STUDIES ON ENTERIC PATHOGENS ASSOCIATED WITH ADULT DIARRHOEAL OUT-PATIENTS IN SOME HOSPITALS AND CLINICS OF ADDIS ABABA: ANTIBIOTIC RESISTANCE AND PLASMID PROFILE ANALYSIS

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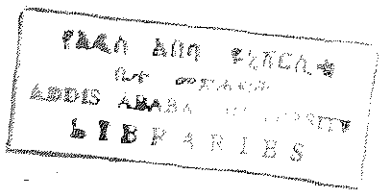

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TABLE OF CONTENTS

LIST OF TABLES

PAGE

LIST OF FIGURES

PAGE

ABSTRACT

700 diarrhoeal samples from adult out-patients, 335 from males and 365 from females, were collected from 4 hospitals (Tikur Anbesa, Zeuditu, Yekatit 12 and Menelik II) and Arada Clinic in Addia Ababa. Of the total samples, 394 (56.3%) yielded one or more etiologic agents. Of the positive samples, 191 (57%) were from males and 203 (55.6%) from females. Among 304 single infections, *Salmonella* was encountered in 13.2%, *Shigella* in 13.5%, *Entamoeba histolytica* in 33.5%, *Giardia lamblia* in 28%, *Strongyloides stercoralis* in 7.6% and *Trichuris trichiura* in 4.3%. In addition 75 double and 15 triple infections were found. No *Cryptosporidium* were identified in any of the samples. Differences in infection status among age groups and between sex were insignificant ($p>0.01$).

Among 45 *Salmonella* isolates, serogroup C comprised 31.1%, B 24.4%, D 13.3%, S.typhi 15.6%, A 8.9% and E 6.7%. Susceptibility of *Salmonella* isolates to tetracycline was 28.9%, to ampicillin 31.1%, to cephalothin 33.3%, to trimethoprim-sulfamethoxazole 42.2%, to chloramphnicol 53.3%, to kanamycin 46.1%, to carbenicillin 62.2%, to gentamicin 91.1% and to polymyxin B was 100%. Susceptibility of individual serogroups ranged from 0% to ampicillin and tetracycline up to 100% susceptibility to polymyxin B in serogroup A. Of the *Salmonella* isolates, 93% were resistant to one or more drugs with a total of 31 distinct resistance patterns (antibiograms). Frequently encountered antibiograms were those combinations which contained ampicillin, tetracycline, cephalothin, kanamycin and trimethoprim-sulfamethoxazole. From 2 to 4 drug resistant strains from each serogroups of *Salmonella* were selected for plasmid profile analysis. All strains contained

multiple plasmids which were larger than 21.2 kilobases (Kb) and several others ranging in sizes from 21.2 Kb to 1.8 Kb.

Among 50 *Shigella* isolates, serogroup A comprised 28%, B 44%, C 18% and D 10%. Susceptibility of *Shigella* isolates to tetracycline was 26%, to ampicillin 30%, to cephalothin 36%, to trimethprim-sulfamethoxazole 48%, to chloramphenicol 50%, carbencillin 72%, to kanamycin 80%, to nalidixic acid 86%, polymyxin B 90% and to gentamicin was 100%. Among all *Shigella* serogroups, least susceptibility was encountered to tetracycline and ampicillin while greatest susceptibility was observed to gentamicin, polymyxin B and nalidixic acid. Of all Shigella isolates, 98% were resistant to one or more drugs with a total of 32 distinct antibiograms. Frequently encountered antibiograms were those combinations which contained tetracycline, ampicillin, cephalothin and trimethoprim-sulfamethoxazole. From 2 to 6 drug resistant isolates from each serogroups of *Shigella*, were selected for plasmid profile analysis. As in *Salmonella*, these also contained multiple plasmids which were larger than 21.2 Kb and others ranging in sizes from 21 Kb to 1.4 Kb.

The importance of environmental sanitation, upgrading the knowledge of personal hygiene among the public and the use of prescribed antibiotics from proper pharmacies during necessities is recommended. The need for further research especially on plasmid profile analysis is stressed.

I. INTRODUCTION

The syndrome of gastroenteritis, the classic symptoms being nausea (loathing for food and an inclination to vomit), vomiting, abdominal pain and diarrhoea, is very common in most tropical countries. Infections of the intestinal tract, especially infectious diarrhoea, are among the most common debilitating diseases in underdeveloped heavily populated areas. They account for as much as 20 to 30% of the attendances in most out-patient clinics, with an estimated rate of 5.0×10^8 infections per year (Chen *et al.*, 1991). It encompasses a wide variety of symptom complexes and can be caused by a wide variety of recognized infectious agents.

Enteric infections have played an important role in the majority of the world's populations, including children, the aged, the malnourished, military personnel and persons from industrialized regions travelling to developing areas. The magnitude of the problem has been profound in areas of the world with reduced economic development where there exists a greater reservoir of enteropathogens and a large susceptible populations with nutritional deficits (WHO, 1992). Morbidity from enteric infections in developing areas exceeds that of the industrialized countries by several fold with attack rates ranging from two to twelve or more illnesses per person per year in developing countries. In addition, diarrhoeal illnesses account for an estimated 12,600 deaths each day in children in Asia, Africa and latin America. Considering the magnitude of the problem in developing countries, however,

availability of information on its incidence, prevalence and epidemiology is relatively scarce (Guerrant *et al.*, 1990).

In recent years there have been numerous advances in the understanding of the increasingly varied range of microorganisms that infect the gastrointestinal tract. These agents include the newly identified pathogens such as *Helicobacter pylori, Cryptosporidium parvum* and still the growing range of types of *E.coli* and also various other bacteria, viruses, protozoa, algae, fungi and helminths, many of which have been recognized only in the last decade or two (Guerrant and Bobak,1991). Diarrhoea may even be dietetic in origin when drinking water and food are readily contaminated with enteric pathogens in areas where methods of faecal disposal may be quite primitive or entirely lacking. Only where laboratory facilities are adequate, can an accurate etiological diagnosis be made. In fact many cases of diarrhoeal etiology remain undiagnosed in developing countries and is usually reported under the general heading of "diarrhoeal" or "dysenteric" diseases due to inadequate laboratory facilities (Amal *et al.*, 1990).

Transmission of enteric pathogens occurs in many ways. Some of the casual organisms are found in many domestic and wild animals including birds. The unhygienic method of handling cooked and uncooked food in market places, passive carriers and domestic animals contribte to the high frequency of diarrhoeal diseases in the tropics (Gilligan, 1986). A proportion of the population of developing countries lives in deprived conditions characterized by ramshackle housing, lack of piped water and sanitation and wide spread faecal

contamination of the environment. Enteric infections, particularly due to bacterial pathogens are readily transmitted under these circumstances. In contrast, the majority of inhabitants of industrialized countries live in a sanitary environment that generally prevents the transmission of enteric pathogens, particularity bacteria. However, in both ecologic niches, changes in human ecology and behaviour can lead to the emergence of certain enteric infections. Relevant factors in developing areas include urbanization leading to periurban slums, diminished breast feeding and political upheaval that results in population migrations. In industrialized areas, large scale food production (e.g., enormous poultry farms), distribution and retailing (e.g., fast-food chains) create opportunities where wide-spread and extensive out-breaks of food-borne enteric infections can ensue if break down in food hygiene measures occur (Levine and Levine, 1994).

Enteric infections in Ethiopia are not only widely spread in the general population but also occur in epidemic forms which indicates the low living standards and the level of socio-economic development (Seyoum et al., 1981; WHO, 1987; Helmut and Zein, 1988). About $3.9x10^7$ diarrhoeal episoides per year with approximately 230,000 deaths and with annual incidence ranging from 3 to 5 episodes per child per year occurs in children under 5 which probabily underestimates the actual morbidity and mortality rates (Helmut and Zein, 1993). In adults both etiologies as well as morbidity and mortality rates are not studied well (Helmut and Zein, 1988). In addtion, diagnostic problems associated with multiple infections lead to gross errors of reporting, which further

complicates the prescription of drugs that are with potential side effects. As long as the low level of sanitation and living standards are not improved, these infections will remain as a serious morbidity and mortality problem in this country (Tesfa-Michael and Tekle-Mariam, 1983; Helmut and Zein, 1993).

Adequate and current information concerning the levels of diarrhoeal associated enteric infections, their drug resistance and, plasmid profile pattern among adult population is not available in Ethiopia. Investigations concerning drug resistance and/or prevalent serogroups among *Shigella* and/or *Salmonella* were conducted by Messele and Alebachew (1979, 1981, 1982), Afeworki and Yetnebersh (1980), Mogessie (1983), Afeworki and Eyassu (1984) and Afeworki (1985). Their findings showed the dominance, in the order of, *Shigella flexneri, Sh.dysenteriae, Sh.boydii* and *Sh.sonnei among Shigella* and *Salmonella typhi* and *S.typhimurium* among *Salmonella* (Messele and Alebachew, 1981; Afeworki, 1985).

Almost all major drug resistance mechanisms have been described in Enteric bacterial pathogens. Drug resistance usually emerges as a result of resistance plasmids which carry their own genes for replication and transfer. These are called resistance (R) factors which carry genes for resistance to one and often several antimicrobial drugs (Grinsted and Bennett, 1988). The study of plasmids either as an epidemiological marker or as a genetic element encoding drug resistance or virulence factors, has greatly increased the understanding of enteric pathogens. In most circumstances the presence of a particular plasmid and its plasmid profile is indicative of pathogenicity and used in epidemiological investigation and

clinical microbiology (Mark *et al.*, 1992). In Ethiopia the plasmid profile of multiple drug resistant bacterial species has not been studied extensively, except for a few studies conducted by Afeworki and Drasar (1988; 1990a; 1990b; 1991) on *Shigella flexneri and Shigella dysenteriae* types. No plasmid profile studies have been conducted on *Salmonella* strains in this country.

Similarly no study has been conducted concerning the relative importance of bacterial and parasite (both protozoal and helminth) related diarrhoea among adult diarrhoeal patients in Ethiopia. Multiple etiologies which are common problems in the diagnosis of most diarrhoeal patients have not been dealt well. Faced with declining resources, expanding population and spread of acquired immunodeficiency syndrome (AIDS), human beings encounter the newly emerging pathogens, among which *Cryptospridium* may be one. Most clinical laboratories abroad routinely screen for this parasite (Cook, 1988) but no such attempt has been made in this country (personal communication with laboratory heads of Black Lion and Zeuditu hospitals). Therefore, the study was conducted with the objective to :-

1. Determine the extent of involvement of bacterial (particularly *Salmonella* and *Shigella*) and parasitic diarrhoeal etiologies including *Cryptosporidium* as well as their simultaneous occurrence in the adult out-patients in Addis Ababa.
2. Determine the susceptibility of *Salmonella* and *Shigella* isolates to commonly used antimicrobial drugs and their current drug resistance patterns.
3. Determine plasmid profiles of selected drug

resistant *Salmonella* and *Shigella* isolates from adult out-patients in Addis Ababa.

Rotavirus, *Yersinia* species, *Campylobacter* and various forms of *E.coli* (Enteropathogenic, Enterotoxigenic, Enteroinvasive, Enteroadherent and Enterohemorrhagenic groups which are with complex and interrelated antigens) have been excluded from this study mainly due to technical problems associated with their identifications.

II. LITERATURE REVIEW

1. The Epidemiology, Aetiology and Impact of Diarrhoea

Diarrhoea is usually defined as the passage of three or more loose or watery stools in a 24 hour period or occurrence of unformed bowel movement at least twice that of the usual daily rate, a loose stool being one that would take the shape of a container (WHO,1992). Diarrhoea could be acute (with Sharp onset and usually lasts less than 14 days) or persistent (which lasts for 14 days or longer) with or without dysentery (blood in the stool). Both types of diarrhoea could be caused by viruses, bacteria, parasites or fungi but acute diarrhoea is usually associated with bacterial, viral and fungal aetiologies while persistent diarrhoea is mostly associated with parasites (WHO, 1992; Blake *et al.*, 1993). In tropical regions diarrhoea is frequent during warmer, rainy seasons while in temperate regions highest frequency is observed during summer (WHO, 1992).

Most enteric infections are asymptomatic, and the proportion that is asymptomatic increases beyond 2 years of age owing to the development of active immunity (Blake *et al.*, 1993). During asymptomatic infections, which may last for several days, weeks or months, stools contain infectious agents and people with such infections play an important role in the spread of many enteric pathogens, especially as they are unaware of their infection, take no special hygienic precautions and move normally from place to place.

Diarrhoea is an important cause of malnutrition. This is

because people with diarrhoea eat less and their ability to absorb nutrients is reduced. Moreover, their nutrient requirements are increased as a result of the infection (due to metabolic demands associated with fever, need to repair damaged gut epithelium and the need to replace serum protein lost through damaged intestinal mucosa) (Guerrant *et al.*, 1990). Thus, diarrhoea and malnutrition combine to form a vicious circle and if the right measure is not taken, can eventually result in death which is more prominent among children than adults. It also affects a country's economy by reducing the health of its work force and increased cost of medication.

2. Parasite Related Diarrhoea

The tremendous health impact of parasite related diarrhoea on both morbidity and mortality in many developing areas must be recognized and controlled along with correction of food shortages in order to improve the nutrition, growth, and survival of an impoverished population (Gross *et al.*, 1989). The most important diarrhoeal related parasitic infections include *Entamoeba histolytica, Giardia lamblia, Cryptosporidium parvum, Trichuris trichiura, and Strongyloides stercoralis* which are found through out the world (WHO, 1980; Guerrant *et al.*, 1990). The rational management of diarrhoeal infections requires the highly selective use of laboratory methods for varied aetiologic agents depending on the clinical and epidemiological settings.

Parasites causing diarrhoea can be grouped according to their site of infection. Some parasites (e.g., *G.lamblia, Cryptosporidium, S.stercoralis, and Capillaria philippinensis*) cause diarrhoea primarily in the small bowel. Others inhabit the large intestine (e.g., *E.histolytica, Balantidium coli (B.coli), and T.trichiuria*) and in general cause an exudative type of diarrhoea though some times diarrhoea can be caused by decrease in absorptive capacity of the colon especially when large mucosal areas are altered structurally as a result of an intense infection.

Some of the chronic diarrhoea of parasitic origin (e.g., amoebic dysentery, intensive trichuriasis, cryptosporidiosis) are complicated by bacterial enteric infections. Co-existence of malnutrition and parasitic infection is not infrequent and functional intestinal disorders can be concomitant with intestinal parasitic infections or can result from past infection, complicating the classification of parasitic diarrhoea and the understanding of the mechanisms of pathogenesis (WHO, 1986/87; Mcadam, 1989).

In general, infection with parasites that cause diarrhoea follow an endemic pattern similar to that of infections with other commonly occurring intestinal parasites. However, focal epidemic can occur when large groups are exposed (e.g., in water-borne epidemics of amoebiasis, giardiasis, and balantidiasis). Some parasites are related to the occupational activity of the host,e.g., balantidiasis among pig breeders and intestinal capilariasis among fishermen.

