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**BACTERIAL AND ROTAVIRAL CAUSES OF  
DIARRHOEA IN CHILDREN UNDER 5 YEARS AND  
THE GENETIC BASIS OF ANTIBIOTIC  
RESISTANCE.**

BY

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of Science in Microbiology of Kenyatta University.**

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## ABSTRACT

Diarrhoea causes significant morbidity and mortality among children in Kenya. The illness is caused by a variety of pathogenic organisms, some which are difficult to identify immediately. This results in the use of enormous amounts of antibiotics and as a result, some of the pathogens develop resistance. This study was carried out between June 2003 and January 2004 to investigate the main causes of diarrhoea in children below the age of five years. It also sought to evaluate the problem of antibiotic resistance among these isolates, the role of plasmids in the resistance and how much of this resistance was transferable. A total of 402 sample stool samples were collected from Kenyatta National Hospital, examined macroscopically to determine the presence of blood, pus, or mucus which are an important lead in determining the causative agents. The specimens were cultured in general and specific media to isolate different bacteria. Biochemical tests were carried out on suspect colonies for proper identification followed by API 20E screening test. A total of 13 different genera of bacteria were isolated some of which were non-pathogenic. The three genera of pathogenic bacteria isolated including 389 *E.coli*, 11 *Salmonella* spp., and 5 *Shigella* spp. Two viral species, 8 rotaviruses and 2 corona viruses, were isolated from the first 100 specimens. Antibiotic sensitivity tests were carried out on the pathogenic isolates using the Kirby Bauer disc diffusion method. *E.coli* ATCC 25922 was used as control to test the potency of the antibiotics. A total of 12 commonly used antibiotics for treatment of Gram-negative bacteria were used for the sensitivity tests. Plasmid fingerprinting was carried out using Horizontal Agarose Gel Electrophoresis (HEG) on the *E.coli* isolates. *In vitro* conjugation experiments were also undertaken to determine possible transfer of resistance to *E.coli* K 12 F- Na<sup>r</sup> that has no plasmids. Results from sensitivity tests indicated multidrug resistance on all three pathogenic strains. The highest resistance was shown by *E. coli*, where four isolates were resistant to 11 of the 12 antibiotics used. The order of resistance by *E.coli* was highest on Trimethoprim-sulphamethaxazole (89%), and lowest on ceftazidime (4.6%). *Salmonella* and *Shigella* showed similar resistance, with highest resistance shown on Trimethoprim-sulphamethoprim (90.6%), while nalidixic acid, ciprofloxacin and gentamicin were effective on all the isolates of bacterial pathogens isolated. In addition, all *Shigella* isolates were sensitive to ceftazidime. The drug resistant *E.coli* had plasmids ranging between 2.0 and 98 megadaltons (Mda). Conjugation experiments showed that the ability to transfer resistance phenotypes by the larger plasmids existed for ampicillin, chloramphenicol and tetracycline. The presence of ciprofloxacin resistant *E.coli* raises serious concerns and hence need for its frequent monitoring. These results indicate that there is need for constant surveillance on all antibiotics used for treatment of diarrhoea in children and to sensitize the public on the proper use of antibiotics.

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## CHAPTER 1

### 1.0 INTRODUCTION

#### 1.1 Diarrhoea

Diarrhoea is defined as an increase in the frequency of stools as well as decrease in consistency (WHO, 1993). It is also defined as 3 or more loose stools in 24 hour duration. Diarrhoea is infectious when it is caused by infectious agents and non-infectious when it is as a result of other reasons such as sensitivity to gluten or inherited metabolic disorders. It is divided into acute, chronic or persistent. Acute diarrhea is defined as one lasting less than 14 days duration, while persistent diarrhoea is that lasting for more than 14 days, and chronic diarrhoea is when symptoms have been present for more than 30 days.

The main dangers of diarrhoea are dehydration, malnutrition, and death (WHO, 1993). Death occurs during diarrhoeal episodes when the body rapidly loses fluids together with some electrolytes that are discharged with the stool. The essential electrolytes that are lost are sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ), chloride ( $\text{Cl}^-$ ) and bicarbonate ( $\text{HCO}_3^-$ ).

The intestines may lose the capacity to absorb fluids and electrolytes taken by mouth (WHO, 1992). About 10% of diarrhea episodes result in dehydration due to the excessive loss of fluids and electrolytes. Infants and young children are much more susceptible to dehydration and its consequences than adults. Diarrhoea can also cause malnutrition, or make existing malnutrition worse because nutrients are

lost from the body, or are used to repair damaged tissues rather than growth. In addition a person with diarrhoea may not be hungry and mothers may not feed children normally while they have diarrhoea or even for some days after recovery (WHO, 1993).

