids were more common and each was found in 12 and 11 isolates respectively (Table. 4.6). Plasmids of sizes 5, 8, 9, and 105Kb, usually occurred together.

All the NTS that had plasmids were multi-drug resistant and specific plasmids were significantly associated with resistance to a specific antimicrobial agent. The plasmid size 105Kb was significantly ($p<0.05$) associated with resistance to streptomycin. Isolates with the plasmid 105Kb were 5.8 (odds ratio) times more likely to be resistant to streptomycin. The plasmid size 8Kb was significantly associated ($p<0.05$) with resistance to both trimethoprim and streptomycin. Overall, 16/16 (100%) of the NTS with plasmids were multi-drug resistant to the antimicrobials tested and 65/67 (97%) of the NTS without plasmids were resistant to at least one of the antimicrobial agents; the proportions of NTS that were MDR to statistically different indicating that the presence of plasmids alone did not confer resistance.

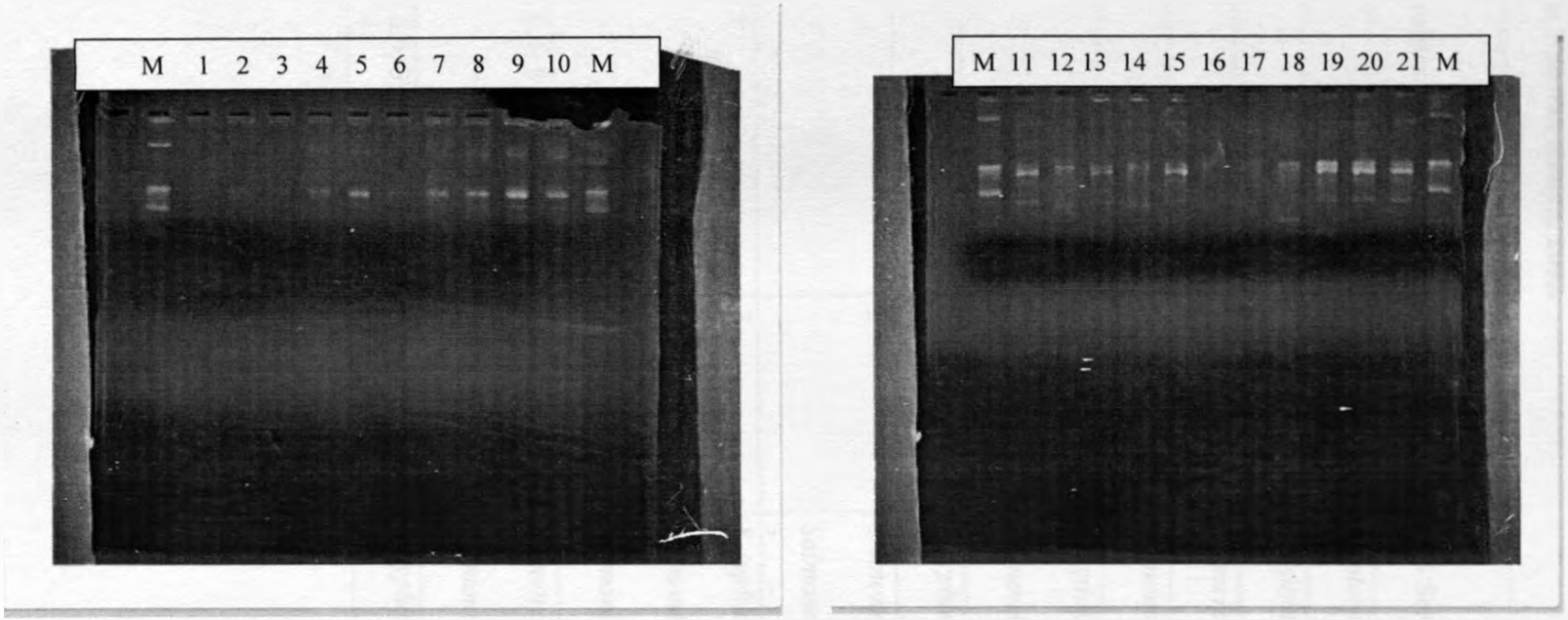

Plate 4.1: Gel electrophoresis photographs showing plasmid bands of 16 non-typhi *Salmonella* isolates from children hospitalized in Nairobi hospitals, Kenya, 2000. Lanes 1,2,3 and 16 show no plasmid bands.

Key: Lanes M are for the marker plasmids 147, 63, 43.5, 6.9 Kb of *E. coli* 39R861 (NCTC 50192).

Table 4.5: Plasmid DNA profiles of 16 non-typhi *Salmonella* isolates from hospitalized children in Nairobi, Kenya, 2002.