The severity of parasite related diarrhoeal manifestations are related with intensity of infection and can be grouped on

the basis of their clinical and public health importance as amoebiasis, giardiasis, cryptosporidiasis, trichuriasis, and strongyloidiasis whose infections are with a global distribution in which diarrhoea is a common symptom, where as balantidiasis, schistosomiasis, and capillariasis are also infections in which diarrhoea is a common symptom, but are more of regional or local importance and are related with occupation.

Amoebiasis is a ubiquitous disease which is particularly important problem in developing tropical countries. The causative agent, *Entamoeba histolytica*, was first described in 1875 by Losch, a Russian physician, while in 1903, Schaudinn named the species (Beaver *et al.*, 1984). According to WHO (1987), it has been estimated that probably 480 million people carried *E. histolytica* in their intestinal tract, 36 million developed invasive forms and 40,000 died as a consequence.

Its transmission is by the faecal-oral route, usually through contaminated food or water. In some countries, as many as 80% of persons with intestinal infection are asymptomatic; the remainder have symptoms ranging from chronic mild diarrhoea to fulminant dysentery. Among extra-intestinal complications the commonest is hepatic abscess, which may rupture in to peritoneum, pleura, lung or pericardium with or without other intestinal complications (Guerrant and Bobak, 1991). Amoebiasis may be more severe during pregnancy, lactation, in persons with immunodeficiency, homosexuals and among travellers (WHO, 1980).

The epidemiology of amoebiasis has been well studied in several countries. The prevalence of *E.histolytica* infection was found to be from 3.6 to 47.4% in India; 11.2% in Lagos,

Nigeria; 7% in Bangkok, Thailand; 50% in Colombia; 4% in USA; 6% in Argentina; 7.5% in Brazil, 15% in Mexico city and 2 to 5% in England (WHO, 1980; Guerrant *et al.*, 1990). Amoebiasis is a common infection in Ethiopia with a prevalence rate ranging from 4 to 36% (Seyoum *et al.*, 1981; Shibru, 1986).

The exact mechanism of disease pathogenesis in amoebiasis is unclear (Beaver *et al.*, 1984). Encystation occurs in the lower ileum and colon. Colonization is dependent on several factors including the number of active trophzoites present. Virulence of *E.histolytica* has been associated with a number of factors like strain variation, viral and bacterial association, hosts iron level and physiological states (Dupont and Pickering, 1980; Beaver *et al.*, 1984).

Human *Cryptosporidium* (organisms with hidden sporocysts) infection was considered to be both rare and zoonotic before advent of AIDS and Clarke (1895) was the first to observe this organism (Cook, 1988). Prior to 1980, infection with species of *Cryptosporidium* were considered rare in animals and in man (Nime *et al.*, 1976). They were thought to be the result of a little-known opportunistic pathogen of immune-deficient individuals out-side its normal host range. Since 1982, the concept of these protozoan parasites began to change in to that of important, wide spread causes of diarrhoeal illness in several animal species, including humans (Cook, 1988).

The first cases of human cryptosporidiosis were reported in 1976 (Nime *et al.*, 1976) and subsequent reports were rare until it was recognized that *Cryptosporidium* (now believed to be *C. parvum*) may produce a short term diarrhoeal illness in immunocompetent persons and a prolonged, life-threatening,

cholera-like illness in immune-deficient patients, especially those with AIDS (Mcadam, 1989).

The epidemiologic features of cryptosporidiosis is transmission by environmentally resistant oocysts. Existence of numerous potential reservoir hosts for zoonotic transmission like calves and other mammals; person to person and water borne transmission due to oocysts resistant to chlorination and other common disinfectants has been documented (Mcadam, 1989).

The prevalence study of cryptosporidiosis shows world wide distribution with highest infection observed in poorly developed regions. For example, prevalence rates reported in surveys from Europe (1-2%) and North America (0.6-4.3%) were lower than those reported from Asia, Australia, Africa and Central and South America (3-20%) (cook, 1987). Children Usually had a significantly higher prevalence than adults and that infections were often seasonal with a higher prevalence during warmer, wetter months (Cook, 1988).

Characteristically, the diarrhoea is profuse and watery. It may contain mucus but rarely blood and leucocytes and often associated with weight loss, abdominal pain, nausea, vomiting and low-grade fever. The duration of symptoms and the out come usually vary according to the immune status of the host (Mcadam, 1989). AIDS and immune-compromised patients usually experience a prolonged life-threatening illness while most immunocompetent patients with good nutritional status experience a short term illness which lasts from 3 to 12 days with spontaneous recovery (Fleming, 1990).

Although, the pathological mechanisms of *Cryptosporidium* induced diarrhoea are poorly defined at present, studies in

calves infected with *C. parvum* suggest that malabsorption and impaired digestion in small bowel coupled with malabsorption in the large intestine are major factors responsible for diarrhoea (Mcadam, 1989). A change in the osmotic pressure across the gut wall causes an influx of fluid into the lumen of the intestine. However, the secretory (cholera-like) diarrhoea common to immune-deficient patients with cryptosporidiosis suggests a toxin mediated hypersecretion into the gut (Cook, 1987; 1988).

Giardia lamblia was described by Lambl in 1859, however, Leeuwenhoek actually may have been the first to describe the agent in his own diarrhoeal stool (Beaver, 1984). *G. lamblia* is the most commonly isolated intestinal parasite throughout the world and is especially prevalent in children's of developing countries. The *Giardia* cyst is highly infectious for humans and patent infections can be established by the ingestion of as few as 10 viable cysts (Dupont and Pickering, 1980). The cyst can be ingested from a variety of different sources like contaminated water and food, sewage, from asymptomatic carriers, from reservoir animals like beavers and through anal-lingual sex among homosexuals (Beaver, 1984).

According to Mcadam (1989), 200 million *Giardia* infections and 500 thousand diseases per year are encountered in Africa, Asia and Latin America, where as in global scale, prevalence rates range from less than 1% to more than 50% (Gutierrez, 1990). Between, 1965-1981, 53 water borne outbreaks of giardiasis were reported, affecting over 20,000 people in the USA (Brock and Madigan, 1988). Taylor *et al.* (1991) reported 20% prevalence of *Giardia* infection among institutionalized

orphans in Thailand. In Ethiopia, the prevalence rate of *G.lamblia* was reported as ranging from 9.3% to 23% (Seyoum *et al.*, 1981; Shibru, 1986).

The average incubation period of giardiasis is 8 days, with a range of 3-42 days (Dupont and Pickering, 1980). Clinical manifestations vary from a total lack of symptomatology to an acute short-lasting diarrhoea syndrome to a chronic malabsorption syndrome with a low grade fever, nausea, cramping,abdominal pain, bloating and flatulence (Gutierrez, 1990).

Malabsorption is common in giardiasis and may be due to mucosal injury or due to a physical barrier to absorption created by the shear numbers of trophozoites lining the intestinal surface or due to altered intestinal motility (WHO, 1980). The sucking disk of the trophozoite has been shown by electron microscopy to produce damage to the small bowel microvilli, with atrophy of jejunal mucosa. Depression of other intestinal enzymes , defects in vitamin B_{12} absorption disaccharidase deficiency have been described in patients with giardiasis (Beaver *et al.*, 1984). An overgrowth of bacteria may occur in the upper gut of patients with giardiasis which contribute to the malabsorption and malnutrition complicating pathogenesis.

Strongyloides stercoralis was first found by Normand (1876) from a patient who had been suffering from uncontrollable diarrhoea in Cochin, China and coined by Bavay in 1876 (Beaver., 1984). It is a soil transmitted intestinal nematode causing infection in the human duodenum and jejunum. While Man is the important host of *S.stercoralis*, dogs and cats

have been found naturally infected with strains indistinguishable from those in Man (Banwell, 1989). The infective stage larvae are developed in the soil or faeces, from which man become infected typically through skin contact. Autoinfection may be responsible for strongyloidiasis in persons who have had no direct contact with infested soil for years (Gutierrez, 1990).

Strongyloides stercoralis is widely distributed in the tropics and sub-tropics. It is more prevalent in the adults than in children in the general population but it has been reported as highly prevalent in homes for retarded children, even in temperate regions (Mcadam, 1989). Dogs are readily susceptible to infection with strains of *S.stercoralis* in humans, and dogs as well as humans can acquire infection through faecal contamination by infected dogs (Gutierrez, 1990). The distribution and prevalence of *S.stercoralis* was found to range from 1.4 to 44% (Seyoum *et al.*, 1981; Shibru, 1986).

Gastrointestinal symptoms, particularly diarrhoea and central epigastric pain are common in strongyloidiasis. Diarrhoea is due to malabsorption but the cause of the epigastric pain is not clear but may be due to invasion of adult worms to the stomach, causing ulceration (Beaver *et al.*, 1984). In the most severe infections, there is ulceration, villous enlargement and mucosal atrophy. The diarrhoea may be profuse and may cause dehydration and electrolyte disturbance. Malnutrition, concomitant infections, some malignant diseases and immunosuppressive therapy can intensify strongyloides infection and even make the disease fatal (Gutierrez, 1990).

Trichuris trichiura was first described by Lennaeus in 1771 (Beaver *et al.*, 1984). It is an intestinal nematode and belongs to the group of soil transmitted helminths. Although it has been shown in a volunteer that *T.suis*, parasitizing pigs, can infect man, infections of zoonotic origin probably play a minor role, if any, in the epidemiology of human trichuriasis (WHO, 1980).

Trichuriasis is a common infection, especially in the warm parts of the world where there is much rain fall. In some tropical countries, the prevalence rate is over 90%, in others it is frequently between 30 and 60% (WHO, 1980). The prevalence rate was 50% in Colombia; 36% in Poland (WHO, 1980) and to % in Ethiopia (Seyoum *et al.*, 1981; Shibru, 1986). Infections with over 5000 *T. trichiura* eggs per gram of stool are usually symptomatic and most of those with more than 20,000 eggs per gram of stool develop severe diarrhoea or dysentery (WHO, 1980).

The morbidity associated with trichuriasis is due to the worms unique mode of attachment to the wall of large intestine (Gutierrez, 1990). The worm has a thin anterior part with which it burrows into the intestinal wall where it feeds on the intestinal tissues. Light infections do not produce significant lesions in the large intestine. Heavy infections with loads of adult worms produce necrosis of mucosal cells around the parasite, intensive cellular infiltration and inflammatory reaction of the mucosa which results in diarrhoea and dysentery (Beaver *et al.*, 1984; Gutierrez, 1990).

In many other intestinal parasitic infections, diarrhoea is not a characteristic symptom, but may occur occasionally in

severe hook worm disease, ascariasis and some other intestinal nematode and cestode infections (WHO, 1980; Mcadam, 1989).

3. The Family Enterobacteriaceae, Particularily the Genera *Salmonella* and *Shigella*

The Enterobacteriaceae comprise a relatively homogenous phylogenetic group within Eubacteria and are characterized phenotypically as follows: gram negative, non-sporulating rods, non-motile or motile by peritrichous flagella, facultative aerobes. They are oxidase negative with relatively simple nutritional requirements, fermenting sugars to a variety of end products with many other complex biochemical reactions (Collee *et al.*, 1989).

Many species of enterobacteria have strains that are pathogenic to humans and probably more is known and remains about *Salmonella and Shigella*. Because of the medical importance of these bacteria, an extremely large number of isolates have been studied and characterized. Since these organisms are frequently cultured from diseased states, some means of recognizing and designating them is necessary largely for practical reasons. In many diagnostic tests performed to help identify an organism, different biochemical reactions are conducted to detect the presence or absence of enzymes involved in the catabolism of the substrate added to the medium (Brock and Madigan, 1988)

Positive identification of enteric bacteria often presents considerable difficulty because of the large number of

strains that have been characterized. Almost any small set of diagnostic characteristics will fail to provide clear-cut distinctions between genera if a large number of strains are tested, because some exceptional strains will probably be isolated. In advanced clinical laboratories, identification is now based on computer analysis of a large number of diagnostic tests, carried out using miniaturized rapid diagnostic media kits, with consideration being given for variable reactions of exceptional strains (Krivoshein, 1989).

3.1 *Salmonella*

3.1.1 Definition and Classification

The genus *Salmonella*, named after an American veterinarian D.E.Salmon conform to the definition of family Enterobacteriaceae and tribe *Salmonellae* (Edwards and Ewing, 1972). The bacterium is gram negative and with few exceptions, is motile. Members of the genus *Salmonella* are usually pathogenic, either to man or to other warm blooded animals. The *Salmonella* are characterized immunologically on the basis of three cell surface antigens; the O or cell wall (somatic) antigen; the H or flagellar antigen; and the Vi (outer polysaccharide layer and analogous to the K antigens) antigen. At present, approximately 2200 serotypes are known to exist based on the O-antigen groups (which are heat stable) and the numerous H-antigens (Which are proteins and labile to heat and acid treament) recognized so far (Michel and Leon, 1992).

The classification of *Salmonella* species has been controversial for several years and according to Edwards and Ewing (1972), the genus contains only three species:*Salmonella*

cholerae-suis, S.typhi and *S.enteritidis*. Recently, it has been proposed that the genus consists of a single species (*Salmonella* enterica) which can be subdivided in to six separate but related subspecies (Michel and Leon, 1992).

The confusing nomenclature associated with this genus may never be eliminated because, many of the serotype designations (e.g., *S.typhi, S.cholerae-suis, S.enteritidis*) are well established in the medical literature and are likely to remain in common usage. However, the taxonomy of this genus is now established as a single species and a corresponding nomenclature will be proposed based on the DNA-DNA hybridization, biochemical, and serological characters (Michel and Leon, 1992).

From an epidemiological point of view, *Salmonella* can be classified into three main groups. The first group comprises *Salmonella typhi and paratyphi A and C*, which infect only man and are spread either directly or indirectly (via food and water) from person to person. The second group includes serovars that are host adapted for particular species of vertebrates, e.g., *S.gallinarium* in poultry, *S.abortus equi* in horses, *S.abortus ovis* in sheep and *S.cholerae-suis* and *S.typhi-suis* in swine. The third group consists of the majority of other *Salmonella* serovars with no particular host preference that infect both man and animals (WHO, 1988)

3.1.2 Epidemiology Of Salmonella Infection

Salmonellosis is one of the most prevalent zoonoses in

developed countries. For example, in USA, 40,000 cases of Salmonellosis in man are reported by physicians each year, and some epidemiologists believe that the actual number of persons infected annually with zoonotic *Salmonella* may be as many as $4x10^6$ with 18,000 hospitalizations and 500 deaths (Cohen and Tauxe, 1986). In England and Wales more than 80% of cases reported under the surveillance programme for food borne diseases, are due to *salmonella* infection. Reports from other developed countries support the conclusion that salmonellosis alone or together with other zoonotic diseases, poses a significant health problem through out the developed world (Cohen and Tauxe, 1986).