In developing countries childhood diarrhoea is among the first four causes of hospital attendance, hospital admission and even death among infants and children (Kinoti *et al.*, 1987). In these countries diarrhoea remains a major cause of morbidity and mortality averaging 26 episodes per child per year for children less than five years of age and five episodes per child per year for children less than one year of age (Bern *et al.*, 1992). Diarrhoeal incidences among children less than five years of age vary according to geographic region and age. Younger children have higher incidences than older ones. Mortality due to diarrhoea and dehydration has been highest among children less than one year of age with a global average of 19.6 deaths per 1000 live births versus 4.6 deaths per 1000 among children 1-4 years of age (Bern *et al.*, 1992, Black *et al.*, 1982, Walsh and Warren 1979). Global mortality due to diarrhoea among children less than 5 years of age during the period of 1978-1987 has been estimated at 3.3 million deaths per year (Bern *et al.*, 1992). This estimate was after a diarrhoeal disease control programme was set up by WHO in 1978 to carry out planned surveys on diarrhoea in children including the treatment and prevention of the disease (Cheng-Rong *et al.*, 1990). In Kenya infectious diarrhoea is a major cause of morbidity and mortality particularly in pre-school children (Fujita *et al.*, 1988).

## 1.2 Bacterial causes of diarrhoea

The bacterial causes of infectious diarrhoea include *E. coli*, *Salmonella* and *Shigella* (WHO, 1990). *E. coli* bacteria were discovered in human colon in 1885 by a German bacteriologist Theodor Escherich (Riley *et al.*, 1983). He showed that certain strains of the bacteria were responsible for infant diarrhoea and gastroenteritis. A large majority of *E. coli* are non-pathogenic and play an important role in the synthesizing of vitamins in the gut especially vitamin K. However the diarrhoeagenic *E. coli* (DEC) is a leading cause of pediatric diarrhoea (Fujita *et al.*, 1988). Several different types of DEC *E. coli* are capable of causing diarrhoeal diseases such as enteropathogenic (EPEC), enterotoxigenic (ETEC), enteroaggressive (EggEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC) and enteroadherent (EAEC) diarrhoea (Gillespie, 1998).

*Salmonella* species usually produce a mild, self limiting intestinal infection, characterized by fever and diarrhoea (WHO, 1993). Infections may however take the form of enteritis resulting in large volume stools which do not contain blood. Using "O" and "H" serological markers, more than 2200 serotypes have been described (Gillespie, 1998). Among these, infections by *Salmonella non-typhi* are most common. Between 5 and 10 % of normal subjects will excrete the organism in stool for an extended period.

*Shigella* are non-motile organisms which are classified into four species: *S. dysenteriae* (serological group A), *S. flexneri* (serological group B), *S. boydii*

(serological group C) and *S. sonnei* (serological group D). Shigellosis has a global distribution with highest prevalence in countries where hygiene is poor (WHO, 1982). It usually causes bloody diarrhoea. In developed countries *S. sonnei* occurs more frequently followed by *S. flexneri* infections, with *S. boydii* and *S. dysenteriae* infections occurring only rarely. In developing countries *S. flexneri* usually accounts for the greatest number of infections (WHO, 1993). Other important causes of infectious diarrhoea in children include *Campylobacter*, *Yersinia* and *Cholera*.

### 1.3 Mechanisms by which bacteria cause diarrhoea

There are several mechanisms by which bacteria cause diarrhoea (WHO, 1992). One of them is by mucosal adhesion. Bacteria that multiply within the small intestines must first adhere to the mucosa to avoid being swept away. Adhesion is through superficial hair-like antigens termed pili or fimbriae that bind to receptors on the intestinal surface. This occurs for example with enterotoxigenic *E.coli* (ETEC). In some instances mucosal adherence is associated with changes in the gut epithelium that may reduce its absorptive capacity or cause fluid secretion such as in infections with enteropathogenic or enteroaggressive *E.coli* (EPEC and EggEC). A second pathogenic mechanism is production of toxins. Enterotoxigenic *E. coli* for example, produces toxins that alter epithelial cells functions. These toxins reduce the absorption of sodium by the villi and may increase the secretion of chloride in the crypts causing secretion of water and electrolytes into the intestinal lumen.

A third mechanism is by mucosal invasion, for example by enteroinvasive *E. coli*, *Shigella* and *Salmonella*. This can result in bloody diarrhoea due to invasion and destruction of mucosal epithelial cells. This occurs mostly in the colon and the distal part of the ileum. Invasion may be followed by the formation of micro abscesses and superficial ulcers, hence the presence of red and white blood cells or visible blood in stool. Toxins produced by these organisms cause tissue damage and also mucosal secretion of water and electrolytes. Abdominal cramps, fever and shock may also result. Ulceration due to the death of the epithelial cells is also a common feature of invasive bacterial infections.