Plasmid profiles	Number of isolates	NTS Serotypes
2	1	*S. enteritidis*
4	1	*S. typhimurium*
8	1	*S. enteritidis*
9; 7.5	1	*Salmonella* spp
105; 8	3	*S. typhimurium* *Salmonella* spp
16; 6.9; 4	1	*S. typhimurium*
105; 8; 5	2	*Salmonella* spp *Salmonella* spp
105; 9; 8; 5	3	*S. typhimurium* *Salmonella* spp *Salmonella* spp
105; 9; 8; 6.9; 5	2	*Salmonella* spp *Salmonella* spp
105; 9; 8; 6.9; 5; 2	1	*S. typhimurium*

Table 4.6: Frequency of plasmids isolated from 16 non-typhi *Salmonella* isolates from hospitalized children in Nairobi, Kenya, 2002.

Plasmid size (Kb)	Number of NTS Isolates
2	2
4	2
5	8
6.9	4
7.5	1
8	12
9	7
16	1
105	11

CHAPTER 5

DISCUSSION

In the current study, *S. enteritidis* was the most common serotype among the hospitalized children. The number of *S. enteritidis* isolates was more than double that of *S. typhimurium*; 28/74 (37.8%) of *S. enteritidis* compared to 11/74 (14.9%) of *S. typhimurium*. These findings were in contrast to results from other studies in Central Africa where *S. typhimurium* was the most frequently isolated NTS (Lepage *et al.*, 1987). However, in the USA as well as in many other parts of the world, *S. enteritidis* infections have been on the increase and have frequently rivalled *S. typhimurium* as the most common cause of salmonellosis (Mishu *et al.*, 1994). Wong *et al.* (1994) reported a gradual but significant increase in *S. enteritidis* in Hong Kong, and had indeed become the most common *Salmonella* serotype isolated from extra-intestinal sources by 1996 (Ling *et al.*, 1998). In addition, the proportion of *S. enteritidis* in the United States also increased from 9.9% in 1985 to 26.1% in 1994, making it the most common serotype (CDC, 1996). In conformity with the findings in this study, worldwide the incidence of *S. enteritidis* has increased dramatically (Rodrigue *et al.*, 1990).

Most of the NTS cases whose homes were visited were from slum areas where sanitary conditions were poor. These slums were characterized by the absence of running water, leaking and free-flowing sewage, dirty communal toilets, and garbage dumps. This finding appears to indicate that the source of the NTS infections to humans is from environmental contamination coupled with poor hygiene. In such scenarios, children, with the tendency to soil themselves in the dirty environments, would be expected to be the highest group at risk of contracting NTS infections and especially considering the low level of hygiene in the slums. Indeed, in this study, children less than five years old constituted the highest proportion (75.7%) of the NTS isolates.

Seventy-two out of 74 (97.3 %) of the NTS isolates in this study were resistant to one or more of the twelve antimicrobials tested. All the *S. typhimurium* and *S. enteritidis* isolates were resistant to at least one antimicrobial agent. These findings are in contrast to other studies in various parts of the world, including England and USA, which showed that, most *S. enteritidis* strains to be susceptible to a wide range of antimicrobial agents (Rampling *et al.*, 1989; Ward *et al.*, 1990; Rodrigue *et al.*, 1992; Nair *et al.*, 1995). Eighty four percent of the NTS isolates in the study by Kariuki *et al.* (1996) were found to be resistant to at least one antimicrobial agent. This study showed a high frequency of isolation of NTS that were resistant to one or more antimicrobials, a finding in conformity with that of Mayer (1988) who concluded that multi-drug resistant (MDR) NTS are common.

Reports of increasing incidences of MDR NTS have been reported in the United Kingdom (Low *et al.*, 1997) and in the United States (Glynn *et al.*, 1998). This study also recorded high resistance percentages among NTS isolates to commonly used antimicrobials such as sulphamethoxazole (81.1%), streptomycin (48.6%), ampicillin (36.5%) and tetracycline (31.1%). The results of this study conform to those of others in the region. Urassa *et al.* (1997) concluded that not only is antimicrobial resistance prevalent in the region, but also resistance to the first line of drugs for treatment. In accordance with the findings of Mache *et al.* (1997) who found *Salmonella* isolates with simultaneous resistance to seven and eight antimicrobial agents, this study also found some NTS isolates with simultaneous resistances to seven (5 isolates), eight (4 isolates) and even nine (1 isolate) antimicrobial agents. A penta multi-drug resistance of the DT104 *S. typhimurium* that was resistant to chloramphenicol, streptomycin, ampicillin, tetracycline and sulfonamide among other antimicrobial agents, has been recorded (Angulo, 1997; Low *et al.*, 1997; Sandvang *et al.*, 1998; Threlfall *et al.*, 1994a). This study showed three isolates, one *Salmonella spp* (O4: *: 1,2) and two *S.*