In the developing countries of South America, diarrhoeal diseases, especially due to salmonellae are one of the leading causes of morbidity and mortality. In other developing countries the extent of the enteric infections, including salmonellosis, is still unclear, although the incidence of diarrhoeal diseases is wide spread. Studies in some of these countries showed that salmonellae are isolated from 1 to 8% of people with diarrhoea and in some urban areas of developing countries where industrially processed food is commonly consumed, cases and outbreaks of *Salmonella* infection in man, especially in adults, are found to be more common than in rural areas (Guerrant *et al.*, 1990).

A special problem associated with salmonellae, and one that is increasing in importance through out the world, is that of nosocomial infection, which often causes explosive outbreaks of salmonellosis in hospitals, particularly in maternity, paediatric, and geriatric units and among chronically sick and

immunodepressed patients. These outbreaks often result in high mortality rate, are difficult to control, and can persist for long periods of time. In such outbreaks, the salmonellae are usually spread by indirect transmission. The original source of infection could be man, animal, food, or the environment (Levine and Levine, 1994).

The prominent epidemiologic factor is the common carrier status in the animals. The absence of symptoms in the majority of infected animals and the technical difficulties in detecting such carriers before or during meat inspection make them a continuous source of contamination of the environment and of animal products. A great number of mammals, birds, poultry and reptiles are responsible for the maintenance of the chain of the infection. Once these animals are killed, the products made from them help the spread of infection if conditions permit the survival and multiplication of the bacteria (Richter and al-Sheddy, 1990). Salmonellae may also be isolated from the environment, including surface waters, sewage effluent, sewage sludge and agricultural products contaminated by polluted water, and man may be infected by any of these sources (WHO, 1988).

3.1.3 Pathologic Mechanisms Of *Salmonella* Infection

After the organissm have been swallowed, they must reach the intestine before they can cause disease. This means getting through the stomach, *Salmonellae* can be destroyed by the acid conditions there. Having survived the passage through the stomach, *salmonellae* must now establish themselves in the

small intestine. To do this, they must adsorb to the intestinal epithelial cells, and one intestinal property which tends to prevent this is intestinal motility or peristalsis. So chemicals or drugs that slow down bowel movement increase the severity of Salmonellosis (Christie, 1980)

After adsorption to the epithelial cells of small intestine and colon, they induce fluid secretion and exsorption, probably due to enterotoxin production. Murry *et al.* (1990) has reported that *S.typhimurium* can produce a cholera like enterotoxin that causes elongation of chinese hamster ovary cells and exhibits vascular permeability factor activity in the rabbit skin, and one can assume similar pathogenesis in man too. Histologic changes ranging from disruption of surface epithelium and multifocal microabscesses have been observed in *Salmonella* gastroenteritis.

In experimental infection, rhesus monkeys fed *Salmonella* developed symptoms similar to those seen in humans with *Salmonella* gastroenteritis (Brock and Madigan, 1988). The primary sites of monkey infection were found to be the colon and ileal mucosa. Mild focal lesions were also noted in the stomach and jejunum. Histologically, the inflammatory lesions was nonspecific, fluorescence studies showed that the *Salmonella* were within colonic epithelial cells and lamina propria. *S.typhimurium* was frequently recovered from mesenteric lymph nodes, blood and rarely in low density from liver and spleen. Bacteria were invariably found to be deeper in the mucosa and were less confined to the epithelium (Murray *et al.*, 1990).

In typhoid fever of humans, a non specific enteritis has

been shown to develop in the upper gut soon after ingestion of *S.typhi*. Random invasion by the organism produces degenerative and proliferative changes in the epithelial lining of villi, crypt cells and lamina propria. After the early invasion of the intestinal epithelium, there is rapid clearing of the invading strain, and extensive multiplication of the organism occurs in the regional lymph follicles and mesenteric lymph nodes. From these sites, *S.typhi* spreads to the thoracic lymph nodes and then into the general circulation. The typhoid fever which developed in chimpanzees after intravenous or intramesenteric lymph node challenge with *S.typhi* was identical to the illness produced in these chimpanzees after oral inoculation. These studies verify the systemic nature of the infection and suggests that an initial intestinal phase is not necessary for the establishment of typhoid fever (Dupont and Pickering, 1980; Krivoshein, 1989).

3.1.4 Survival and Current Drug Resistance Pattern

When compared with other gram negative rods, salmonellae are relatively resistant to various environmental factors. They grow at temperatures between eight and forty five and in a pH range of four to eight in an environment with a low level or no oxygen. They have been shown to be resistant to drying, even for years, especially in dried faeces, dust, and other dry materials such as feeds and certain foods. Processes such as salting and smoking have a limited effect on the survival of salmonellae; several months survival has been observed in brine containing more than 20% salt and in smoked dry meat. In

addition to this, resistance to freezing explains why these organisms survive in many kinds of food (Collee *et al.*, 1989).

Since the discovery that *Salmonella* strains were inhibited by antimicrobial agents *in vitro*, these drugs have been employed in the treatment of human salmonellosis. However, resistance to various antimicrobial agents among food-poisoning strains of *Salmonella* has emerged with time in various geographic locations. Non-administered drug usage and routine sub-therapeutic use of antimicrobials as growth promoters in animal feed have led to the emergence of multiple drug resistance (Diaz-de-Aguayo *et al.*, 1992).

Transferable plasmid mediated resistance has been found among *Salmonella* strains to streptomycin, tetracyclines, sulfonamides, chloramphenicol, ampicillin and kanamycin (Santoes *et al.*, 1989). Studies in Great Britain have shown that antibiotic resistance in *S.typhimurium* has emerged among calves fed tetracyclines and such resistant strains could be shown to spread to dairy cattle and to humans (WHO, 1988).

The drug of choice in salmonellosis is chloramphenicol and prior to 1972 (Santoes *et al.*, 1989), only a few strains of *Salmonella* had been reported as resistant to chloramphenicol. However, a major concern has been the development of chloramphenicol resistance among strains of *Salmonella* responsible for large number of cases in Mexico, India, Vietnam, Thailand, Spain, U.S.A and Europe (Santoes *et al.*, 1989). It is clear that drug resistance among *Salmonella* strains has developed in many parts of the world and the situation is more complex and disappointing in the treatment of patients and the carrier state with already available drugs.

3.2 *Shigella*

3.2.1 Definition and Classification

The genus *Shigella*, named after a Japanese bacteriologist K.Shiga conform to the definition of family *Entero*bacteriaceae and the tribe *Escherichieae* (Edward and Ewing, 1972). The *Shigellae* are a group of gram negative, non-motile organisms which are differentiated from other enterobacteria more because of their ability to cause specific disease in man than by the reason of any definite pattern of fermentative activity. These organisms form a fairly homogeneous group of non-lactose fermenters, with the exception of *Shigella sonnei* which ferments lactose late. *Shigella* are non-sporulating, aerobic or facultatively anaerobic, non-aerogenic bacteria with a few exceptions (Collee *et al.*, 1989). These are divided into four sub-groups (serogroups) and each subgroup is differentiated into different serotypes based on biochemical characteristics, agglutination reaction, intragroup antigenic relationships and in part upon tradition (Collee *et al.*, 1989; Krivoshein, 1989).

Group A consists of organisms which do not ferment mannitol. The group name is *Sh.dysenteriae* and there are 10 serotypes. Group B organisms ferment mannitol and the group name is *Sh.flexneri* with 6 serotypes and 9 subtypes. Group C contains a group of mannitol fermenting organisms which resemble *Sh.flexneri* in their biochemical reactions, but are antigenically distinct and the group name is *Sh.boydii* with 15 serotype. Group D contains only *Sh.sonnei*, a mannitol and late lactose fermenter (Barbara *et al.*, 1987).

3.2.2 Epidemiology of *Shigella* infection

Primates, including humans, are the only hosts naturally susceptible to infection by *Shigella*. The majority of bacillary dysentery cases which occur in the developed and developing countries are spread from person to person by the faecal-oral route. In developing countries where personal hygiene practices are primitive, sanitary conditions are poor and malnutrition common, shigellosis is usually endemic and is an important cause of morbidity and mortality. Occasionally epidemic forms also occur. In developed countries, shigellosis is endemic in mental institutions and among disadvantaged, living in crowded conditions such as in the inner cities (Ferreccio *et al.*, 1991).

Faecal excretion of the infecting strain of *Shigella* usually lasts for one to four weeks if not treated. Long-term excretion (greater than a year) rarely occurs but has been documented in patients residing in custodial institutions and in others, often with co-existent intestinal parasitic infections. A study carried out in a residential institution for mentally retarded in Mexico demonstrated a role in hand transmission in shigellosis. Of people with positive stool cultures for *Shigella*, 10% had positive finger cultures for the same strain, while other patients had *Shigella* organisms recovered from cultures of fingers and hands at a time when stool findings were negative (Taylor *et al.*, 1991). The finding of *Shigella* in stools of asymptomatic students in Mexico suggests that healthy transient carriers may be a

reservoir of these organisms and a potential source of infection (Santos *et al.*, 1989).

Transmission rates of shigellosis are increased by crowding and by reduced income and personal hygiene. The unusually high communicability of shigellosis is partially explained by the finding of a low inoculum of bacteria (10^1 to 10^2 organisms) necessary to produce illness in humans (Ferrecco *et al.*, 1991). Such a low dose is unique to shigellosis among bacterial pathogens and helps to explain why shigellosis is the most common form of bacterial diarrhoea acquired by microbiology laboratory workers. Flies may play a role in disease transmission, especially in tropical climates. Dysentery in tropical parts of the world is most prevalent during peak fly season and bacteriologic studies of flies indicate their potential role in disease transimission (Krivoshein, 1989).

Man is both the reservoir as well as the natural host of *Shigella*. Water and toilet facilities are highly associated with transmission of *Shigella*, especially in developing countries (Ferrecio *et al.*, 1991). A newly identified mode of *Shigella* infection is thought to be via faecal ingestion during analingus (venereal) transmission. Currently, *Sh.sonnei* accounts for between 60 and 80% of the reported cases in the developed countries, and *Sh.flexneri* serotype accounts for most of the remaining cases. In developing countries *Sh.flexneri* is the dominant serogroup although others also co-exist (Guerrant *et al.*, 1990).

3.2.3 Pathologic Mechanisms of *Shigella* Infection

The primary virulence property of *Shigella* is invasiveness, whereby the infecting strain is capable of penetrating the epithelial lining of the gut (Taylor *et al.*, 1991). The bacilli penetrate intact epithelial lining and reach the lamina propria within a few hours. Epithelial changes range over a wide spectrum from severe degenerative alterations to increased cellular activity. Prior to invasion, the bacilli attach to the surface of epithelial cells in which invasion unevenly affects the cells, with disappearance of goblet cells and extrusion of epithelial cells, leading to the formation of micro-ulcers at the luminal surface. Crypt abscesses develop secondary to a focal accumulation of inflammatory cells. Treatments which decrease stomach acidity increase the rate and severity of the disease (Wachsmuth and Morris, 1989 ; Taylor *et al.*, 1991).

Shigellosis is a biphasic illness which depends upon the site of intestinal localization of the infecting strain. Twelve hours after ingestion of a virulent *Shigella* strain, the bacteria have been shown to transiently multiply in the small intestine to the concentration of 10^7 to 10^9 cells/ml of luminal content (Wachsmuth and Morris, 1989). Increased stool volume and higher water content (without dysentery) in early shigellosis reflect active secretion of electrolytes and fluid from the jejunal surface, and is characterized by abdominal cramps and fever. After one to three days the *Shigella* will be confined to lower abdominal quadrants. Urgence, tenesmus, and dysentery commonly are seen at this time when the organisms are

confined to the colon (Guerrant and Bobak, 1991). Thus the disease appears to be an expression of a descending infection of the intestinal tract, with the early symptoms corresponding to small bowel infection and later ones to colitis.

The importance of toxin production in the pathogenesis of the diarrhoeal syndrome has not been resolved entirely in Shigellosis. In the early part of the 20th century, the Shiga bacillus was shown to elaborate a neurotoxin which produced paralysis and death in mice and rabbits. More recently, the toxin of Shiga bacillus has been shown to have enterotoxic and cytotoxic properties (Brock and Madigan, 1989), a factor which may relate to the unusual severity of the illness. Release of the toxin in the small bowel, leading to out-pouring of fluid and electrolytes, may explain the watery diarrhoeal phase of early infection, or the toxin may be active after mucosal penetration by virulent organisms which contributes to the tissue damage and inflammatory reaction. It has also been observed that, both virulent and avirulent *S*higella strains are equally susceptible to phagocytosis by macrophages, but virulent strains are highly lethal to the macrophages (Dupont and Pickering, 1980; Murray *et al.*, 1990).

3.2.4 Survival and Current Antibiotic Resistance Pattern of *Shigella*

Shigellae are sensitive to heat and are killed in one hour at 55°c. At laboratory temperatures below this level, they survive for various periods of time, and the survival period increases with inoculum size, high relative humidity and shade.

Based on temperature and other conditions they survive very readily in milk, water and other foods, while they are easily killed by chemicals like phenol (Collee *et al.*, 1990).

In many countries high incidence of antibiotic resistance has been observed in *Shigella* and is almost always due to R-factors. Resistance to tetracyclines, streptomycin, sulfonamides, ampicillin and to other drugs also have been widely reported. Chloramphenicol resistance has been generally less common in the U.S.A.and Europe although frequent in Japan, central America, and Mexico. Resistance to quinoline drugs including nalidixic acid has been reported to be very low (Santos *et al.*, 1989; Murry *et al.*, 1990). From 1982 to 1988, *Shigella* strains isolated in Metro Manila, Philippines were tested for drug resistance and exhibited resistance to ampicillin, chloramphenicol, tetracyclines and streptomycin (Leano *et al.*, 1990).

A strain of *Shigella* isolated in Somalia in 1976 was resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracyclines. The eventual spread of this strain to Zaire and other central African countries was demonstrated by the presence of an identical plasmid. The strain was later noted to have acquired an additional plasmid conferring resistance to trimethoprim-sulphamethoxazole (Edward *et al.*, 1993). *Shigella* species isolated during the 1991/92 winter season at the French military hospital in Djibouti were highly resistant to amoxicilln, tetracyclines, trimethoprim-sulphamethoxazole (Edward *et al.*, 1993). The most common isolates have been *Sh.flexneri and Sh.boydii*. A number of isolates have been resistant to all commonly used antibiotics

like tetracycline, ampicillin, trimethoprim-sulfamethoxazole, clavulanic acid,amoxicillin/clavulanic acid and chloramphnicol.

In Mexico 100 *Shigella* strains isolated from stool cultures of dysenteric patients were tested for 8 antimicrobial agents and the highest susceptibility rates were observed with furazolidone (98%), nalidixic acid (97%), and gentamicin (90%). The same strains were significantly less susceptible to other antimicrobials like ampicillin and trimethoprim-sulfamethoxazole for which susceptibility rates of 40% and 64%, respectively were observed (Santoes *et al.*, 1989).

Reports from other countries indicate that drug resistance is posing a great problem in Thailand (Taylor *et al.*, 1991), Latin America (Santos *et al.*, 1989), Somalia (Casalino et al., 1994) and Brazil (Blake *et al.*, 1993). In Ethiopia, Afeworki (1985) suggested the emergence of drug resistant strains of *Salmonella* and *Shigella* which were not common before.