Rotaviruses are also an important cause of severe diarrhoea worldwide (Steele, 1998). In developing countries, rotaviruses may cause up to one million deaths each year, accounting for an estimated 20-25% of all deaths due to diarrhoea and 6% of all deaths among children less than five years old (Cook *et al.*, 1990). From studies in infants and young children in both developed and developing countries, rotaviruses accounts for over one third of severe cases of diarrhoea that require hospitalization and that can potentially lead to death. In rotavirus infections, most public health interventions such as clean water and improved sanitation are unlikely to decrease the incidence of the disease and hence vaccines are being developed as the first strategy of prevention (Steele, 1998).

#### 1.4 Transmission of diarrhoeal bacteria agents

Transmission of all these organisms to man is mainly by faecal oral route. Material contaminated by the faeces of animals or humans must get into the persons mouth. In developing countries food and water borne transmission of diarrhoeal pathogens are also common and in areas with inadequate sanitary disposal facilities, flies may be an important vector (WHO, 1982). Lack of adequate water supply for people living in rural areas has been a major cause of diarrhoeal episodes especially in children (Waiyaki *et al.*, 1985). Most of the people use water which collects in dams, ponds and unprotected wells. Water is also got from rivers, springs and streams for domestic needs. The same water sources are used for other activities such as bathing, swimming and playing. Livestock are brought to the water sources to drink. It is not uncommon to see several water related activities being carried out side by side and sometimes at the same site. The problem of environmental sanitation is due to inadequate and often unsafe methods of waste disposal. Thus faecal contamination of the environment is prevalent and becomes a potential cause of large scale gastrointestinal infections. In many different parts of the world it has been demonstrated that there is a close association between inadequate sanitation, and high prevalence of diarrhoeal diseases and certain enteric pathogens (WHO, 1983).

In developed countries, food and water borne disease outbreaks are unusual (WHO, 1982). However the disease is often endemic in institutions such as day

care nurseries, and in geriatric and other chronic care wards. These sometimes constitute foci from which the community at large may be infected.

### **1.5 Control and treatment of diarrhoeal diseases**

There are many strategies for controlling transmission of the common diarrhoeal diseases. These include personal and domestic cleanliness, hygienic food preparation and storage, clean and plentiful water supply and sanitary waste and refuse disposal. Breastfeeding for younger children also plays an important role in protection against intestinal infections especially during the first six months of life. The protective capacity of breast milk is due to the presence of immunological as well as non-immunological factors (WHO, 1992). In some cases breastfeeding is substituted by use of infant feeding bottles, which easily become contaminated with faecal bacteria and can cause diarrhoea. When milk is added to an unclean bottle it becomes contaminated. If it is not consumed immediately bacterial growth occurs. Storing cooked food at room temperature can also easily cause contamination for example by contact with contaminated surfaces or container. If food is kept for several hours at room temperature bacteria in it can multiply many times (WHO, 1992). The single most effective preventive measure is through regular hand washing with soap and warm water after handling animals, after visiting the toilet and before any meal (Heymann, 2002). Therefore in order to alleviate the problem of diarrhoeal diseases in developing countries there must be promotion of improvements in living standards, which has played a

major role in reducing prevalence of diarrhoeal illness in Europe and North America (WHO, 1995).

A large proportion of diarrhoeal episodes are self-limiting (WHO, 1990). However, diarrhoeal diseases need treatment especially when dehydration is noticed and when they involve bloody stool. In bacterial diarrhoea, treatment should not be given before proper analysis of stools has been done to establish the causative agents. Antibiotics should therefore only be given to specific cause of diarrhoea and susceptibility tests carried out (WHO, 1990). This is because numerous problems are associated with the misuse of antibiotics. Adverse reactions are common and an extensive use of antimicrobials contributes to widespread antibiotic resistance. This represents an additional side effect especially in poorer countries. In many countries, a high incidence of antibiotic resistance has been observed (Murray, 1986). Widespread outbreaks of shigellosis due to antibiotic resistant *Shigella*, for example, has been documented in central America, Asia and Africa (Mikhail *et al.*, 1990, Edward *et al.*, 1993). Most pathogenic bacteria continue evolving mechanisms essential for species survival, particularly by the selection and propagation of resistant phenotypes (Kalama, 1999). Of particular importance in this regard is multidrug resistance demonstrated in many infectious bacteria such as *Shigella*, *E.coli* and *Salmonella*. Multidrug resistance on *Shigella* isolated from patients with diarrhoea was shown in Kwale province (Sang *et al.*, 1985). Twenty nine per cent (29%) of *Shigella flexneri* were resistant to tetracycline. Multiple drug resistance was observed in

some *Shigella flexneri* 2a serotype. Similar studies in Nyanza province on enteropathogens from community water sources, showed multiple drug resistance of faecal coliforms (Waiyaki *et al.*, 1985). The resistance was shown on ampicillin, tetracycline, chloramphenicol, and streptomycin. Antimicrobial susceptibility on *Salmonella* and *Shigella* isolates from Aids patients in Nairobi, Kenya, also indicated resistance to commonly used antibiotics, namely, amoxycillin, tetracycline, streptomycin and cotrimoxazole (Kariuki *et al.*, 1994). Other studies on *Shigella dysenteriae* showed multidrug resistance on ampicillin, tetracycline and chloramphenicol (Oundo *et al.*, 1996). The study was conducted following an outbreak of dysentery in three areas in Kenya, namely, Kisumu, Mombasa and Nairobi.