enteritidis, that had the penta-multi-drug resistance of DT104 *S. typhimurium* in addition to being resistant to both trimethoprim and amoxycillin-clavulanic acid. These results are consistent with those of Evans and Davies (1996) who found 13% of *S. typhimurium* isolates resistant to both trimethoprim and amoxycillin-clavulanic acid in addition to the penta MDR of DT104. Chromosomally located genes encoding resistance in *S. typhimurium* DT104 (Threlfall *et al.*, 1994a) may have been responsible for the resistance in these three seven-drug multi-resistance isolates in this study since one of the three isolates had no plasmids, and those that were extracted from two of the isolates were of different molecular sizes, and could therefore not be attributed to the seven-drug resistance. Hence, resistance to antimicrobials is related to but not restricted to the presence of plasmids. This study showed that the penta MDR is not restricted to DT104 *S. typhimurium*.

The causes of the high MDR in this study may be attributed to the practice of indiscriminate dispensing of antimicrobials in this country. Indalo (1997) found that 64% of retail chemists in Nairobi and the peri-urban areas sold antibiotics without prescription from authorized persons. In addition, some chemists accept to sell under-dose drugs on request, thus disregarding the Pharmacy and Poisons Act of the Laws of Kenya (1972). Furthermore, some chemists sold antimicrobials in unlabelled envelops in total disregard of The Kenya National Drug Policy (1994) that requires that the dispensed medicines be put in a package bearing the information: name and address of the chemist supplying, name of patient, name of product, instructions for use and any precautions to be taken. This kind of dispensing practice may have very easily contributed to under-dosing and indiscriminate use of antimicrobial agents, both of which are factors contributing to antimicrobial resistance (Waiyaki, 1993). Over the counter purchases of injectable and intramammary antibiotics for use by farmers may be the

other contributing factors (Waiyaki, 1997), especially when withdrawal periods are not observed.

The very high percentage of isolates resistant to sulphamethoxazole (81.1%) may also be due to the fact that coupled with the misuse, ease of availability and low cost, it is a commonly used antimicrobial indicated for the treatment of respiratory, gastrointestinal, urinary tract and genital infections (Harvey *et al.*, 1992; Indalo, 1997). One of the *S. enteritidis* isolates in this study was resistant to ciprofloxacin in addition to resistantce to cefuroxime both of which are antimicrobial agents newly introduced into the market. This finding underlines the need for continuous surveillance of antimicrobial susceptibility patterns of NTS and reviews of treatment guidelines as recommended by Waiyaki (1993) and Omari *et al.* (1997).

Higher percentages of antimicrobial resistance were recorded among the drugs most commonly used in animal treatment including sulphamethoxazole (81.1%), streptomycin (48.6%), ampicillin (36.5%) and tetracycline (31.1%). This scenario may be due to the indiscriminate use of antimicobials for veterinary use leading to selection of resistant NTS, whereby, if these resistant NTS infect humans they will be resistant to the antimicrobials that had been used in the animals. Antimicrobial residues in foods of animal origin may also contribute in selection of resistant NTS in humans. These antimicrobials are also commonly used in humans thereby exacerbating the problem of selective survival of resistant NTS in humans. Epidemiological investigations have demonstrated that in the USA, the use of antimicrobial agents in livestock is the principal cause of the emergence and dissemination of resistance to antimicrobial agents in strains of non-typhoid salmonellosis (Cohen and Tauxe, 1986; van den Bogaard and Stobberingh, 1999).

The plasmid-mediated antimicrobial resistance appeared not to be associated with spread of NTS. This study, however, showed specific plasmids (105Kb and 8Kb) to be involved significantly ($p<0.05$) in the resistance of NTS to the antimicrobials streptomycin and trimethoprim, both of which are among the commonly used antimicrobials. The epidemiological association of the plasmids with resistance to commonly used antimicrobials, which are not only easily available but also cheap, meant that occurrence of such plasmids made treatment more expensive.

Although the method of plasmid profile analysis (PPA) has been found useful by several investigators for strain differentiation (Hampton *et al.*, 1995; Threlfall *et al.*, 1996), this was not the case in this study, where plasmids of equal sizes were found in different *Salmonella* serotypes. Moreover, similar plasmid profiles were also found in different serotypes. Olsen *et al.* (1994) has shown that the same plasmid profile might be present in strains that are clearly different. The PFGE of XbaI-digested DNA combined with PPA was found useful by Lindqvist *et al.* (1999) in discriminating between an endemic and feed-borne *S. infantis* in cattle. The feed-related *S. infantis* was clonally related to the endemic infection by the ribotype, IS200-type and XbaI-profile but had a distinctive plasmid that appeared in pulsed-field gel electrophoresis as a 60kb band when cleaved with XbaI or linearized by SI-nuclease. Plasmids are, however, regarded as fairly unstable genetic markers (Chrichton *et al.*, 1996). In this study, the low number of isolates with plasmids appeared to indicate that the plasmids were not responsible for virulence; otherwise they would be conserved in the isolates. This finding contrasted sharply with that of Chiu *et al.* (2000) who reported that all those serovars preferentially causing primary bacteraemia such as *S. enteritidis*, harboured a virulence plasmid.