4. Origin of Drug Resistance and Analysis of Plasmid Profiles

The origin of drug resistance may be genetic or non genetic. In non-genetic drug resistance, microorganisms that are metabolically inactive (non-multiplying) or those lost (lack) the specific target structures for a drug or those become impermeable to the antibiotic due to impaired cell wall or cell envelope penetration and thus be resistant. The nature of the medium in terms of nutrient contents can have modifying influence. However, the bases of such resistance is not understood in many cases (Hugo and Russell, 1992).

Most drug resistant microbes have emerged as a result of

genetic changes and subsequent selection processes. Genetic changes may be chromosomal or extrachromosomal and can be transferred from one bacterial species to another by a variety of mechanisms like conjugation, transformation and transduction (Jawetz *et al.*, 1991).

Chromosomal resistance develops as a result of spontaneous mutation in a locus that controls susceptibility to a given antimicrobial drug. The presence of the antimicrobial drug serves as a selecting mechanism to suppress susceptibles and favour the growth of drug resistant forms. Antibiotic resistance mediated by chromosomal genes can arise because of a modification of the target of antibiotic action (change in receptor, transport protein, ribosome or other structural changes) or by producing a gene product with a reduced or absent affinity for the drug in question or by reducing a cell's need for a particular metabolic product or by reducing cellular permeability to the antibiotic resulting in its exclusion from the bacterium (Barbara *et al.*, 1987; Hugo and Russel, 1992). Such mutation occurs with a low frequency (10^{-8} to 10^{-12} cells per cell division) thus is an infrequent cause of emergence of clinical drug resistance in a given patient.

Extrachromosomal drug resistance is due to genetic elements called resistance (R) plasmids (factors). These are circular, double stranded DNA molecules that contain specialized genes, and have the ability to be replicated in the bacterial cell and are generally dispensable. They carry genes for resistance to one and often several antimicrobial drugs and are composed of two parts:the resistance transfer factor (RTF), responsible for transfer to other bacteria and various drug

resistance cistrons, comprising the R factor (Grinsted and Bennet, 1988). They are most common in bacteria from clinical and veterinary sources, where exposure to antibiotics is likely to be high. Genes from two or more R-factors can be integrated in one via recombination and is one means by which multiple drug resistant forms might have first arisen. Thus a single R-plasmid can contain a variety of genes coding for different antibiotic inactivating enzymes.

R-factors were first discovered in Japan from epidemic strains of *Shigella* in the late 1950's, and have since been found in other parts of the world (Grinsted and Bennet, 1988). Although specific evidence for the origin of multiple drug resistant R-factors is not available, a number of lines of circumstantial evidence suggests that plasmids with R-factor type character exist in the natural bacterial population before the antibiotic era and the wide spread clinical use of antibiotics provided selective pressure for their evolution and rapid spread (Brock and Madigan, 1988). The acquisition of drug resistance factors is a more efficient way of developing antimicrobial resistance than chromosomal mutation and is probably the most important way by which resistance is acquired in clinical isolates.

Transfer of short DNA sequences (transposons, transposable elements or insertion sequences that may carry resistance genes) occurs between one plasmid and another or between a plasmid and a portion of the bacterial chromosome and has helped to explain the wide distribution of resistance determinants among the plasmids and chromosomes of many different species. It is now known that transposons can insert

into plasmids or chromosomes in areas where no homology exists and independent of the presence of a recombination gene, thus facilitated the movement of resistance determinants among R-factors and chromosomes (Barbara *et al.*, 1987).

The dramatic rapidity with which R-plasmid mediated resistance spreads through bacterial population, especially among different genera and species of enteric pathogens like *Salmonella, Shigella and E.coli* coupled with frequent detection of R-positive bacteria associated with clinical infections, aroused serious fears for the future antibiotic treatment of diseases caused by gram negative bacteria. Under certain circumstances like epidemiologic studies the presence of a particular plasmid and its plasmid profile is indicative of pathogenicity (Mark *et al.*, 1992).

Plasmid profile analysis is a relatively new typing method and in this technique, bacterial strains are lysed and their plasmids are extracted in a standard way. The plasmids are then separated by agarose gel electrophreses and the size of the plasmid is calculated by comparison with molecular weight standards.

Plasmid profile analysis of *Salmonella* and *Shigella* has been used by several researchers for molecular epidemiological investigations. Tanabe *et al.* (1988) in Japan and Wu *et al.* (1988) in China analyzed plasmid profiles of selected multiple drug resistant *Salmonella* and *Shigella strains (isolates)* obtained from outbreaks. *Salmonella* isolates were resistant to commonly used drugs (chloramphenicol, ampicillin, tetracycline, streptomycin, carbencillin and co-trimoxazole) and contained 3-4 plasmids per strain with 192 and 96 Kba (Kilobase) plasmids

being the commonest. *Shigella* isolates were resistant to chloramphenicol, ampicillin, tetracycline and carbencillin and contained 3 to 6 plasmids per strain with 192, 115 and 20 Kba plasmids being the commonest. The method is recommended for the identification of the outbreak strains (those associated with epidemics) of nosocomial (hospital) and community acquired enteropathogenic infections as being rapid and efficient.

Nakamura *et al.* (1986) conducted plasmid profile analysis of *S.typhimurium* of animals reared in the same and different areas in Japan. They concluded that, when *S.typhimurium* strains isolated from animals reared in the same areas exhibit identical or similar plasmid patterns, they are most probably derived from the same source. When strains isolated in the same area exhibit quite a different plasmid pattern, these strains are most likely derived from independent sources.

Lin and Chang (1992) in Taiwan analyzed plasmid profiles of clinical isolates of *Shigella strains* with multiple resistance to ampicillin, chloramphenicol, tetracycline and streptomycin and found to harbour a large transmissible plasmid of 72 to 120 Kba. Schuman *et al.* (1989) in Illinois, USA, characterized multiple antibiotic resistant *Salmonella* strains and their plasmid analysis which revealed that the strains possessed plasmids of sizes from approximately 6 kilobase pairs up to 158 Kbp.

In Ethiopia, Afeworki and Drasar (1990a, 1990b, 1991) determined plasmid profiles from drug resistant *Sh.dysenteriae* and *Sh.flexineri* serotypes and found that, the former harboured a large plasmid of about 192 Kba, while the latter harboured a large plasmid of about 216 Kba. *Shigella dysenteriae* strains

which were isolated from diarrhoeal cases contained from 5 to 9 plasmids per strain ranging in sizes from 192 to 2.6 Kba in which 192 and 9.6 Kba plasmids were common features of all isolates while *Shigella flexneri* isolates contained 3 to 6 plasmids per strain ranging in sizes from 216 to 10.2 Kba in which 3.5 and 4.8 Kba plasmids were found to be the commonest. They concluded that plasmid profile analysis of the above strains in Ethiopia showed a high degree of uniformity within individual serotypes.

III. MATERIALS And METHODS

1. Collection of Stool Specimens

Diarrhoeal stool specimens were collected from selected Hospitals and Clinics of Addis Ababa using sterile buffered glycerol saline solution and buffer treated swabs (Collee *et al.*, 1989) for bacteriological isolation while for parasitological identification clean plastic vials with caps were used for collection. Name, sex and age of patients were labelled on both specimen containers.

2. Parasitological Investigation

2.1 Direct Wet Smear

This method was used to observe protozoal (cyst and trophzoites) and helminth (larva and ova) infections. A drop of saline (0.85%) and aqueous iodine solution (1% aqueous solution of potassium iodide saturated with iodine crystals) were placed separately on the same slide. About 2 mg (2 cubic mm) of faeces was placed separately on each and stirred until completely suspended. Then gross fibres and particles were removed and covered with a 22x22 mm cover slip. The preparation was first observed with middle power (x100) and then with high power (x400) objectives of the microscope. All direct smear preparations were examined within one and half hour of collection.

2.2 Modified Formol-Ether Concentration Technique

This technique described by Casmore *et al.* (1985) was used especially for the recovery of *Cryptosporidium* Oocysts and also cysts and ova of parasites as follows. About 1 ml (equivalent of diarrhoeal stool) of fluid stool specimen was emulsified in about 3 ml of 10% formalin in a beaker using a vortex mixer. Then 3 ml of ether was added and shaken vigorously for 30 to 40 seconds. Then 10% formalin was added to make the volume 15 ml, remixed and poured through a double layer of cheese cloth gauze into a centrifuge tube. This was centrifuged at 1000 rpm for one minute, then using a pipette, the fluid beneath the upper debris layer was carefully transferred to another centrifuge tube, while the pelleted sediment was used for examining cysts and ova of other parasites. Formalin (10%) was added to the transferred fluid to the level required for balancing the tubes and centrifuged at 3000 rpm for 10 minutes, and then the sediment was used for examination of *Cryptosporidium* Oocyst.

To examine *Cryptosporidium* oocysts, the modified acid fast staining procedure of Garcia *et al.* (1983) was used as follows. Sedimented material was placed on a slide and heat fixed at 70°C for 10 minutes before staining. The smear was then placed on a staining rack and flooded with carbolfuchsin. The slide was heated to steaming and allowed to stain for 5 minutes and the smear was rinsed with distilled water and decolorized with 5% Sulfuric acid solution for 30 seconds. The smear was rinsed again with distilled water, drained and the slide was flooded with methylene blue counterstain for 1 minute, rinsed with

distilled water, drained and allowed to dry. The preparations were then observed using high power (x400) and oil immersion objective.

3. Bacterial Isolation and Biochemical Characterization

The specimens were brought to the Microbiology laboratory of Biology department (Science Faculity), Addis Ababa university. Each specimen was plated directly on Oxoid primary media (Oxoid, England): MacConkey agar, *Salmonella-Shigella* agar (SS) and Brilliant green (BG) and streaked using a sterile loop. The same swab was then dipped in selenite enrichment broth and all were incubated at 37°C for 24 hours. Enrichment broth cultures were streaked on the same sort of agar plates used for primary isolation and incubated as above. Characteristic colonies were selected from plated media and using a straight sterile wire, a single colony was picked and inoculated in about 4 ml nutrient broth and incubated at 37°C for four hours until growth was ascertained by turbidity. (Edwards and Ewing, 1972; Collee *et al.*, 1989).

For biochemical characterization the broth cultures were inoculated using a straight sterile wire to the following tubes: triple sugar iron agar slant and butt to observe the fermentation of constituent sugars and H_2S production, to lysine iron agar to observe oxidative deamination and decarboxylation of lysine on the slant and butt respectively, urea agar slant to determine hydrolysis of urea, Simmon's citrate as a sole source of carbon, semi-solid agar medium to

determine motility, mannitol broth (1%) with Andrade's indicator to determine fermentation of mannitol, glucose broth (1%) with inverted Durham tubes and Andrade's indicator to observe fermentation of and gas production from glucose, peptone water for indole production test using Kovac's reagent. All biochemical tests were incubated at 37°C for 24 hours except indole production test for 48 hours (Edwards and Ewing, 1972; Collee *et al.*, 1989).

Cultures were made from broth inoculum on nutrient agar to check their purity after incubation for 24 hours at 37°C. Bacterial isolates suspected for *Salmonella and Shigella* characters during biochemical typing were inoculated onto nutrient agar slants and after overnight incubation at 37°C were stored at 4 to 6°C in screw capped test tubes sealed with parafilm and preserved as a stock culture.

4. Serogrouping

Bacterial isolates with biochemical tests identified as possible *Salmonella or Shigella* were serogrouped by slide agglutination tests as in Collee *et al.*(1989), using *Salmonella* and *Shigella* polyvalent and group antisera (*Salmonella* group A, B, C, D, *S.typhi* and E antisera as well as *Shigella* group A, B, C and D antisera) from Difco laboratories Inc, USA. A drop of sterile saline (0.85%) was placed on one side of clean microscope slide. A small amount of culture from triple sugar iron agar slant was emulsified in it by means of sterile loop. It was then examined using a hand lens (X10) to ascertain evenness of suspension and lack of clumps. A drop of the

antiserum was placed on the bacterial suspension and mixed by tilting to and fro for 30 to 60 seconds while viewing for possible clumping using desk top light. For control purpose, a drop of saline was placed on another slide and bacterial culture was emulsified with out antiserum as well as *Salmonella* and *Shigella* strains whose serogroups were known were used.

5. Antibiotic Sensitivity Testing

The Kirby-Bauer (Bauer *et al.*, 1966) standard and recognized agar disk diffusion procedure for drug susceptibility testing was used for the following antibiotics with stated amounts and abbreviations obtained from Difco, USA. Ampicillin (AM, 10 μg), carbenicillin (CB, 100 μg), cephalothin (CE, 30 μg), chloramphenicol (CL, 30 μg), gentamicin (GM, 10 μg), kanamycin (K, 30 μg), polymyxin B (PB, 300 units), tetracycline (TE, 30 μg), trimethoprim-sulfamethoxazole (SxT, 25 μg). Nalidixic acid (NA, 30 μg) was tested only for *Shigella* strains.

Each isolate was subcultured on a plate of MacConkey agar and about 5 to 6 colonies were transferred randomly to a test tube containing 4 ml of tryptic soy broth (TSB) and incubated at 37°C until evenly spread growth was attained and standardized using sterile physiological saline to recommended turbidity level of 0.5 McFarland. Sterile Muller-Hinton Agar (Oxid, England) was poured in amounts of 60 to 70 ml onto 150x15mm petridishes placed on a level surface. Then using a sterile cotton swab dipped in to broth culture, the entire surface of a Muller-Hinton agar was swabbed evenly and left at

room temperature for 5 minutes. Antibiotic disks were then placed on the surface of Muller-Hinton plates with spacing of about 24 mm between disks and pressed down gently with sterile forceps for even contact and incubated at 37°C for 24 hours. The diameters of inhibition zones were then measured by a graduated metal calliper to a nearest millimetre and the results were interpreted as resistant, intermediate and susceptible, according to the standard interpretative table of Bauer *et al.*, (1966). A few intermidiate readings observed were assumed as susceptible for convenience. A standard reference strain of *E.coli* (ATCC 25922), which is sensitive to all drugs was used for a quality control.

6. Plasmid Profile Analysis

Thirty multiple drug resistant *Salmonella and Shigella strains,* 15 from each, (with representative strains from each serogroups of both) resistant to the same or different combinations of tetracycline, ampicillin, chloramphenicol and two or three other drugs from above mentioned ones were used for plasmid profile analysis. Firstly, a rapid alkaline plasmid DNA extraction procedure of Birnboim and Dolly (1979) with 10 ml culture volume was used. However, this was found to be less efficient than a modified alkaline lysis protocol of using 100 ml culture followed by plasmid purification with a QIAGEN resin tips (Qiagen Inc, USA) (Sambrook *et al.*, 1989; Anonymous, 1992) and the latter protocol was followed.