Most infections are treated on an empirical basis, and therefore, clinical experience has indicated that this has caused resistance to conventional chemotherapy (Atif *et al.*, 2000). Current knowledge of the prevailing patterns of antibiotic resistance is important for the proper selection and use of antimicrobial drugs. The presence of infectious diarrhoeal diseases therefore has been viewed as a factor that can undermine national and international security (Heymann, 2002). The threats posed by infectious diseases have global causes and effects that can only be managed by strong National public health capacity. This includes constant analysis of stools before prescribing any antibiotics and susceptibility testing to control multidrug resistance.

## **1.6 Justification**

Since diarrhoea causes significant morbidity and mortality among children in Kenya, the causative agents should be properly identified, classified and antibiotic sensitivity tests done. In addition a lot of health facilities in Kenya are dispensing drugs indiscriminately hence creating a lot of resistance gene pools. This study set to determine the level of this antibiotic resistance and hence help in the choice of drugs for treatment.

## **1.7 Hypotheses**

1. Most diarrhoea in children have a bacterium or several bacteria as the causative agent (s).
2. The antibiotics being used are effective in treating bacterial diarrhoea.
3. There are specific genes that confer resistance to routinely used antibiotics on the diarrhoea causing organisms or pathogens.
4. These antibiotic resistant genes are transferable through *in-vitro* conjugation.

## **1.8 Objectives of the study.**

### **1.8.1 General objectives**

To determine the bacterial causes of diarrhoea in children, below five years of age in Nairobi and to examine resistance patterns of these pathogens to commonly used antibiotics.

### 1.8.2 Specific objectives.

1. To examine stool samples, isolate and identify the causative agents of diarrhoea in children.
2. To determine the antibiotic susceptibility of the isolates to commonly available antibiotics.
3. To characterize antibiotic resistant strains by plasmid DNA profiles.
4. To determine if R- resistance factors in bacterial isolates are transferable by *in-vitro* conjugation tests.

## CHAPTER 2

### 2.0 LITERATURE REVIEW

#### 2.1 Global burden of diarrhoeal diseases

Globally, diarrhoea is one of the principal causes of morbidity and mortality among children, especially in the developing world (Kosek *et al.*, 2003). In 1992, a review of studies conducted in the 1980's suggested that mortality due to diarrhoeal diseases was about 3.3 million annually. There has been an increase in food related diarrhoeal diseases over the past decade (Gillespie, 1998). This is due to intensive methods of animal husbandry in which animals are kept in close proximity with humans, facilitating the spread of enteric organisms. In the U.S.A., *Salmonella* infections are common in infants (Chhabra and Glacer, 1994). The incidence in the first month of life is estimated by the Centres for Disease Control and Prevention (CDC) to be approximately 75 per 100,000 live born infants.

The diverse range of pathogens which may affect the gastrointestinal tract, together with the large number of individual species that form the normal flora of gastrointestinal tract, means that the investigation of faeces is difficult (Madigan *et al.*, 2000). Specific methods should therefore be used for each pathogenic microorganism

Various studies have been carried out in many parts of the world relating to children's pathogens (WHO, 1993). The studies involve all the pathogens in general or a specific selected type. Isolation of the pathogen is important in order for treatment to be effected. Chhabra and Glacer, (1994) isolated *Salmonella spp.* from children with bloody diarrhoea

who were treated and recovered in ten days. Effective treatment may also require determining the etiology, sources and risk factors of the infection (Indar-Harrinauth *et al.*, 2001). This can help to initiate public health education on the risk factors involved in infection.

The prevalence of enteropathogens in diarrhoeal diseases should be determined during isolation. An example is a study in the United States (Talan *et al.*, 2001) involving 877 episodes of bloody diarrhoea. *Shigella*, *Campylobacter* and *Salmonella* were isolated in 30.6% of the cases and this assisted in effectively treating the patients. In Israel prevalence of *Shigella* species found that the relative prevalence of *Sh. sonnei* in patients with shigellosis increased over the years from 60% in 1986 to 91% in 1991, while the prevalence of, *Sh. flexneri* decreased from 29% to only 8% (Ashkenazi *et al.*, 1993). This information helped to initiate control measures for *Sh. Sonnei*.