Most NTS isolates of the cases were similar serologically, and also in terms of antimicrobial susceptibility, to those isolated from family members. It is probable that the children cases and the adults, from whom NTS were isolated, got the NTS from the same source, but adults, with a stronger immunity than the children, did not develop bacteraemia. It has been suggested that bacteraemia due to NTS in young children might be related to the immaturity of their immunological defenses, the latter increasing the risk of such bacteraemia (Meadow *et al.*, 1985; Wittler and Bass, 1989; Cohen *et al.*, 1987; Riley *et al.*, 1984). In addition, as stated above, children have a tendency to play in dirty environments thus exposing them to infections.

The problem of multi-drug resistance of NTS to commonly used antimicrobials compounded by the increasing prevalence of NTS infections among children is a real threat to the survival of children below five years of age. Concerted efforts by all stakeholders must be made to find the causes as well as the solution to multi-drug resistance to NTS. Needless to say, the indiscriminate use of antimicrobials by both human and veterinary doctors must be stopped. Further studies, preferably case control study types, are required to positively identify the potential risk factors of NTS infections in the high-risk age-group of children. This study lacked control groups and therefore chance may have played a role in the identification of the risk factors.

CHAPTER 6

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1: Conclusions

The following conclusions can be made from this study:

1. Among the NTS that cause bacteraemia in children in Nairobi, Kenya, *S. enteritidis* (37.8%) and *S. typhimurium* (14.9%) were the most common serotypes;

2. All the *S. enteritidis* and *S. typhimurium* that cause bacteraemia in children were resistant to at least one commonly used antimicrobial agent;

3. All the NTS were susceptible to ciprofloxacin and it may be the drug of choice in NTS bacteremia of children;

4. Multi-drug resistance was common among the NTS of hospitalized children in Nairobi, Kenya;

5. Plasmids were associated with resistance to trimethoprim and streptomycin;

6. Resistance to antimicrobial agents was associated but not restricted to presence of plasmids;

7. There is no association between plasmids and virulence;

8. There is no correlation between plasmid profile and serotype of NTS;

9. The vast majority (75.7%) of the NTS cases were children less than 5 years old;

10. A high proportion (77.4%) of the NTS cases were from slum areas characterized by very poor sanitary conditions.

6.2: Recommendations

The following recommendations are made from this study:

(i) The cause of the high incidence of MDR *S. enteritidis* in children be investigated.

(ii) The role of human carriers in the transmission of NTS that infect children causing bacteraemia be further investigated;

(iii) There is a need for continued monitoring of antimicrobial susceptibility of the NTS of children in order to keep pace with the changing susceptibility patterns in the treatment of *Salmonella* bacteraemia in children;

(iv) In view of the high frequency of isolation of *S. enteritidis,* whose main food animal reservoir is poultry, the impact of the rapidly growing poultry industry on the incidence of MDR *S. enteritidis* be further investigated;

(v) In view of the increasing incidence of MDR NTS, it is recommended that the usage of antimicrobial agents in livestock be more controlled;

(vi) There is a need for continued surveillance for antibiotic resistant NTS in animals and to monitor the development of resistance to antimicrobial agents in use at any one particular time;

(vii) Studies using genomic fingerprinting, for example pulse field gel electrophoresis (PFGE) profiles, need to be carried out to establish if any genetic relatedness of the MDR NTS exists;

(viii) In view of the finding of plasmids of equal sizes, and also similar patterns, among different S*almonella* serotypes, the genetic relatedness of the plasmids should also be determined by, for example, restriction enzyme fingerprinting;

(ix) Further studies are needed to investigate the transmission methods of the plasmids and genes encoding for resistance of NTS that cause bacteraemia in children.

(x) Enforcement of the law (Cap. 244, Pharmacy and Poisons act) to prevent misuse of drugs.

(xi) Poverty eradication to ensure good sanitary conditions in the slums as well as proper sewage disposal as a way to reduce the risk of salmonellosis in the slums.

(x) Public education on the problems caused by antimicrobial abuse and misuse.

CHAPTER 7

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CHAPTER 8

APPENDICES

APPENDIX 8.1: Consent form for people who participated in the study.