A single bacterial colony from MacConkey agar plate was inoculated to a 100 ml of nutrient broth with appropriate

recommended concentrations of powder antibiotics from Difco laboratories Inc, USA (AM, 50 μg/ml; K, 10 μg/ml (filter sterilized); TE, 15 μg/ml; CL, 20 μg/ml; the latter two antibiotics were first dissolved in ethanol; Sambrook *et al.*, 1989). The culture was allowed to grow for about 14 to 16 hours in a shaking incubator at 180 revolutions per minute (Rpm) shaking at 37°C and all remaining steps were performed at room temperature.

The bacterial cells were then harvested by centrifugation at 5000 rotations per minute (rpm) for 15 minutes using a Sorvall Rc-2B superspeed centrifuge (Bio-RAD, USA), the supernatant was discarded and the remaining fluid was drained with an aspirator. The pellet was resuspended in 4 ml of buffer P1 (solution of 50 mM tris base with 10 mM EDTA, pH 8) with 1 mg/ml RNase A. Then the bacteria were lysed in 4 ml of buffer P2 (solution of 200 mM NaOH with 1% sodium dodecyl sulfate (SDS)) and neutralization buffer P3 (solution of 3 M potassium acetate with glacial acetic acid, pH 5.5) were added (to precipitate protein, chromosomal DNA and cellular debris), mixed and incubated for 15 minutes at room temperature and then centrifuged for 15 minutes at 8000 rpm. The supernatant was removed promptly and filtered using a folded Whatman filter paper (Sigma, USA). The QIAGEN-tip 100 was then equilibrated by applying 4 ml of QBT equilibration buffer (solution of 800 mM NaCl, 50 mM MOPS (Morpholino propane Sulfonic acid), 15% ethanol and 0.15% Triton x-100, pH 7) which facilitates flow through QIAGEN-tip and the above filtered supernatant was added onto a QIAGEN-tip and allowed to flow through by gravity. The QIAGEN-tip was then washed twice with 10 ml of buffer QC

(solution of 1 M NaCl, 50 mM MOPS, 15% ethanol, pH 7) to remove the plasmid DNA contaminants like carbohydrates. The plasmid DNA was then eluted to collect the plasmid DNA from QIAGEN-tip resin with 5 ml of buffer QF (solution of 1.25 M NaCl, 50 mM Tris base, 15% ethanol, pH 8.5) in to Sorvall plastic tubes (using QIArack 2; Cat no.19014, USA) by gravity and then the DNA was precipitated with 3.5 ml of isopropanol at 8000 rpm for 15 minutes using Sorvall RC-2B superspeed centrifuge. The supernatant was discarded and the pellet was washed with 5 ml of 70% ethanol (centrifuged for 2 minutes at 5000 rpm) and the supernatant was discarded and the DNA pellet was allowed to air dry and dissolved in 35 μl of TE (10 mM Tris-1 mM EDTA, pH 8)buffer.

To analyze the plasmid profile, agarose gel electrophoresis was conducted using 0.5% (w\v) agarose (FMC, Bioproducts, USA) in 1xTAE (40 mM Tris base, 0.1% acetic acid, 10 mM EDTA, pH 8) buffer which was boiled in a microwave oven (Sigma, USA) for about 5 minutes with frequent shaking. When the temperature of agarose solution was lowered to about 60°C, 0.5 μg/ml of Ethidium bromide (Sigma, USA) was added to stain the gel, poured on a horizontal casting tray (BRL, Model H4, USA) with a comb and left for about 20 minutes to solidify. The gel was placed in 1xTAE electrophoresis buffer (with 0.5 μg/ml EtBr). Then 10 μl of plasmid DNA was mixed with 2 μl of 5x loading buffer (0.25% bromophenol blue with 50% (v/v) glycerol in water), the mixture was loaded into the wells and allowed to run for 2.5 hours at 120 volts. Plasmids were visualized using transilluminated ultraviolet (UV) light at 310 nm and photographed using a polaroid camera (with black and

white type 667 film, Sigma, USA). For molecular weight determination of plasmid DNA, EcoRI and Hind III digested bacterophage lambda DNA standard fragments (21.227, 5.148, 4.973, 4.268, 3.530, 2.027, 1.904, 1.584, 1.375, 0.974, 0.831, 0.564 and 0.125 Kb pairs) (Sigma, USA) were used. It would have been preferable to use addtional molecular weight markers covering the whole range of possible Salmonella and Shigella plasmid sizes, however, these lambda molecular weight marker were the highest molecular weight markers that were available.

7. Statistical Analysis

X^2 (Chi-square) test was conducted to evaluate possible relation of infection frequency with sex and age groups.

IV. RESULTS

1. Diarrhoeal Aetiologies Identified and their Frequencies

Among 700 diarrhoeal samples collected from both male and female adult out-patients, 237 were from Tikur Anbesa hospital, 195 were from Zeuditu hospital, 145 were from Menelik II hospital, 78 were from Yekatit 12 hospital, and 45 were from Arada clinic. In 394 (56.3%) samples, one or more etiologic agents were identified, while in 306 (43.7%) samples the etiologic agents could not be identified. About 57% of samples collected from males and 55.6% of samples collected from females were found to be positive for one or more etiologic agents associated with diarrhoea (Table 1). The etiologic agents identified in single, double and triple infections were *Salmonella, Shigella, Entamoeba histolytica, Giardia lamblia, Strongyloides stercoralis, and Trichuris trichiura.* No quadruple infection was encountered. Ascaris and Hook worm ova were also observed, but their egg counts were much lower than the level recognized as heavy ascariasis (greater than 50,000 eggs per gram of stool) or heavy hook worm infection (greater than or equal to 20,000 eggs per gram of stool) and cannot reasonably be accounted as diarrhoeal etiologies (WHO, 1980). *Cryptosporidium* oocyst was not found in this investigation.

Among a total of 304 single infections, *Salmonella* was encountered in 13.2% of the infections, *Shigella* in 13.5%,

Entamoeba histolytica in 33.5%, *Giardia lamblia* in 28%, *Strongyloides stercoralis* in 7.6% and *Trichuris trichiura* in 4.3% of the infections. From 75 double infections, different combinations of mixed infections were encountered, of which the dominant ones were those combinations which contained bacterial and protozoal agents. Among triple infections, combinations of protozoal and helminth infections were dominant. Of all diarrhoeal infections observed in this study, it seems that bacterial and protozoal infections are most common in both single and multiple (more than one etiology in single individual) infections (Table 2).

Out of 394 positive samples for etiologies, 48.5% was from males and 51.5% was from females. 304 single aetiologies were encountred, of which 48% was from males and 52% was from females, while out of 75 double aetiologies, 48% was from males and the remaining was from females and also 15 triple aetiologies were encountred. For comparison of differences in infection status of males and females, Chi-square (X^2) test was conducted and found to be statistically insignificant ($p>0.01$) (Table 3).

Table 1. Number of specimens found positive for one or more etiology/ies of diarrhoea from some hospitals and clinics of Addis Ababa.

Hospital or clinic	Male			Female			Total		
	NOSC*	SPFE+		NOSC	SPFE		NOSC	SPFE	
		No	%		No	%		No	%
Tikur Anbesa hospital	105	54	51.4	132	65	49.2	237	119	50.2
Zeuditu hospital	94	47	50	101	48	47.5	195	95	48.7
Menelic hospital	75	55	73.3	70	50	71.4	145	105	72.4
Yekatit 12 hospital	35	16	45.7	43	24	55.8	78	40	51.3
Arada clinic	26	19	73.1	19	16	84.2	45	35	77.8
total	335	191	57	365	203	55.6	700	394	56.3

* Number of specimen collected.
\+ Specimen positive for aetiologic agents.

Chi-square analysis for determination of possible association of single, double and triple infections with age group was found statistically insignificant ($p>0.01$). However, 62.7% of total infections, 61.5% of single infections, 66.1% of double infections and 73.4% of triple infections were encountered in age groups of 14 to 35, while least infections of all categories were observed in age groups of 36 to 57 (Table 4).

2. Bacterial Aetiologies Identified

In bacteriological identification, a total of 45 (6.4% of etiologic agents) *Salmonella* and 50 (7.1% of etiologies) *Shigella* Isolates were identified, of which 5 of the former and 9 of the latter were found in association with other parasitic infections (Table 2, 5 and 9).

Table 2. Frequency of single, double and triple etiologies encountered among diarrhoeal patients in Addis Ababa.

Single etiology	Number encountered	% among single etiology	% among total aetiologies
Sal	40	13.2	10.2
Shi	41	13.5	10.4
Eh	102	33.6	25.9
Gl	85	28	21.6
Ss	23	7.6	5.8
Tt	13	4.3	3.3
Total	304		
Double etiologies	Number encountered	% among double etiologies	% among total etiologies
Sal and Eh	1	1.3	0.25
Sal and Gl	2	2.7	0.51
Sal and Ss	1	1.3	0.25
Shi and Eh	2	2.7	0.51
Shi and Gl	2	2.7	0.51
Shi and Ss	1	1.3	0.25
Shi and Tt	2	2.7	0.51
Eh and Gl	17	22.7	4.3
Eh and Ss	15	20	3.8
Eh and Tt	13	17.3	3.3
Gl and Ss	10	13.3	2.5
Gl and Tt	8	10.7	2.0
Ss and Tt	1	1.3	0.25
Total	75		
Triple etiologies	Number encountered	% among triple etiologies	% among total etiologies
Sal,Eh and Gl	1	6.7	0.25
Shi,Eh and Gl	1	6.7	0.25
Shi,Eh and Tt	2	13.3	0.51
Eh,Gl and Ss	4	26.7	1.00
Eh,Gl and Tt	7	46.7	1.8
Total	15		

Sal=Salmonella;Shi=Shigella;Eh=Entamoeba histolytica
Gl=Giardia lamblia;Ss=Strongyloides stercoralis
Tt=Trichuris trichiura

Table 3. Sex-wise frequency of single, double and triple aetiologies.

Sex	Single aetiologies		Double aetiologies		Triple aetiologies		Total	
	No	%	No	%	No	%	No	%
Male	146	48	36	48	9	60	191	48.5
Female	158	52	39	52	6	40	203	51.5
Total	304		75		15		394	

X^2cal^* = 0.85 (p>0.01).

Table 4. Age-wise frequency of single, double and triple aetiologies.

Age group	Single aetiologies		Double aetiologies		Triple aetiologies		Total	
	No	%	No	%	No	%	No	%
14-24	101	33.2	22	30.1	7	46.7	130	33
25-35	86	28.3	27	36	4	26.7	117	29.7
36-46	41	13.5	5	6.7	1	6.7	47	11.9
47-57	23	7.6	7	9.3	0	0	30	7.6
$\geq$ 58	53	17.4	14	18.7	3	20	70	17.8
Total	304		75		15		394	

X^2cal^* = 6.634 (p>0.01).

X^2cal=Chi-square calculated.

2.1 *Salmonella*

2.1.1 Frequency of *Salmonella* Isolates

Among 45 *Salmonella* isolates, the frequency of occurance of serogrousp C, B, D (apart from *S.typhi)*, *S.typhi*, A, and E was found to be 31.1%, 24.4%, 13.3%, 15.6%, 8.9% and 6.7% respectively (Table 5).

2.1.2 Antimicrobial Susceptibility of *Salmonella* Isolates

Antimicrobial drug susceptibility of *Salmonella* isolates to 9 drugs is shown in Table 5. Susceptibility of all serogroups to polymyxin B and gentamicin was found to be 100% and 91.1%, respectively. Others were found to fall between 28.9% for tetracyclines and 62.2% for carbenicillin. All strains of serogroup A were not Susceptible to ampicillin and tetracyclines. Among the isolates, 16.7% of serogroup D, 21.4% of serogroup C, 33.3% of serogroup E and 45.6% of serogroup B were susceptible to ampicillin. All strains of serogroup E were not susceptible to tetracyclines. While for others, susceptibility to tetracyclines varied from 28.6% of serogroup C and *S.typhi* upto 50% of serogroup D. Susceptibility of all serogroups to cephalothin and trimethoprim-sulfamethoxazole was below 50% except serogroup B with 54.5% susceptibility to both drugs and 57.1% susceptibility of *S.typhi* to the latter drug.

A susceptibility of less than 50% was exhibited in serogroups C and E with respect to chloramphenicol and

kanamycin where as similar response was observed in serogroups A and B with kanamycin and chloramphenicol respectively. about 18.2% of serogroup B and 7.1% of serogroup C were susceptible to all antibiotics. From drug susceptibility studies of *Salmonella* strains, polymyxin B, gentamicin and, to less extent, carbenicillin were found to be drug of choice at least *in vitro*, while tetracyclines, ampicillin and kanamycin were least effective for all serogroups.

Of all *Salmonella* isolates, 93.3% were resistant to one or more drugs (Table 6). Among serogroups, 100% of A, D, E including *S.typhi* as well as 81.8% of serogroup B and 92.9% of C isolates showed resistance to one or more drugs. Among all *Salmonella* strains isolated, resistance to one drug was observed only in 4.4%, while the highest resistance encountered was for 5 drugs (33.3%), followed by 3 and 4 drugs (15.6% each) and to 6 drugs (11.1%). No strain was found to be resistant to all drugs tested. However, 7.1% of serogroup C showed resistance to 7 and 8 drugs each, while 9.1% of serogroup B to 7 drugs tested. Resistance of each serogroup to one or more drug is tabulated in Table 6.

Resistance to two or more drugs was detected in 100% of serogroups A, D, S.typhi and in 72.7% and 85.7% of serogroup B and C respectively.

Table 5. Antimicrobial Susceptibility of *Salmonella* isolated from diarrhoeal out-patients in Addis Ababa.

Sal* SGI	NOS# (%)	Susceptibility to : + Number of strains (%)									
		AM	CB	CE	CL	GM	K	PB	TE	SxT	All
A	4 (8.9)	0 (0)	3 (75)	1 (25)	4 (100)	4 (100)	1 (25)	4 (100)	0 (0)	1 (25)	0 (0)
B	11 (24.4)	5 (45.6)	7 (63.6)	6 (54.5)	5 (45.6)	10 (90.9)	7 (63.6)	11 (100)	4 (36.4)	6 (54.5)	2 (18.2)
C	14 (31.1)	3 (21.4)	9 (64.3)	3 (21.4)	6 (42.9)	13 (92.9)	5 (35.7)	14 (100)	4 (28.6)	5 (35.7)	1 (7.1)
D	6 (13.3)	1 (16.7)	3 (50)	1 (16.7)	3 (50)	4 (66.7)	3 (50)	6 (100)	3 (50)	2 (14.3)	0 (0)
S. typhi	7 (15.6)	4 (57.1)	4 (57.1)	3 (42.9)	5 (71.4)	7 (100)	4 (57.1)	7 (100)	2 (28.6)	4 (57.1)	0 (0)
E	3 (6.7)	1 (33.3)	2 (66.7)	1 (33.3)	1 (33.3)	3 (100)	1 (33.3)	3 (100)	0 (0)	1 (33.3)	0 (0)
All	45	14 (31.1)	28 (62.2)	15 (33.3)	24 (53.3)	41 (91.1)	21 (46.1)	45 (100)	13 (28.9)	19 (42.2)	3 (6.7)

* *Salmonella* serogroups identified; # number of strains
\+ Ampicillin (AM);Gentamicin (GM);Carbenicillin (CB);Kanamycin (K)
Cephalothin (CE);Polymyxin B (PB);Chloramphenicol (CL)
Tetracycline (TE);Trimethoprim-Sulfamethoxazole (SxT)

Table 6. Single and multiple antibiotic resistance of *Salmonella* isolated from diarrhoeal out-patients in Addis Ababa.