The virulence of some enteropathogens can be increased considerably by the presence of internal factors such as production of enterotoxins (Echeveria *et al.*, 1987, Ansaruzzan *et al.*, 2000). Some species have also been associated with persistent diarrhoea more often than others, for example a study in Bangladesh (Baqui, 1990) found *Shigella spp.* to be significantly associated with the occurrence of acute diarrhoea compared with other pathogens.

Annual incidence of diarrhoea has been found to be associated with the age of the children, being highest in younger children below 2 years and decreasing progressively

with age (Black *et al.*, 1982, Cheng-Rong *et al.*, 1990). Infant mortality has consequently been found to be highest in the younger children below 2 years (Fikree, 2000). This mortality has been associated with delayed hospitalization and treatment rather than to differences in host factors or in the virulence of the pathogen (Carmeli *et al.*, 1993). Mortality can therefore be reduced by hastening hospitalisation and treatment.

## 2.2 African's burden of diarrhoeal diseases.

Africa has had various studies on diarrhoea conducted involving single species or general enteropathogens. Such a study on a single species involved a retrospective analysis of the occurrence of *Sh. flexneri* serotypes in Algeria, Libya, Ethiopia, Kenya and Uganda (Mutanda, 1987). In general, *Sh. flexneri* type 2 was found to be the most frequently isolated infective agent. However, a changing frequency among the serotypes had taken place in Uganda, Kenya and Ethiopia. The proportion of type 2 increased in Uganda from 1968 to 1982 at the time when the records were reviewed. In Kenya in the 1960s, type 2 was dominating, but changed to type 6 by 1977 and back to type 2 by 1982, while in Ethiopia, the change from type 1 to 2 occurred in 1982. Similar studies in Ghana, (Akpedonu, 1994, Nkurumah, 1992, Pithe *et al.*, 1993) aimed at identifying the enteropathogens associated with paediatric persistent diarrhoea identified a variety of bacteria, but *E. coli* was the most common, followed by *Salmonella spp*, *Campylobacter jejuni* and *Shigella spp*.

In many isolation studies (Geyer *et al.*, 1993, Nkurumah, 1992), mixed infections are more often encountered than single infections and both pathogenic bacteria and parasites

are commonly isolated. Although the ages of the patients varied from one month to three years those aged between four to six months were the most susceptible to severe diarrhoea, requiring hospitalization. Although more male than female children below one year were hospitalized the trend was reversed in children above one year. In addition, babies less than one month of age, where maternal antibodies should still provide protection, were also admitted to the gastroenteritis unit.

### 2.3 Kenyan incidences of diarrhoeal diseases.

In a study carried out in Nderu, Lusigiti, Kamirithu and Rironi of Kiambu district (Kinoti *et al.*, 1987), mixed infections involving several bacterial species were identified including *Campylobacter*, enterotoxigenic *E. coli* (ETEC), *Aeromonas*, and *Yersinia enterocolitica*. Chungu *et al.*, 1989 also identified mixed infections in over 80% of the cases with a maximum of 7 potentially pathogenic organisms occurring together in a single specimen. Mixed infections have also been found in other studies (Fugita *et al.*, 1988, Sang *et al.*, 1985). During epidemic outbreaks children are more often affected than adults (Shiroya, 1984), for example, in a cholera outbreak in southern coast of Kenya children below 5 years were found to account for 11% of the cases.

Sometimes inaccurate assessment of true diarrhoea status produces biased estimates of its occurrence (Thomas *et al.*, 1989), sensitivity and specificity of the mother's report of diarrhoea is 0.79 and 0.94 respectively, when compared to an independent observation of loose stool consistency. Analysis of all stool specimens should therefore be properly analysed before implementing any treatment.

#### 2.4 Involvement of *Hafnia alvei* in diarrhoeal diseases

*Hafnia alvei*, a non-pathogenic organism has been isolated from stool of children with diarrhoea (Gatheru *et al.*, 1983). This bacterium is a facultative anaerobe originally described in 1943 as *Paracolobactrum aerogenoides* but later placed in the genus *Hafnia* (Brenner *et al.*, 1977). For decades *H. alvei* was regarded as non-pathogenic but there have been reports of sepsis (Englund, 1969 and Mobley, 1971), meningitis (Mojtabace and Siadati, 1978), urinary tract infections (Andronova, 1978) and postoperative wound infection (Frick *et al.*, 1990) attributed to it. *H. alvei* associated with gastroenteritis has been recognised in several studies (Kalashnikova *et al.*, 1975; Albert *et al.*, 1991; Ginsberg and Goldsmith, 1988). Studies by Carvalho and McMillan (1990), Westblom and Milligan, (1992) and Gunthard and Pennekamp, (1996) also identified *H. alvei* as the cause of different infections. *H. alvei* has also shown antibiotic resistance to cephalothin and tetracycline but susceptibility to ampicillin, second and third generation cephalosporins, aminoglycosides, aztreonam, imipenem and trimethoprim-sulphamethaxazole (Westblom and Milligan, 1992).