The center for Microbiology research, Kenya Medical Research Institute is carrying out a study to try to understand how a bacterium called salmonella is spread and from where it comes. In order to do this we are asking you to allow us to obtain stool samples from your children and other members of your household. We would also like to take samples from animals if you have any.

Should we find the bacterium you will be informed but there is no need to be concerned. We will provide simple information on hygiene and cooking to prevent any problems. Should any treatment be required, (which is very unlikely), it will be provided by us.

In case you do not give consent for participation of your child there will be no penalty and the doctor will proceed to treat your child as required.

If you have understood what I have explained to you, I ask you to confirm and sign up for participation of your child and other members in the study.

Declaration

I understand that participation in the study is purely voluntary. I agree that my child may participate. I further understand that I may decline to the participation of my child at any time without suffering any consequences such as denial of medical attention. I am a parent/guardian who cannot sign my name I need a space for thumb printing

Name of child

Parents/Guardian signature	Date	Thumb print
Witness signature	Date	
Interviewer/investigator	Date	

APPENDIX 8.2: Questionnaire form for household characteristics of non-typhi *Salmonella* cases

Please answer ALL questions appropriately

PATIENT (INDEX CASE) NAME ---------- HOSPITAL NO. ----------

LAB NO. ----------

Is any of the following foods consumed in the home

Milk Yes / No (Tick)

Eggs Yes / No

Beef Yes / No

Fish

Other animal protein foods, please specify ----------

How are the foods above consumed (Tick)

a. Milk Fresh boiled

Raw fermented

Yogurt

Other (please specify) ----------

b. Eggs boiled hard

Boiled soft

Fried hard

Fried soft

Raw

Other (please specify) ----------

c. Beef Large pieces Yes / No (Tick)

Minced Yes / No

d. Fish Fry Yes/No

Boiled Yes/No

Other (please (specify)----------

3. What is the source of these foods if they are consumed in the home?

a. Milk Home, own animal (cow, goat, other) - Tick appropriately.

Neighbour

Vendors (Kiosk/hawkers)

Dairy plant

Packaged

Other (specify) ----------

b. Eggs Home

Neighbour

Kiosk

Other (specify) ----------

c. Beef (Minced) Butchery

Home-minced

Other (Specify)

4. a. Is milk (raw or pasteurized) used at home, to make any of the following? (Tick)

	Raw	Pasteurized
Mala	Yes / No	Yes / No
Yogurt	Yes / No	Yes / No
Cakes	Yes / No	Yes / No
Ice-cream	Yes / No	Yes / No

b. Are any of the following animals found in the home? (Tick)

Cattle	Yes / No
Pigs	Yes / No
Chicken	Yes / No
Goats	Yes / No
Sheep	Yes / No
Dogs	Yes / No
Cats	Yes / No

Other (specify) ----------

c). If cattle are present in the home, are any of them in milk? Yes / No.

d). History of recent calving, kidding, farrowing, or lambing Yes / No

e). Following the end of the milking process:

Milk is sieved before being taken into the house. Yes / No

Milk is taken into the house where it is sieved. Yes / No

f). All utensils used for milking are cleaned outside the house. Yes / No

g). Milk utensils are cleaned inside the house. Yes / No

h). Milk utensils are separate from other household utensils. Yes / No

i). Milking is done by: A family member Yes / No

An outsider Yes / No

j) Are any commercial feeds used for the animals? Yes / No

If yes, which ones?

k) Has the presence of rats been detected in or around the home? Yes / No

5. a). Is the index case a baby Yes / No

Is a sieve used in the preparation of the baby's foods/fruits? Yes / No

Is a blender used in the preparation of the baby's foods/fruits? Yes / No

b). Has the index case travelled recently? Yes / No

c). If Yes, to where? (Specify) ----------

d). For how long? (Days, Weeks, months) ----------

e). If yes, did any other family member(s) travel then? ----------

6. What is / are the water sources for the family?

Tap

River

Well

Borehole

Other (specify)----------

b) Is water boiled before drinking? Yes / No

7.Is animal manure used at all in the farm? Yes / No

If yes, what is the source?

8. a)What are the occupations of the parents/guardians of the index case?

Father ----------

Mother ----------

Guardian ----------

b) What are the occupations of other people living in the home?