To (only)	Resistance of serogroup number of strains (%)						
	A 4	B 11	C 14	D 6	*S.typhi* 7	E 3	All 45
One drug	0 (0)	1 (9.1)	1 (7.1)	0 (0)	0 (0)	0 (0)	2 (4.4)
Two drugs	0 (0)	1 (9.1)	1 (7.1)	0 (0)	1 (14.3)	0 (0)	3 (6.7)
Three drugs	1 (25)	2 (18.2)	1 (7.1)	1 (16.7)	2 (28.6)	0 (0)	7 (15.6)
Four drugs	0 (0)	0 (0)	1 (7.1)	1 (16.7)	4 (57.1)	1 (33.3)	7 (15.6)
Five drugs	3 (75)	3 (27.3)	4 (28.6)	3 (50)	0 (0)	2 (66.7)	15 (33.3)
Six drugs	0 (0)	1 (9.1)	3 (21.4)	1 (16.7)	0 (0)	0 (0)	5 (11.1)
Seven drugs	0 (0)	1 (9.1)	1 (7.1)	0 (0)	0 (0)	0 (0)	2 (4.4)
Eight drugs	0 (0)	0 (0)	1 (7.1)	0 (0)	0 (0)	0 (0)	1 (2.2)
All	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
One or more drugs	4 (100)	9 (81.8)	13 (92.9)	6 (100)	7 (100)	3 (100)	42 (93.3)
Two or more drugs	4 (100)	8 (72.7)	12 (85.7)	6 (100)	7 (100)	3 (100)	40 (88.9)

Table 7. Resistance antibiograms of *Salmonella* isolated from diarrhoeal out-patients in Addis Ababa.

SSG+ (No)#	Resistance antibiogram*	No.
A (4)	AM,CB,TE	1
	AM,CE,SxT,TE,K	3
B (9)	AM	1
	CE,TE	1
	AM,TE,CL	1
	AM,CB,SxT	1
	CB,CE,CL,TE,SxT	1
	CL,GM,K,TE,SxT	1
	AM,CB,CL,TE,K	1
	AM,CE,CL,TE,K,SxT	1
	AM,CE,CL,TE,K,SxT,CB	1
C (13)	TE	1
	CB,CE	1
	AM,CE,SxT	1
	AM,CE,SxT,K	1
	AM,CE,CB,K,TE	1
	AM,CE,K,TE,CL	1
	AM,CE,K,TE,SxT	1
	AM,CB,TE,CL,SxT	1
	AM,CE,K,TE,CL,SxT	3
	AM,CE,K,TE,CL,SxT,CB	1
	AM,CE,K,TE,CL,SxT,GM,CB	1
D (6)	AM,CE,SxT	1
	AM,CE,K,CB	1
	AM,CE,K,CL,SxT	1
	AM,CE,K,TE,SxT	1
	AM,CB,TE,CL,GM	1
	CE,CB,TE,GM,CL,SxT	1
S.typhi (7)	CE,TE	1
	AM,CE,K	1
	CE,K,SxT	1
	CE,K,AM,TE	1
	CB,TE,CL,SxT	2
	CE,TE,AM,SxT	1
E (3)	AM,CB,TE,CL	1
	AM,CE,K,TE,SxT	1
	CL,CE,K,TE,SxT	1

* Abbreviation of antibiotics is as in materials & methods.
\+ Salmonella serogroup
\# No. of strains

Of all strains of *Salmonella* tested, 88.9% were resistant to two or more drugs (Table, 6).

A total of 31 distinct antibiograms (resistance patterns) were found in all *Salmonella* strains tested (Table 7). While among individual serogroups, 2 antibiograms were observed in group A, 9 in group B, 11 in group C, 6 in group D and *S.typhi* each and 3 in group E. Different combinations of antibiograms were observed, of which frequently encountered antibiotics were ampicillin, tetracyclines, cephalothin, trimethoprim-sulfamethoxazole (Bactrim) and kanamycin. The major antibiograms were those with 5 antibiotic combinations, followed by 4 and 3 antibiotics. One strain in group B and C each showed simultaneous resistance to seven antibiotics and Only 1 strain in group C showed resistance to 8 antibiotics (Table 6).

2.1.3 Plasmid profiles of *Salmonella* Isolates

Figure 1 shows results of plasmid DNA gel electrophoresis and plasmid size determination of representative selected multiple drug resistant *Salmonella* strains. A total of 15 isolates, of which, 2 strains from serogroup A, 3 strains from serogroup B, 4 strains from serogroup C, 2 strains from serogroup D, 2 strains from *S.typhi* and 2 strains from serogroup E were used for plasmid profile analysis. Almost all the strains contained several plasmids with sizes significantly greater than 21.2 Kb (kilo bases) (large plasmids, for convenience) and also several plasmids less than 21.2 Kb (saml1 plasmids). Lanes 4, 7 and 15 lack large plasmids (>21.2 Kb).

Approximate sizes of plasmids and resistance patterns are tabulated in Table 8 with positive signs indicating possession of a plasmid with stated molecular size. From 4 upto 8 plasmids per strain were isolated from *Salmonella* isolates. All isolates contained 5, 4, and 1.5 Kb plasmids. Large and 6.2 Kb plasmids were possessed by the majority of the Salmonella isolates while 4.5 and 1.6 Kb plasmids were identified from one isolate in serogroup E and *S.typhi* respectively.

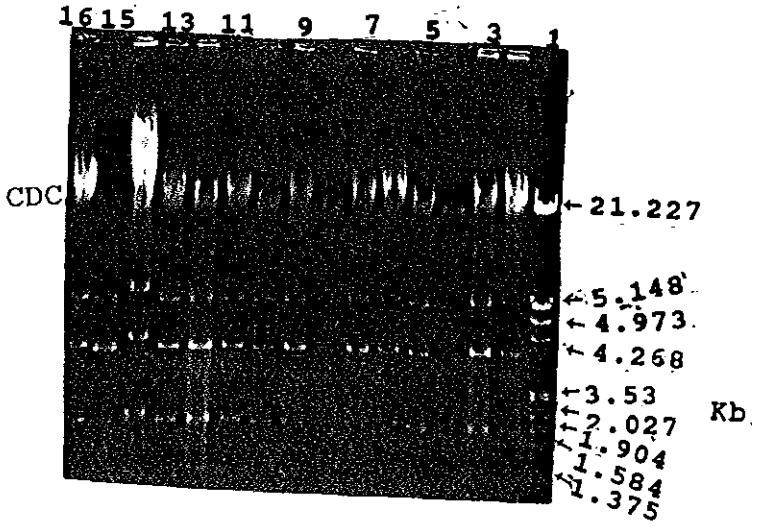

Figure 1. Agarose gel electrophoresis of plasmid DNA from Salmonella strains isolated from diarrhoeal patients. From right to left; sizes in kilobases (Kb). Lane 1, Lambda size marker; lane 2 and 3 (from serogroup A); lane 4, 5 and 6 (from serogroup B); lane 7, 8, 9 and 10 (from serogroup C); lane 11 and 12 (from serogroup D); lane 13 and 14 (from *S.typhi*), and lane 15 and 16 (from serogroup E). CDC (chromosomal DNA contamination with about 21.2 Kb plasmid).

Table 8. Drug resistance pattern and approximate sizes of Plasmids from *Salmonella* isolated from adult diarrhoeal out-patients in Addis Ababa.

SS[t]	Resistance pattern*	CLI[#]	Size of plasmids (Kb) (plasmid profile)								
			Lps[+]	6.2	5	4.5	4	3.8	3.5	1.6	1.5
A	AM,TE,K,CE,SxT	2 & 3	+	+	+		+	+	+		+
B	TE,K,CL,GM,SxT	4		+	+		+		+		+
	AM,TE,K,CL,CE,SxT	5	+	+	+		+	+			+
	AM,TE,K,CL,CB	6	+		+		+	+			+
C	AM,TE,K,CL,CE,SxT	7		+	+		+		+		+
	AM,TE,K,CL,CE,SxT	8	+		+		+				+
	AM,TE,K,CL,CE,SxT	9	+	+	+		+	+			+
	AM,TE,K,CL,CE,SxT,CB,GM	10	+	+	+		+	+			+
D	TE,CL,CE,CB,GM,SxT	11	+	+	+		+	+			+
	AM,TE,K,CE,SxT	12	+	+	+		+	+	+		+
St[b]	AM,TE,K,CE	13	+	+	+		+		+		+
	TE,CL,CB,SxT	14	+		+		+			+	+
E	AM,TE,K,CE,SxT	15		+	+	+	+		+		+
	AM,TE,CL,CB	16	+	+	+		+				+

t *Salmonella* serogroups; b *S.typhi*
\# corresponding lanes in Fig.1; + large plasmids (>21.2 Kb).
* abbreviation of antibiotics is as in materials & methods.

2.2 Shigella

2.2.1 Frequency of *Shigella* Isolates

Among 50 *Shigella* isolates, serogroup A comprised 28%, serogroup B 44%, serogroup C 18% and serogroup D 10% (Table,9).

2.2.2 Antimicrobial suscptibility of *Shigella* Isolates

Antimicrobial drug susceptibility of *Shigella* isolates to 10 drugs is shown in Table 9. Susceptibility of all isolates to gentamicin was 100%, to polymyxin B 90%, to nalidixic acid 86%, kanamycin 80%, to carbenicillin 72% and for others ranges from 26% susceptibility to tetracyclines to 50% susceptibility to chloramphenicol. No strain in serogroup D was susceptible to tetracyclines. The susceptibility of all strains in each serogroup to ampicillin, tetracyclines and cephalothin was below 50%, except a 60% susceptibility of serogroup D to cephalothin. Susceptibility of all strains in serogroup A and D to chloramphenicol and trimethoprim-sulfamethoxazole was found to be below 50%. Only one strain in serogroup B was susceptible to all antibiotics tested. From drug susceptibility studies of *Shigella*, gentamicin, kanamycin, polymyxin B, nalidixic acid and to some extent carbenicillin were found to be most effective at least *in vitro* whereas tetracyclines, ampicillin and cephalothin were least effective for all serogroups.

Ninty eight percent of all *Shigella* isolates were

resistant to one or more drugs as shown in Table 10. Among serogroups, 100% of serogroup A, C and D as well as 95.5% of serogroup B isolates showed resistance to one or more drugs. Resistance to one drug was encountered in 6%, while the highest resistance was observed for three drugs (32%), followed by five and six drugs (18% each) and to two drugs (10%). No strain was resistant to all drugs tested but one strain each from serogroup A and B was resistant to seven drugs.

Resistance to two or more drugs was detected in 100% of serogroup D, 92.9% of A, 90.9% of B and 88.9% of serogroup C. Of all strains of *Shigella* tested, 92% was resistant to two or more drugs (Table 10).

A total of 32 distinct antibiograms were encountered in all *Shigella* strains (Table 11). While among individual serogroups, 12 antibiograms were observed in group A, eighteen in group B, eight in group C, and four in group D. Like in *Salmonella*, different combinations of antibiograms were observed, of which frequently encountered antibiotics were ampicillin, tetracyclines, cephalothin and trimethoprim-sulphamethoxazole. The major antibiograms were those with three antibiotic combinations, followed by five and six antibiotic combinations. No strain was resistant to all antibiotics tested but one strain of serogroup A and B each were found to be resistant to seven antibiotics (Table 11).

Table 9. Antimicrobial susceptibility of *Shigella* isolated from diarrhoeal out-patients in Addis Ababa.

Shi SGI*	NOS# (%)	Susceptibility to: number of strains (%)										
		AM	CB	CE	CL	GM	K	PB	TE	SxT	NA	All
A	14 (28)	3 (21.4)	8 (57.1)	4 (28.6)	4 (28.6)	14 (100)	9 (64.3)	12 (85.7)	3 (21.4)	6 (42.9)	12 (85.7)	0 (0)
B	22 (44)	7 (31.8)	17 (77.3)	9 (40.9)	12 (54.5)	22 (100)	19 (86.4)	20 (90.9)	8 (36.4)	11 (50)	19 (86.4)	1 (4.5)
C	9 (18)	3 (33.3)	8 (88.9)	2 (22.7)	7 (77.8)	9 (100)	7 (77.8)	8 (88.9)	2 (22.2)	5 (55.6)	7 (77.8)	0 (0)
D	5 (10)	2 (40)	3 (60)	3 (60)	2 (40)	5 (100)	5 (100)	5 (100)	0 (0)	2 (40)	5 (100)	0 (0)
All	50	15 (30)	36 (72)	18 (36)	25 (50)	50 (100)	40 (80)	45 (90)	13 (26)	24 (48)	43 (86)	1 (2)

* Shigella serogroups identified; # Number of strains
\+ Ampicillin (AM);Gentamicin (GM);Carbenicillin (CB);Kanamycin (K)
Cephalothin (CE);Polymyxin B (PB);Chloramphenicol (CL)
Tetracycline (TE);Nalidixic acid (NA)
Trimethoprim-Sulfamethoxazole (SxT)

Table 10. Single and multiple antibiotic resistance of *Shigella* isolated from diarrhoeal out-patients in Addis Ababa.

To (only)	Resistance of serogroup, No of strains (%)				
	A,14	B,22	C,9	D,5	All,50
One drug	1 (7.1)	1 (4.5)	1 (11.1)	0 (0)	3 (6)
Two drugs	0 (0)	2 (9.1)	2 (22.2)	1 (20)	5 (10)
Three drugs	3 (21.4)	10 (45.5)	2 (22.2)	1 (20)	16 (32)
Four drugs	1 (7.7)	2 (9.1)	1 (11.1)	1 (20)	5 (10)
Five drugs	2 (14.3)	4 (18.2)	1 (11.1)	2 (40)	9 (18)
Six drugs	6 (49.2)	1 (4.5)	2 (22.2)	0 (0)	9 (18)
Seven drugs	1 (7.1)	1 (4.5)	0 (0)	0 (0)	2 (4)
Eight drugs	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Nine drugs	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
All	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
One or more drugs	14 (100)	21 (95.5)	9 (100)	5 (100)	49 (98)
Two or more drugs	13 (92.9)	20 (90.9)	8 (88.9)	5 (100)	46 (92)

Table 11. Resistance antibiograms of *Shigella* isolated from diarrhoeal out-patients in Addis Ababa.