#### 2.5 Rotaviruses in diarrhoea

Apart from bacteria, rotaviruses have been identified as the principal cause of diarrhoea especially in children (Bishop *et al.*, 1974, Middlestone *et al.*, 1974, Kapikian *et al.*, 1976). Viral particles especially rotavirus are common causes of the gastroenteritis, responsible for 50% of children admitted with acute gastroenteritis (Bishop *et al.*, (1974;

Davidson *et al.*, (1975); Flewett *et al.*, (1973). The viral particles in the stools of children with acute gastroenteritis were identified using the electron microscope which was found to be an easy method of detecting rotavirus (Coiro *et al.*, 1988) and was found to be as sensitive as the conventional methods.

Rotaviruses are associated with high morbidity in developed countries and mortality in less developed countries (Leung and Pai, 1988). In the United States, rotavirus infections account for approximately one half of all paediatric patients hospitalised with gastroenteritis (Kapikian *et al.*, 1976). Although the proportion of mortality due to rotavirus diarrhoea in children is not well established, an estimate puts the number of rotavirus associated deaths to 500,000 a year (Vesikari, 1985). Studies in Ethiopia also showed rotaviral diarrhoea as an important cause of morbidity and mortality in children (Assefa, 1993).

Rotaviral infections have highest frequency in children below 12 months (Candeias *et al.*, 1989, Panigrahi *et al.*, 1985, Olusanya and Taiwo, 1989). In Kenya isolation rate in children aged 1-12 months ranged from 14%-54% and peaked in the 6-12 month age group (Mutanda *et al.*, 1984). Studies by Huq *et al.*, (1988), on incidences of asymptomatic rotavirus infections in neonates identified presence of rotavirus antigens in their stool.

Prevalence of rotavirus is common from results of various studies in various parts of the world including Thailand (Hasegawa *et al.*, 1987), Nigeria (Coker *et al.*, 1987). In Kenya

rotaviruses have been isolated (Chunge *et al.*, 1989; Kinoti *et al.*, 1987) where detection rate of rotavirus in children with diarrhoea was higher in urban than rural patients.

Coinfection of rotavirus with bacteria and parasites is common and has been documented (Cascio *et al.*, 2001, Essers *et al.*, 2000) with both bacteria (*Salmonella*, *Campylobacter*, *Shigella*, *Yersinia* and *Aeromonas*) as well as parasites (*Giardia* and *Cryptosporidium*). Other bacteria involved with coinfection include enteropathogenic *E.coli* and *Vibrio cholerae* (Black *et al.*, 1982).

## **2.6 Antibiotic resistance in Enterobacteriaceae**

### **2.6.1 Biochemical basis for antibiotic resistance in Gram negative bacteria**

The development of antibiotic-resistant (AR) bacteria in any country is of global importance (WHO, 1992). After their initial selection and dissemination, antibiotic resistant bacteria can be transferred across international borders by human travellers, animal and insect vectors, agricultural products, and surface water. The sources and routes of importation of strains of antibiotic resistant bacteria are most often unknown or undetected, because many bacteria carrying resistance genes do not cause disease, and routine surveillance often does not detect them (Okeke and Edelman, 2001). Control of international dissemination of antibiotic resistant bacteria depends on methods to reduce selection pressure for the development of such bacteria and improved surveillance to detect their subsequent spread.

Bacteria have evolved sophisticated biochemical mechanisms to evade the lethal effects of antibiotics. These mechanisms include target overproduction of resistant genes or modification, permeability barriers, enzymatic inactivation and sequestration (Davis, 1994). Antibiotic resistance can be primary (associated with a biosynthetic pathway naturally present in the organism) or secondary (associated with the acquisition of a resistant gene) (Davis, 1992). These resistance mechanisms can exist singly or in combination. Enzymatic inactivation is highly specific because a precise stereochemical fit must be present for enzymes to effectively inactivate antibiotics. Target alterations can affect the binding of either single or multiple antimicrobials because some serve as targets for several different classes of antibiotics. Similarly permeability barriers can be either general or specific, depending upon the manner of antimicrobials that traverse the altered pathway (Nikaido, 1994).