Name	Occupation
1. ----------	----------
2. ----------	----------
3. ----------	----------
4. ----------	----------

Name of interviewer: ----------

Sign: ----------

Date----------

APPENDIX 8.3: Interpretation table for the API 20 E system

TESTS	SUBSTRATES	REACTIONS/ENZYMES	RESULTS	
			NEGATIVE	POSITIVE
ONPG	Ortho-nitro-phenyl-galactoside	Beta-galactosidase	Colourless	Yellow (1)
ADH	Arginine	Arginine dyhydrolase	Yellow	Red / orange (2)
LDC	Lysine	Lysine decarboxylase	Yellow	Orange
ODC	Ornithine	Ornithine decarboxylase	Yellow	Red / orange (2)
CIT	Sodium citrate	Citrate utilization	Pale green/Yellow	Blue-green / green (3)
H_2S	Sodium thiosulfate	H_2S production	Colourless/greyish	Black deposit / thin line
URE	Urea	Urease	Yellow	Red / orange (2)
TDA	Tryptophane	Tryptophane desaminase	**TDA** / immediate	
			Yellow	Dark brown
IND	Tryptophane	Indole production	**JAMES** Reagent / immediate or IND / 2 min	
			JAMES Pale green-yellow	**JAMES** Pink
			IND Yellow ring	**IND** Red ring
VP	Sodium pyruvate	Acetoin production	**VP 1** + **VP 2** / 10 min	
			Colourless	pink / red
GEL	Kohn's gelatin	Gelatinase	No diffusion of black pigment	Diffusion of black pigment
GLU	GLUCOSE	Fermentation / oxidation (4)	Blue / blue-green	Yellow
MAN	MANNITOL	Fermentation / oxidation (4)	Blue / blue-green	Yellow
INO	INOSITOL	Fermentation / oxidation (4)	Blue / blue-green	Yellow

APPENDIX 8.3 continued

SOR	SORBITOL	Fermentation / oxidation (4)	Blue / blue-green	Yellow
RHA	RHAMNOSE	Fermentation / oxidation (4)	Blue / blue-green	Yellow
SAC	SUCROSE	Fermentation / oxidation (4)	Blue / blue-green	Yellow
MEL	MELIBIOSE	Fermentation / oxidation (4)	Blue / blue-green	Yellow
AMY	AMYGDALIN	Fermentation / oxidation (4)	Blue / blue-green	Yellow
ARA	ARABINOSE	Fermentation / oxidation (4)	Blue / blue-green	Yellow

1. A very pale yellow should also be considered positive
2. An orange colour after 24 hours of incubation must be considered negative
3. Reading made in cupule (aerobic)
4. Fermentation begins in the lower portion of the tubes, oxidation begins in the cupule.
5. A very pale yellow should also be considered positive
6. An orange colour after 24 hours of incubation must be considered negative
7. Reading made in cupule (aerobic)
8. Fermentation begins in the lower portion of the tubes., oxidation begins in the cupule

APPENDIX 8.4: NCCLS 2002 guidelines for zone diameters (mm) and MIC (ug/litre)

		Susceptibility				
		Susceptibility/Zone sizes (mm)			MIC µg/ml	
Antimicrobial	Disc Content (µg)	**R**	**I**	**S**	**R**	**S**
Ampicillin 10mg	10	≤13	14-16	≥17	≥32	≤8
Amoxycillin-clavulanic acid	20/10	≤13	14-17	≥18	≥32/16	≤8/4
Chloramphenicol	30	≤12	13-17	≥18	≥32	≤8
Streptomycin	10	≤11	12-14	≥15	-	-
Sulphamethoxazole	250/300	≤12	13-16	≥17	≥350	≤100
Trimethoprim	5	≤10	11-15	≥16	≥16	≤4
Cefuroxime	30	≤14	15-17	≥18	≥32	≤8
Ceftazidime	30	≤14	15-17	≥18	≥32	≤8
Ciprofloxacin	5	≤15	16-20	≥21	≥4	≤1
Nalidixic acid	30	≤13	14-18	≥19	≥32	≤8
Tetracycline	30	≤14	15-18	≥19	≥14	≤4
Gentamycin	10	≤12	13-14	≥15	≥8	≤4
Cefotaxime	30	≤14	15-17	≥18	≥64	≤8

S-Sensitive I-Intermediate R-Resistant

APPENDIX 8.5: Antimicrobial susceptibility of 74 NTS isolated from children hospitalized in Nairobi, Kenya, 2002.