SSG+ (NOS)#	Resistance antibiogram(s)*	No.
A (14)	TE	1
	AM,CB,K	1
	AM,TE,CE	1
	CL,SxT,TE	1
	AM,CE,CL,PB	1
	AM,CB,TE,CL,SxT	2
	AM,CE,TE,K,SxT,NA	1
	AM,CE,TE,CB,CL,SxT	2
	AM,CE,TE,K,CL,SxT	1
	AM,CE,TE,K,CL,PB	1
	CE,TE,CB,CE,CL,SxT	1
	AM,TE,CE,K,CL,SxT,NA	1
B (21)	TE	1
	TE,SxT	1
	CL,SxT	1
	AM,CE,PB	1
	AM,CE,TE	3
	TE,CL,SxT	1
	AM,CE,CB	1
	AM,CE,SxT	2
	TE,CL,CB	1
	TE,CE,CB	1
	AM,TE,CE,K	1
	TE,CB,CL,SxT	1
	AM,CE,TE,K,PB	1
	AM,CB,GM,CL,NA	1
	AM,CE,TE,CL,SxT	1
	AM,CB,TE,CL,SxT	1
	AM,CE,TE,CL,SxT,NA	1
	AM,CE,TE,CL,SxT,NA,K	1
C (9)	CE	1
	AM,CE	1
	TE,SxT	1
	AM,CE,TE	2
	TE,CB,CL,SxT	1
	AM,TE,CE,K,NA	1
	AM,TE,CE,K,NA,SxT	1
	AM,TE,CE,CL,PB,SxT	1
(5)	TE,CE,	1
	TE,CL,SxT	1
	AM,TE,CE,SxT	1
	AM,TE,CB,CL,SxT	2

+ Shigella serogroup; # Number of strains
* Abbreviation of antibiotics is as in materials & methods.

2.2.3 Plasmid Profiles of *Shigella* Isolates

Figure 2 shows results of plasmid DNA gel electrophoresis and plasmid size determination of selected multiple drug resistant *Shigella* strains. 4 strains from serogroup A, 6 strains from serogroup B, 3 strains from serogroup C, and 2 strains from serogroup D were used for plasmid profile analysis. Like *Salmonella*, most of the strains contained multiple plasmids, with some of sizes greater than 21.2 Kb (large plasmids, for convenience) and also some others less than 21.2 Kb (small plasmids). In lanes 3 and 11, the plasmid bands are not clearly visible, however, about 4.5 and 4.0 Kb plasmid bands in lane 3 and about 21.2 Kb plasmid in lane 11 are slightly visible. Lanes 3, 4, 5, 8 ,11 and 13 lack those plasmids with sizes greater than 21.2 Kb.

Approximate sizes of plasmids (in Kb) and resistance patterns are tabulated in Table 12 with positive signs indicating possession of a plasmid with stated molecular size. From single upto six plasmids per strain were isolated from *Shigella* isolates. Except one isolate in serogroup B, all isolates possessed 4.0 Kb plasmid. 8 of the isolates contained large plasmids, while 9, 4, 10, 4, 1 and 4 of the isolates contained 21, 5, 4.5, 3.6, 1.9 and 1.4 Kb plasmids respectively.

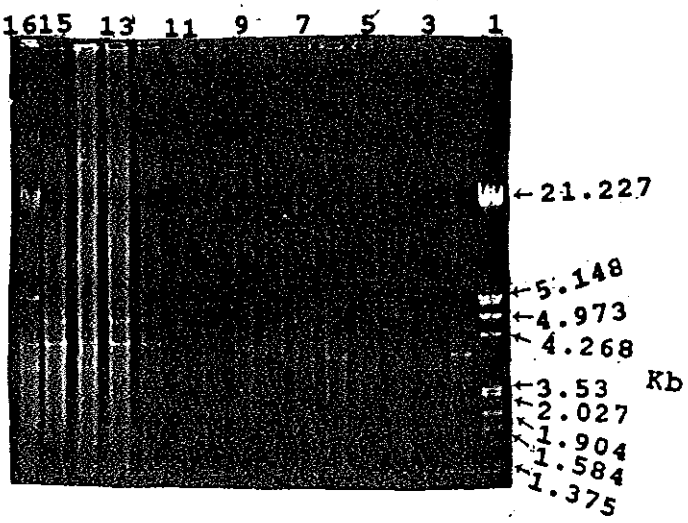

Fig.2 Agarose gel electrophoresis of plasmid DNA from *Shigella* strains isolated from adult diarrhoeal out-patients. From right to left; sizes in kilobase (kb). Lane 1, Lambda size marker; lane 2, 3, 4 and 5 (from serogroup A); lane 6, 7, 8, 9, 10 and 11 (from serogroup B); lane 12, 13 and 14 (from group C); lane 15 and 16 (from serogroup D).

Table 12. Drug resistance pattern and approximate sizes of plasmids from *Shigella* isolated from diarrhoeal out-patients in Addis Ababa.

SS[W]	Resistance pattern*	CLI[#]	Size of plasmids (kb) (plasmid profile)							
			Lp[+]	21	5	4.5	4.0	3.6	1.9	1.4
A	AM,CE,TE,K,SxT,NA	2	+	+	+		+	+		+
	AM,CE,TE,CB,CL,SxT	3				+	+			
	AM,CE,TE,K,CL,PB	4				+	+			
	AM,CE,TE,K,CL,SxT,NA	5					+			
B	AM,CE,TE	6	+	+	+		+	+		+
	AM,CE,SxT	7	+	+	+	+	+			+
	AM,CE,TE,K,PB	8				+	+			
	AM,CB,GM,CL,NA	9	+			+	+			
	AM,CB,TE,CL,SxT	10	+			+	+			
	AM,CE,TE,K,CL,SxT,NA	11		+						
C	TE,CB,CL,SxT	12	+	+		+	+			
	AM,TE,CE,K,NA,SxT	13		+		+	+			
	AM,TE,CE,CL,PB,SxT	14		+		+	+	+		+
D	AM,TE,CE,SxT	15	+	+		+	+			
	AM,TE,CB,CL,SxT	16	+	+	+		+	+	+	

W Shigella Serogroup; # corresponding lane in Fig.2;
\+ large plasmids (>21.2 Kb).
* abbreviation of antibiotics is as in materials & methods

V. DISCUSSION

The identification of single and multiple (mixed) infections of bacterial and parasitic aetiologies in diarrhoea is a common phenomenon especially in developing countries (Chunge *et al.* , 1992) and the identification of one or more etiologic agents in 394 (56.3%) diarrhoeal samples in this study supports such observation. Although not solely from diarrhoeal samples, Shibru (1986) in a nation wide survey in Ethiopia reported that out of 28,696 stool samples, 32.8% were found to be negative for etiologic agent while in 67.2% one or more etiologic agent were identified. Similar identifications were also reported from different parts of the world (Adkins *et al.*, 1987; Hailu, 1989; Chunge *et al.*, 1992; Edward *et al.*, 1993). The observed combinations of bacterial and parasitic infections most probably indicate the co-existence of such infections in the population and unsanitary environmental condition with poor personal hygiene.

In agreement with reports of Chunge *et al.* (1992), *Entamoeba histolytica (E.histolytica)*, *Giardia lamblia (G.lamblia)*, *Shigella* and *Salmonella* were found to be the major etiologic agents of diarrhoeal causes in this study occurring in 33.6%, 28%, 13.5% and 13.2% respectively than *Strongyloides stercoralis (S.stercoralis)* and *Trichuris trichiura (T.trichiura)* encountred in 7.6% and 4.3% respectively of the single infections. The most frequent double infection encountered was *E.histolytica* with *G.lamblia* (22.7%), followed by *E.histolytica* with *S.stercoralis* (20%).

These investigations indicate that bacterial and protozoal aetiologies were more prominent in diarrhoeal cases than helminth infections confirming other reports from elsewhere (Reisberg, 1986; Banwell, 1989; Guerrant *et al.*, 1990).

Triple infections were also exhibited in 3.8% (15) of diarrhoeal cases found positive for aetiologies. Frequently isolated ones were *E.histolytica* with *G.lamblia* and *T.trichiura* (46.7%) followed by *E.histolytica* with *G.lamblia* and *S.stercoralis* (26.7%). On the other hand, bacterial aetiologies were also diagnosed with protozoal agents in triple infections. It was also observed that the severity of diarrhoeal manifestations as well as the deterioration of the physical conditions of the out-patients was increased with multiple infections, especially when *E.histolytica* and bacterial co-infections occur and this was also observed by Tharavanij (1982).

Attempt was also made to investigate possible association of frequency of single, double and triple infections with sex and age groups and found to be statistically insignificant ($p>0.01$). Dupont and Pickering (1980) and WHO (1988) also supported this finding, but noted that women are more likely to be affected than men due to physiological changes during pregnancy and their closer contact with infected children. Eventhough not statistically, most of the infections were encountered in age groups of 14 to 35. Dupont and Pickering (1980) also found similar results in a community study and gave possible reasons like occupation, frequency of exposure to the environment and infective agents for such an observation. The same reason could hold true in this case too.

The effort made to isolate *Cryptosporidium* was not successful in this study, and a number of reasons might have contributed for this. Most importantly, the patients were out-patients, which were most probably immunocompetent and the diarrhoeal cases encountered could be non-Cryptosporidial, or oocysts could have been missed in mixed infections in which their number was small (Nime *et al.*, 1976). It is also evident from the result to conclude the contribution of other diarrhoeal aetiologies known to cause diarrhoea but not included in this study like various forms of *Escherichia coli (E.Coli), Rotavirus and Yersinia enterocolitica*, since in 43.7% of diarrhoeal cases the aetiologies were not identified.

In bacteriological investigations, isolation of 6.4% *Salmonella* aetiologies from adult diarrhoeal out patients in this study is greater than 2.9% isolation reported from Djibouti (Mikhali, 1990), 0.5% from Fujian, China (Chen *et al.*, 1991), 5.3% from Portugal (Cabrita *et al.*, 1990), 1% and 4.5% from Ethiopia by Wallace (1976) and Mogessie (1983) respectively. However, it is less than 9.2% isolation recorded from Manila, Philippines (Adkins *et al.*, 1987), and 23% from Zimbabwe (Simango and Dindiwe, 1987). This difference could be associated with level of endemicity of *Salmonella* in stated regions.

Of all *Salmonella* isolates in this study, 84.4% belong to non-typhi serogroups, which reflects the acute gastroenteritis nature of the majority of *Salmonella* related diarrhoea, while the 15.6% of *S.typhi* isolates may represent post-systemic intestinal phase with diarrhoea. The isolation by Taylor *et al.* (1991) in Thailand, Rivera *et al.* (1991) in Tudela, Spain,

Sobeh *et al.* (1984) in India, Mogessie (1983) in Addis Ababa, Ethiopia are in line with this. Messele and Alebachew (1981) and Afeworki (1985) also isolated *Salmonella* serogroups in Addis Ababa, Ethiopia in which *S.typhi* was dominant, because most of their samples were from blood specimen.

Among all antibiotics, least susceptibility in each serogroups was observed with ampicillin, tetracycline and cephalothin. This observation contrasts with findings of Messele and Alebachew (1981) where more than 98.4% of all the *Salmonella* isolates were susceptible to all drugs and also with those of Mogessie (1983) where more than 77.8% of all the *Salmonella* isolates were susceptible to all antibiotics. The astonishing drug resistance pattern observed in this study agrees with reports from other parts of the world (Rivera *et al.* 1991; Nakamura *et al.* 1986; Santos *et al.*, 1989; Cohen and Tauxe 1986; Mirza and Wamola 1989; Schuman *et al.*, 1989; Nakamura *et al.*, 1989; Hammami *et al.*, 1991) and all pointed out in their respective studies that commonly used antibiotics believed to be most effective in the past for cases related with *Salmonella* are nowadays becoming least effective due to emergence of multiple drug resistant *Salmonella* strains.

About 28.6% of the *S.typhi* isolates were resistant to chloramphenicol in this study while 100% of the S.typhi isolates were susceptible to this antibiotic according to the findings of Messele and Alebachew (1981), Mogessie (1983) and Afeworki (1985). This signifies the emergence of chloramphenicol resistant *S.typhi* in Ethiopia, especially in Addis Ababa. *S.typhi* resistant to Chloramphenicol is well documented in studies elsewhere (Khadilkar *et al.*, 1990; Singh,

1991; Rowe *et al.*, 1990; Mourad *et al.*, 1993; Elshafie and Rafay 1992).

About 93.3% and 88.9% of the *Salmonella* isolates were resistant to one or more drugs and to two or more drugs respectively in this study while 79.4% and 41.2% of the *Salmonella* isolates were respectively resistant to one or more drugs and to two or more drugs in investigations carried out by Messele and Alebachew (1981). Among the isolates, 33.3%, 15.6%, 11.1% and 6.7% of the *Salmonella* were simultaneously resistant to 5, 4 and 3, 6 and 2 drugs respectively in this study (Table 6). This high frequency of multiple antibiotic resistance most probably indicates the ease of access and the extensive use of the antibiotics for therapeutic and prophylactic purposes (both prescribed and unprescribed) in the health centres and the society at large.

Different antibiotic combinations were encountered among resistance antibiograms, of which the major ones were those with 5 antibiotic combinations followed by 4 and 3 antibiotics (Table 7). The most frequently encountered antibiograms in almost all strains of *Salmonella* isolates in this study were tetracycline, ampicillin, cephalothin, kanamycin and trimethoprim-sulfamethoxazole. The frequent occurrence of an antibiotic in antibiogram mostly reflects its frequency in use and level of resistance development among target population (Santoes et al., 1989; Murray, 1986).

The resistance antibiograms observed by Messele and Alebachew (1981) mostly consisted of sulphadiazine and streptomycin combinations, while in this study ampicillin and tetracycline combinations were dominant signifying the

resistance of bacterial isolates to first line antibiotics. Over all, Polymyxin B, gentamicin and carbenicillin were found to be the drug of choice according to *in vitro* susceptibility, but it is important to realize that *in vitro* inhibition or resistance cannot be necessarily taken as sufficient evidence of drug's effectiveness or lack of effectiveness *in vivo*.

Plasmid profile analysis of *Salmonella* isolates in this study revealed the exitence of multiple plasmids among *Salmonella* isolates (Table 8). All isolates contained from 4 up to 8 plasmids per strain. Nakamura *et al.* (1986), Karmaker *et al.* (1991), Finch *et al.* (1992), Ferris *et al.* (1992), Stanley *et al.* (1992), Threlfall *et al.* (1992), Rivera *et al.* (1991), Schuman *et al.* (1989) and Dorn *et al.* (1992) in their respective studies also identified *Salmonella* strains with 1 to 7 plasmids per strain from multiple drug resistant strains.

All analyzed *Salmonella* isolates contained 5, 4, and 1.5 Kb plasmids in this study. Except three isolates, large and 6.2 Kb plasmids were also possessed by all analyzed isolates while 3.8, 4.5 and 1.6 Kb plasmids were possessed by 6, 1 and 1 isolates respectively (Table 8). Finch *et al.* (1992) in Lima, Peru also analyzed plasmid contents of 228 drug resistant salmonella strains and found that 65, 55, 38 and 24 Kb plasmids were possessed by 1, 6, 30 and 25 of the isolates respectively while 90, 80, 14 Kb and 65, 16 Kb plasmids were possessed by 1 and 6 of the isolates, respectively. They pointed out the importance of plasmid profile analysis in Peru since 1984 in tracing the epidemiology of infection and this baseline preliminary study of plasmid profile analysis of *Salmonella* in this country may also be used in epidemiological

investigations.