In the bacterial cell membranes, a complex array of multidrug efflux transporters and permeability barriers can exist in resistant bacterial cells. In Gram-negative bacteria, the peptidoglycan cell wall is enveloped by a lipopolysaccharide outer membrane interrupted at many locations by porins which serve as pathways by which antibiotics can traverse the membrane (Nikaido, 1994). Lipopolysaccharides serve as an efficient barrier against the rapid penetration of certain antibiotics. Penetration of antibiotics through the outer membrane can occur by passive diffusion through porin channels by facilitated diffusion using siderophore receptors, or by self promoted uptake (Chopra, 1990). Porin channels are non-specific aqueous diffusion channels present in the outer membrane of Gram-negative bacteria and exclude antibiotics by their size. Resistance can be due to an altered

lipid bilayer, which forms the basic structure of the outer membrane. Certain antibiotics bind to a non-saturable anionic transporter and are pulled across the cytoplasmic membrane by the internal negative charge of the cell. This is an energy-dependent process and is fuelled by a differential gradient (Hancock and Bellido, 1992).

Active efflux mechanisms are based upon energy dependent system (Levy, 1992) and is due to the presence of specialized membrane proteins. The multidrug efflux proteins are specific export proteins that are encoded on plasmids or in the chromosome. They pump oxytetracyclines, tetracyclines, fluoroquinolones, chloramphenicol, erythromycin,  $\beta$ -lactams and other antibiotics.

Enzymatic modifications of aminoglycosides occur by one of three mechanisms including *N*-acetylation, *O*-nucleotidylation and *O*-phosphorylation (Shaw *et al.*, 1993). Specific enzymes attack certain amino or hydroxyl groups and add an acetyl, adenylyl or phosphate molecules hence modifying the antibiotics.

Another mechanism is ribosomal resistance, which although less common, results in resistance to certain antibiotics such as streptomycin (Meir *et al.*, 1994). Similarly ineffective transport of strict anaerobic bacteria causes resistance to certain antimicrobials due to their lack of an oxygen transport system (Hancock and Bellido, 1992).

### 2.6.2 Genetic mechanisms for antibiotic resistance in Gram negative bacteria

One of the genetic mechanisms of resistance is intrinsic resistance which can be due to decreased binding affinity of the penicillin-binding proteins (PBPs) (Williamson *et al.*, 1985). Resistance also results from impermeability of the bacterial membranes. Multidrug efflux may be increased in intrinsically resistant isolates. Intrinsic factors include production of  $\beta$ -lactamases which destroy penicillins and cephalosporins by hydrolysis and this is the greatest single cause of resistance to antimicrobials (Li *et al.*, 1994).

Another genetic mechanism is due to structural mutations in pre-existing genetic determinants (Heisig and Tschorny, 1994) which occurs in streptomycin, rifampin, and more recently fluoroquinolones such as norfloxacin and ciprofloxacin. Resistance in these instances result from point mutations which is addition, deletion or substitution of nucleotide(s). This results in alteration of protein sequence of the translated gene product, somewhere in the gene encoding the proteins that are targeted by the antibiotic. Mutations occur at random and those that are not recognised and repaired by the host repair systems are passed on to successive generations of bacteria. In the case of streptomycin, one step resistance occurs via point mutations in the gene encoding ribosomal proteins resulting in an inability of streptomycin to bind to its target, the ribosome. Resistance to rifampin which targets cellular DNA-dependent RNA polymerase occurs at a high frequency in most bacteria. In *Escherichia coli*, resistance results from amino acid changes in one of six highly conserved regions of a gene that encodes for RNA polymerase (Jin and Gross, 1988).

Fluoroquinolones such as ciprofloxacin target the host DNA gyrase enzyme, which is essential for cellular maintenance and replication, because it is involved in coiling and uncoiling of chromosomal DNA. Mutations resulting in resistance of *Escherichia coli* to ciprofloxacin most commonly occur in the genes encoding for the enzyme gyrase.

One step mutation to antibiotics resistance can also result from changes in the regulation of pre-existing genes (Bergstrom *et al.*, 1982). All Gram-negative bacteria have been found to possess a chromosomal  $\beta$ -lactamases gene that is primarily a cephalosporinase but that result in resistance to all cephalosporins and penicillins when produced in large amounts. The production of this enzyme can be induced by exposure to antibiotics among them cefoxitin and imipenem.

The number of new antimicrobial agents has led to the isolation of some classes of mutants that have become clinically important due to the presence of an antimicrobial-rich environment. These mutations result in extended-spectrum  $\beta$ -lactamases (ESBLs) (Jácopy and Medeiros, 1991). The development of the ESBLs cephalosporins was fuelled in part by the need for antibiotics that were resistant to hydrolysis by the plasmid-mediated  $\beta$ -lactamases of Gram-negative bacilli. These enzymes were acquired by *E.coli* and became prevalent within the organism and other enterobacteriaceae after the clinical introduction of ampicillin. The cephalosporins avoided hydrolysis by these enzymes via relatively minor alterations in the  $\beta$ -lactam molecular structure that precluded effective interaction with the  $\beta$ -lactamase molecule. Due to this limited modification, the enzymes

are able to restore some degree of activity as has occurred with ceftazidime-hydrolysing ability. The emergence and spread of organisms producing these enzymes have threatened to significantly decrease the ability of the entire cephalosporin class of antibiotics (Meyer *et al.*, 1993).