Lab. No	Type	CXM	RL	TMP	CHL	STR	NAL	CIP	AMP	AMC	CAZ	CN	TCY
3087	O9g	S	R	R	R	R	S	S	R	S	S	S	R
3106	O9g	S	I	S	S	S	S	S	S	S	S	S	S
3165	O67e	S	S	R	S	S	S	S	S	S	S	S	S
3166	O4	S	R	S	S	S	S	S	R	R	S	S	S
3172	O9g	S	I	S	S	S	S	S	S	S	S	S	S
3173	O9g	S	I	S	S	S	S	S	S	S	S	S	S
3175	O9g	S	I	S	S	S	S	S	S	S	S	S	S
3176	O9g	S	R	S	S	S	S	S	S	S	S	S	S
3243	O4	S	I	S	S	S	I	S	R	I	S	R	S
3271	O67	S	I	R	S	S	S	S	S	S	S	S	S
3333	O9g	S	I	S	S	S	S	S	S	S	S	S	S
3353	O9g	S	R	R	R	S	S	S	S	S	S	S	R
3354	O67	S	R	S	S	S	S	S	S	S	S	S	S
3451	O4	S	R	S	S	S	S	S	S	S	S	S	S
3452	O67	S	R	S	S	S	S	S	S	S	S	S	S
3453	O4	S	R	S	S	S	S	S	S	S	S	S	S
3462	O9g	I	R	S	S	S	S	S	S	S	S	S	S
3463	O9g	S	R	S	S	S	S	S	S	S	S	S	S
3495	O9g	S	R	S	S	S	S	S	S	S	S	S	S
3496	O4e	S	R	S	S	S	S	S	S	S	S	S	S
3497	O4r	S	R	S	S	S	S	S	S	S	S	S	S
3544	O9g	S	R	R	S	R	S	S	S	S	S	S	R
3545	O4i,1,2	S	R	R	S	R	S	S	R	I	S	S	S
3546	O9	S	S	S	S	S	S	S	S	S	S	S	S
3568	O67	S	R	S	S	S	S	S	S	S	S	S	S
3615	O9g	I	R	S	S	S	S	S	S	S	S	S	S
3667	O9g	I	R	R	R	R	S	S	S	S	S	S	S
3784	O9g	S	R	R	S	S	S	S	S	S	S	S	S
3785	O4i,1,2	S	R	R	S	S	S	S	S	S	S	S	S
3947	O68	S	R	S	S	S	S	S	S	S	S	S	S
3948	O4i	R	S	R	R	R	R	S	R	R	S	S	S
3949	O68	S	S	S	S	S	S	S	S	S	S	S	S
3950	O9g	I	S	S	S	S	R	S	R	S	S	S	S
3951	O9g	S	S	R	R	R	R	S	S	R	S	S	R
4054	O9g	S	R	S	S	S	S	S	R	S	S	S	S
4055	O4i	S	R	R	S	I	S	S	S	I	S	R	S
4056	O9g	S	R	S	S	S	S	S	R	S	S	S	S
4057	O9g	S	R	S	S	S	S	S	S	S	S	S	S
4058	O4	S	R	S	S	S	S	S	S	S	S	S	S
4099	O4i	S	R	R	S	I	S	S	S	R	S	R	S
4101	O9g	S	R	R	R	R	S	S	R	R	S	S	R
4102	O9g	S	R	R	R	R	S	S	R	R	S	R	R

Appendix 8.5 continued

4103	O9g	I	R	R	R	R	S	S	R	I	S	R	R
4104	O4i	S	R	R	S	I	S	S	R	I	S	R	S
4105	O4i	S	R	R	S	I	S	S	R	R	S	R	S
4106	O4(1,2)	S	R	R	S	I	S	S	S	R	S	R	S
4107	O4(1,2)	S	I	S	S	I	S	S	R	S	S	S	S
4122	O9g	S	S	S	S	I	S	S	R	S	S	S	S
4145	O4i,1,2	S	R	R	S	R	S	S	S	R	S	R	S
4241	O4(1,2)	R	R	R	S	R	S	S	S	R	S	R	S
4243	O4(1,2)	S	R	S	S	I	S	S	S	S	S	S	S
4244	O9g	S	R	S	S	S	S	S	S	S	S	S	S
4314	O4	R	R	S	R	S	S	S	R	R	S	S	R
4315	O4i1,2	S	I	S	I	S	S	S	S	S	S	S	I
4316	O4i,1,2	S	R	S	I	S	S	S	S	S	S	S	S
4317	O4i,1,2	S	R	S	I	S	S	S	R	R	S	S	I
4334	O4i,1,2	S	R	R	S	R	S	S	R	R	S	R	S
4335	O4i,1,2	S	R	R	S	R	R	S	R	R	S	I	I
4392	O67	S	R	R	S	R	S	S	R	I	S	I	S
4393	O67	S	S	S	S	I	S	S	S	S	S	S	I
4421	O4i,1,2	R	R	R	S	R	S	S	R	S	R	R	S
4422	O9g	S	I	S	S	R	I	S	S	S	S	I	I
4423	O67	S	S	S	S	I	S	S	S	S	S	S	S
4424	O4(1,2)	I	R	R	R	R	S	S	R	R	S	R	I
4425	O9g	S	R	R	R	R	S	S	R	R	S	S	R
4426	O67	S	S	S	S	I	R	S	I	I	S	S	R
4427	O4i,1,2	S	S	S	S	R	I	S	S	S	S	S	I
4428	O4(1,2)	I	R	R	S	R	S	S	R	R	S	R	R
4429	O4(1,2)	S	S	I	S	R	I	S	S	I	S	S	I
4486	O9g	S	R	R	R	R	S	S	R	R	S	S	R
4487	O4(1,2)	S	S	S	S	S	S	S	R	S	S	S	S
4488	O9g	S	R	S	S	R	S	S	S	S	S	S	I
4489	O4(1,2)	S	R	R	S	R	S	S	R	R	S	R	R
4490	O4i,1,2	S	S	S	S	I	S	S	S	S	S	R	R