There has not been any previous study in relation to plasmids associated with drug resistance among *Salmonella* isolates in this country, so comparison is not possible. Moreover it was not possible in this study to attribute exact molecular sizes to the plasmids found because appropriate molecular weight markers were not available, so direct comparison with other studies is not possible. In Japan Nakamura *et al.* (1986) from multiple drug resistant *S.typhimurium* found out 126, 10.4 and 6 Kb plasmids from ampicillin, chloramphenicol, kanamycin, streptomycin and sulphadiazine resistant strains, 134 and 5.7 Md plasmids from another strain resistant to the same antibiotics and 189, 149.6, 95, 8, 7.7 and 6.1 Kb plasmids from ampicillin, kanamycin, streptomycin and tetracycline resistant strains. They suggested that larger conjugative plasmids mostly mediate drug resistance and that the isolation of one or more different plasmids from the same strain suggests that they might have been introduced or deleted spontaneously. Piatt and Brown (1986) in Scotland also isolated *Salmonella* strains with 0 to 7 plasmids in which the majority of the isolates were *S.typhimurium* and *S.enteritides* in which 67% of *S.typhimurium* isolates contained 95 and 3.3 Kb plasmids while 76% of *S.enteritides* possessed a 56.7 Kb plasmid.

Karmaker *et al.* (1991) in Calcutta, India, isolated multiple drug resistant *S.typhi* and found out that chloramphenicol, ampicillin and tetracycline resistance was conferred by a 120 Kb plasmid. Finch *et al.* (1992) in Lima, Peru also isolated multiple drug resistant S.typhi and found

that ampicillin and kanamycin resistance was associated with a 90 Kb plasmid while tetracycline resistance was associated with an 80 Kb plasmid.

So the large plasmids (Lp) found in these *Salmonella* isolates are most probably those plasmids associated with drug resistance, while in those isolates which lack large plasmids, probably chromosomal genes or integrated plasmids confer drug resistance or the plasmid was unstable and so could not be isolated. Rankin *et al.* (1995) suggested that molecular weights (sizes) of plasmids which confer resistance to a given drug(s) for the same strain could vary, based on time, geographic location, environmental stress, additional character(s) conferred by a plasmid and other parameters. Plasmid studies of this sort are preliminary and mainly used to see their existence and general profile within serogroups, rather than analysing their role in relation to strains within a serotype (which is needed prior to plasmid analysis) or analysing their exact role in drug resistance.

The isolation of *Shigella* from 7.1% of diarrhoeal adult out patients in this study is less than 14.8% isolation from Portugal (Cabrita *et al.*, 1992), 7.7% from Djibouti (Mikhail, 1990), 10.7% from Fujian, China (Chen., 1991), 11.6% from Manila, Philippines (Adkins *et al.*, 1987), 11% and 9% from Ethiopia respectively by Wallace (1976) and Mogessie (1983).

Among the *Shigella* isolates serogroup B (*Sh.flexneri*) was the dominant one in this study (44%) which is in agreement with other studies from Ethiopia (Messele and Alebachew, 1979; 1982; Afeworki and Yetnebersh, 1980; Mogessie, 1983; Afeworki and Eyassu, 1984) and other developing countries like Somalia

(Edward *et al.*, 1993), Zimbabwe (Simango and Dindiwe, 1987), and the Philippines (Adkins *et al.*, 1987). Serogroup A (*Sh.dysenteriae*) is second in frequency (28%) and followed by serogroup C (*Sh.boydii*) 18% and D (*Sh.sonnei*) 10%, which demonstrates their co-existence in this country. In developed countries, *Sh.flexneri* has given way to *Sh.sonnei* as the dominant serogroup (Guerrant *et al.*, 1992; Patrick *et al.*, 1995).

Less than 50% of *Shigella* isolates were susceptible to tetracycline, ampicillin, cephalothin and trimethoprim-sulfamethoxazole. Their susceptibility ranges from 26% of tetracycline to 48% of trimethoprim-sulfamethoxazole among all *Shigella* isolates tested. Especially for tetracycline and ampicillin the susceptibility of each strain in all serogroups was almost below 40% (Table 9). The frequency of ampicillin susceptibility in this study (30%) is much lower than that reported by Afeworki and Yetnebersh (1980) (78%) and by Messele and Alebachew (1982) (79%). The susceptibility for other drugs in this study is also much lower than that reported by above authors. Murray (1986) also reported that among *Shigella* isolates, the percentage of strains susceptible to commonly used antibiotics varied within the following ranges: ampicillin, 93% (in Bangladesh) to 13% (Thailand); tetracycline, 89% (SriLanka) to 10% (Mexico); and trimethoprim-sulfamethoxazole, 100% (Bangladesh, 1980) to 45% (Bangladesh, 1984). In this study too, the majority of resistant strains from 3 to 6 drugs frequently contained a combination of these drugs.

All *Shigella* isolates in this study were susceptible to

gentamicin, and almost 85% of the strains were susceptible to polymyxin B, nalidixic acid and kanamycin *in vitro*. However, their clinical effectiveness was not appreciated except for nalidixic acid (Burstein and Regalli, 1989). Lima and Lima (1993) also pointed out that multiple antimicrobial resistant strains of *Shigella* make it important to develop new interventions for the treatment and control of shigellosis and recommended nalidixic acid as an effective drug for *Shigella* therapy, supporting the observations of Burstein and Regalli (1989). According to the results of current study too, nalidixic acid was found to be effective *in vitro* and could thus be recommended for therapy of shigellosis in this country.

Among *Shigella* isolates, 98% were resistant to one or more drugs tested in this study and this was much higher than those reported by Afeworki and Yetnebersh (1980) (70%), Messele and Alebachew (1982) (85%) and Mogessie (1983) (82.5%). Among serogroups, 100%, 95.5%, 100% and 100% of A, B, C and D isolates were resistant to one or more drugs, respectively, while 92% of all *Shigella* isolates were resistant to two or more drugs (Table 10). *Shigella* isolates resistant to multiple drugs were reported by number of authors (Lin and Chang 1992; Edward *et al.*, 1993; Blake *et al.*, 1993; Hvams *et al.*, 1991). This high frequency of multiple antibiotic resistant *Shigella* isolates observed in this study most probably indicates the ease of access and the extensive use of antibiotics in Addis Ababa and probably in entire country. It could be concluded from this and previous studies in Ethiopia that relatively recent isolates of *Shigella* strains examined tended to be relatively more resistant than earlier ones to the first line

antibiotics as noted by Murray (1986) and Santos *et al.* (1989).

The most common resistance antibiograms among *Shigella* isolates were those combinations containing 3 drugs (ampicillin, tetracycline and cephalothin) followed by 5 drugs (above ones plus trimetoprim-sulfamethoxazole and chloramphenicol) and 6 drugs (above ones plus carbenicillin) (Table 11). Unlike this study, streptomycin and sulphadiazine were the frequently encountered antibiograms in reports of Messele and Alebachew (1979; 1982) and Afeworki and Yetnebersh (1980) and these drugs nowadays are not among the first-line antibiotics for enteric infections. Santos *et al.* (1989) also pointed out that frequent antibiograms are encountered more with those antibiotics misused or used frequently for therapeutic and/or prophylactic purposes.

Plasmid profile analysis of *Shigella* isolates in this study revealed the exitance of multiple plasmids among *Shigella* isolates (Table 12). Previous studies conducted by Afeworki and Drasar (1988; 1990a; 1990b; 1991) on *Sh.dysenteriae and Sh.flexneri* also demonstrated this fact. Most of the isolates in this study contained 2 up to 6 plasmids per strain, however, some strains contained only single plasmids. This could be due to loss of the other plasmids because of instability or chromosomal integration, which are commonly encountered phenomena among plasmid containing strains (Olukoya and Oni, 1990; Patrick *et al.*, 1995).

Except one strain, all other *Shigella* isolates contained 4.0 Kb plasmid in this study, while large, 21, 5, 4.5, 3.6, 1.9 and 1.4 Kb plasmids were possessed by 8, 9, 4, 10, 4, 1 and 4 of the isolates respectively. Afeworki and Drasar (1990a;

1990b;1991) from *Shigella* strains also isolated multiple plasmids and noted that some plasmids were contained in all of the isolates while some others in a few of the strains. It was also stated that high frequency of isolation of different plasmids from related strains shows lack of epidemiological link among the host or patients (Frost *et al.*, 1985) and the observation in this study probably reflects this fact, since the out-patients come mostly from different routes.

One up to six plasmids were isolated from *Shigella (Sh.) dysenteriae* strains in this study. Afeworki and Drasar (1990a; 1990b) also isolated 1 up to 9 plasmids per strain from *Sh.dysenteriae* serotypes with sizes ranging from about 2.3 Kb up to 191 Kb. They also noted that a 55 Kb plasmids code for ampicillin and tetracycline resistance, while in those *Sh.dysenteriae* strains which lack this plasmid ampicillin, chloramphenicol and tetracycline resistance was mediated chromosomally. Frost *et al.* (1985) reported that ampicillin, chloramphenicol, tetracycline, sulphadiazine and trimethoprim resistance was coded by 120 to 128 Kb plasmids. So large plasmids observed among *Sh.dysenteriae* isolates in this study the observed drug resistance and in those isolates which lack such plasmids, chromosomal mediation could be involved.

An approximately 9.6 Kb plasmid which was thought to be characteristic and serotype specific for *Sh.dysenteriae* type 1 (Shiga bacillus) (Watanabe and Timmiis, 1984; Afeworki and Drasar, 1990b) was not identified in a plasmid analysis of serogroup A. This most likely implies that serogroup A isolates, at least plasmid analyzed ones in this study comprise non-Shiga bacillus (i.e. types 2 to 6) in this study. Afeworki

and Drasar (1990a) pointed out that *Sh.dysenteriae* isolates other than Shiga bacillus contain 3 to 6 cryptic plasmids, mostly 3.5 to 4.8 Kb in size and usually less than 16 Kb. possession of a single cryptic plasmid is characteristic of *Sh.dysenteriae* type 2, and the results obtained in this study agree with their observations.

Sh. flexneri isolates in this study also contained from single up to six plasmids, whose molecular weights varied between strains, probably implying serotype variation (Fig.2, Table 12). Afeworki and Drasar (1988; 1991) in Ethiopia also analyzed plasmid patterns of drug resistant *Sh.flexneri* serotypes and found that all serotypes contained a 217 Kb plasmid with a high degree of uniformity within individual serotypes, while diversity in molecular weights of plasmids was observed between serotypes. Ling *et al.* (1993) in Hong Kong found that among 102 *Shigella* isolates, three quarters were *Sh.flexneri* strains and all harboured multiple plasmids up to a maximum of 10 per strain in some strains. Casalino *et al.* (1994) noted that the genes for resistance to ampicillin, chloramphenicol, tetracycline and streptomycin formed a linkage group located on the chromosome of all *Sh.flexneri* serotypes and this plasmid-chromosome configuration may play a role in the emergence of increased genetic stability of resistance patterns. They also further noted that 80 and 110 Kb plasmids were common among serotypes and further stressed the lack of specific correlation between antimicrobial resistance patterns and predominant plasmid profile.

The study of *Sh.boydii* (serogroup C) both in developed and developing countries is scarce, probably due to its subordinate

prevalence in both regions. Plasmid profile studies of this subgroup are so scarce that comparison of this study with others was not possible. However, it is evident from this study that, this subgroup also contains multiple plasmids which differ in their profile among the strains possibly implying serotype specificity.

Two strains from *Sh.sonnei* were analyzed and both contained also multiple plasmids. Even though this subgroup contains a single serotype, different plasmid profiles were identified, probably implying difference in the epidemiological route of the strains with large plasmids coding for drug resistance. Mendoza *et al.* (1988) in Spain also analyzed isolates of *Sh.sonnei* from outbreaks using antibiotic resistance patterns and plasmid profile analysis and found that different strains carried different plasmids with some common ones. Lin and Chang (1992) analyzed plasmids from predominantly ampicillin, chloramphenicol, tetracycline and streptomycin resistant *Sh.sonnei* strains and found that large plasmids of 72 to 120 Kb were common, together with 3 to 5 small plasmids per strain, while Ling *et al.* (1993) noted the lack of predominant plasmid profile among drug resistant *Sh.sonnei* strains.

Lin and Chang (1992) observed that *Shigella* strains with multiple drug resistance were found to harbour large plasmids of 72 to 120 Kb, while Afeworki and Drasar (1990a) also noted that plasmids with sizes of 80 to 96 Kb code for multiple drug resistance and this was further confirmed by Ling *et al.* (1993) who demonstrated that 92 and 96 Kb plasmids were found to encode resistance to ampicillin, tetracycline, chloramphenicol,

kanamycin, sulphonamide, trimethoprim, cotrimoxazole and gentamicin. So the large plasmids (Lp) in multiple drug resistant *Shigella* strains observed in this study most probably represent high molecular weight plasmids which mediate drug resistance and in those strains which lack comparable plasmids, chromosomal integration (mediation) or loss of a plasmid should be suspected (Patrick *et al.*, 1995; Rankin *et al.*, 1995).

VI. RECOMMENDATION

Based on this study, the following recommendation are made:-

1. In research as well as routine diagnosis of diarrhoeal infections, multiple aetiologies should be suspected before deciding on an aetiology found at first glance. In addition to commonly encountered diarrhoeal aetiologies, suspicion and further investigations should be made for other diarrhoeal cases, since in 43.7% of the agents the aetiologies were not identified, signifying the existence of other possible bacterial, viral and/or parasitic agents of diarrhoea.
2. Since diarrhoeal diseases are mostly associated with personal hygiene and environmental sanitation, these two things should be stressed among the community. Since the samples were collected in Addis Ababa and the majority of the patients were also from the same area, particular attention should be given to sanitation of the city, taking into account overcrowdedness of the dwellers which is further complicated by influx of those "Homeless" to the city.
3. The trend of selling antibiotics in "shops" that facilitate easy access of unprescribed antibiotics which complicate drug resistance and health should be prevented. If antibiotic therapy is necessary, prescription should be made based on current drug resistance pattern of the concerned pathogen. From this study and other studies

elsewhere (Cohen and Tauxe, 1986; Khadilkar *et al.*, 1990; Olukoya and Oni, 1990) nalidixic acid and gentamicin might be the drug of choice for cases related with shigelosis and salmonelosis including *S.typhi* respectively. Generally other measures, particularly oral rehydration therapy (ORT) which is cheap and effective as well as applicable for all age groups (WHO, 1986/87) should be advised rather than antimicrobial therapy.

4. Plasmid profile analysis of specific drug resistant serotypes with appropriate molecular weight markers and conjugal transfer studies of R-plasmids should be done for further molecular epidemiologic studies of *Salmonella* and *Shigella* strains in this country .

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