(1,2) Poly phase 1 and phase 2 positive; S- Fully sensitive; I-intermediate; R-Resistant

CXM- CEFUROXIME

CIP- CIPROFLOXACIN

AMC- AMOXYCILLIN/CLAVULANIC ACID

AMP- AMPICILLIN

CAZ- CEFTAZIDIME

RL- SULPHAMETHOXAZOLE

CN- GENTAMICIN

TMP- TRIMETHOPRIM

TCY- TETRACYCLINE

CHL- CHLORAMPHENICOL

STR- STREPTOMYCIN

NAL-NALIDIXIC ACID

Appendix 8.6: Resistance patterns to antimicrobial agents of 72 non-typhi *Salmonella* isolated from children hospitalized in Nairobi, Kenya, 2002

Resistance patterns	Number of non-typhi *Salmonella* isolates			Total
	Salmonella enteritidis	*Salmonella typhimurium*	*Salmonella species*	
RL	11	0	9	20
TMP	0	0	1	1
RL+TMP	1	1	1	3
RL+AMC	0	0	1	1
RL+CXM	2	0	0	2
RL+AMP	1	0	0	1
CXM+NAL	1	0	0	1
STR+AMP	1	0	1	2
CAZ+AMP	0	0	1	1
RL+CHL+AMP	0	1	0	1
RL+STR+AMP	0	0	2	2

Appendix 8.6 continued

TCY+STR+AMP	0	0	1	1
TCY+STR+CN	0	1	0	1
RL+TMP+CHL+TCY	1	0	0	1
RL+TMP+STR+TCY	1	0	0	1
RL+TMP+STR+AMC	0	1	0	1
RL+CHL+AMP+TCY	0	1	0	1
RL+NAL+AMC+CN	0	0	1	1
STR+AMP+AMC+TCY	0	0	1	1
STR+NAL+AMP+TCY	0	1	0	1
RL+TMP+CHL+STR+TCY	1	0	0	1
RL+TMP+STR+AMC+CN	0	0	5	5
RL+CHL+AMP+ AMC+TCY	0	1	0	1
RL+STR+AMP+CN+TCY	0	0	1	1
RL+STR+AMP+CAZ+TCY	1	0	0	1
RL+TMP+CHL+STR+CXM	1	0	0	1

Appendix 8.6 continued

RL+TMP+CHL+STR +AMC+TCY	1	0	0	1
RL+TMP+STR+AMP+AMC+CN	0	2	1	3
RL+CHL+AMP+TCY+AMC +CXM	0	0	1	1
RL+TMP+STR+AMP+CN+CXM	0	1	0	1
TMP+CHL+STR+NAL+AMC+CXM	0	0	1	1
TMP+CHL+STR+NAL+AMC+TCY	1	0	0	1
RL+TMP+CHL+STR +AMC+CN +TCY	1	0	0	1
RL+TMP+CHL+STR +AMP+ AMC+TCY	1	0	0	1
RL+TMP+STR+AMP+AMC+CN +TCY	0	0	1	1
RL+TMP+STR+AMP+AMC+CN+CXM	0	0	1	1
TMP+STR+AMP+AMC+NAL+CAZ+TCY	0	0	1	1
RL+TMP+CHL+STR +AMC+CN+CXM+TCY	1	0	0	1
RL+TMP+STR +AMP+AMC+CN+NAL+ TCY	0	1	0	1
RL+TMP+STR +AMP+AMC+CN+CXM + TCY	0	0	1	1
RL+TMP+CHL+STR +AMP+AMC+CAZ+TCY	1	0	0	1

Appendix 8.6 continued

RL+TMP+CHL+STR +AMP+AMC+CN+CXM + TCY	0	0	1	1
TOTALS	28	11	33	72

RL - SULPHAMETHOXAZOLE

AMC - AMOXYCILLIN/CLAVULANIC ACID

CHL- CHLORAMPHENICOL

STR- STREPTOMYCIN

NAL- NALIDIXIC ACID

TCY- TETRACYCLINE

AMP- AMPICILLIN

TMP- TRIMETHOPRIM

CAZ- CEFTAZIDIME

CXM- CEFUROXIME

CN- GENTAMICIN

CIP-CIPROFLOXACIN