There are three primary mechanisms by which bacteria exchange DNA; transduction, transformation and conjugation. Transduction is the process of DNA transfer mediated by bacteriophages (Davis, 1990) able to invade and replicate within bacterial cells. Replicated phage genome is packaged into phage heads which are released after lysis of the host cells. They then invade other cells and integrate into bacterial chromosomes after invasion. In some instances the phage genome is excised from the chromosome, along with a neighbouring segment of DNA, which is packaged into the phage head along with the phage genome (Dulbecco, 1990). If the genome is integrated next to an antimicrobial resistance determinant, then the resistance gene can be transferred. If the packaged DNA is a plasmid, it will be capable of replication within the recipient cell as long as the host is permissive for plasmid replication and there are not incompatible plasmids already present.

Transformation is a process by which bacteria are able to take up naked DNA from the surrounding environment, which are incorporated into the bacterial chromosome in a heritable form (Prescott *et al.*, 1999). In a natural transformation the DNA comes from a donor bacterium. The process is random and any portion of a genome may be transferred between bacteria. When bacteria lyse they release a considerable amount of DNA into the

surrounding environment. These fragments may be relatively large and contain several genes. If a fragment contacts a competent cell, one able to take up DNA and be transformed it can be bound to the cell and taken inside. Penicillin resistance in some bacterial strains has been passed in this way.

Conjugation is the process by which different bacterial cells come into contact to exchange genetic material (Davis, 1990). Conjugation represents the general mechanisms by which resistant genes spread between related and some unrelated bacteria. In most cases, the sequences of events that make conjugation possible are encoded on plasmids within the donor strain. Plasmids are extrachromosomal replicating elements that range widely in size and encoded functions. Some exceed 180 kilobases (kb) in size and encode a wide variety of antimicrobial resistance genes (Rice *et al.*, 1990). Others are less than 3 kb and encode a single resistance gene or encode no resistance genes at all (Couturier *et al.*, 1988). The best studied plasmids in Gram-negative bacteria are the F (fertility) factor from *E.coli*. The F-plasmid conjugates at an extremely high frequency resulting in most recipient cells containing a copy of the plasmid within two hours. In some strains, the F factor becomes integrated into the bacterial chromosome. From these strains the F factor may transfer to recipients, followed by large segments of the *E.coli* chromosome. Strains in which the F factor is integrated into the bacterial chromosome are referred to as Hfr (high frequency recombinant) strains (Low, 1987). Most techniques of isolating plasmids take the advantage of plasmids presence within cells in a covalently closed supercoiled state making them more resistant denaturation in alkaline solutions. The ability to

separate plasmids from the chromosomes allows determination of their size by separation on agarose gels.

Most of the antibiotic resistance has been associated with presence of plasmid DNA coding for resistance. Emergence of gentamicin resistant *E. coli* 0111:K58 in Kenya was observed in a nursery ward at Kenyatta National Hospital (Mutanda *et al.*, 1987). Gel electrophoresis of the *E. coli* revealed that the multiple antibiotic resistances were plasmid mediated. This resistance has been transferred from one strain to another through conjugation (Neu, 1985). Anderson and Datta, (1965) found that ampicillin resistance in *Salmonella typhimurium* could be transferred to recipient *E. coli* and that an R-factor (resistant factor) was involved. Venezia *et al.*, (1995) in the USA working on *Klebsiella oxytoca* showed that the resistance to ceftazidime was associated with a conjugative plasmid of ~ 85kb. Similarly plasmid related resistance was found in *Shigella* spp. (Lee *et al.*, 1991) and *Klebsiella pneumonia* (Rice *et al.*, 1996). In the *Shigella* plasmid profiles of approximately 76, 69, 5.1, 4.4, and 3.6 megadaltons were isolated. In the *Klebsiella* many plasmid patterns were observed in the clinical isolates but all of them possessed large plasmids that ranged in size from ~150kb to > 200kb.

Haider *et al.*, (1988) observed ninety two per cent of the *Sh. dysenteriae* type 1 strains showing a typical profile of four plasmids with masses of 140, 6, 4, and 2 megadaltons. There was a strong association between the possession of this plasmid and resistance to chloramphenicol, streptomycin, tetracycline and trimethoprim-sulphamethaxazole. Abimbola *et al.*, (1993) showed six distinct antibiotic resistance patterns exhibited by the

*E. coli* strains where 60% were found to harbor plasmids of molecular weights ranging from 0.62 to 60 kb.

Conjugation has been shown to transfer plasmids conferring resistance from one bacterium to another (Coker *et al.*, 1987). Conjugative plasmids on *E. coli* and *Klebsiella pneumoniae* were shown to confer increased resistance to ceftazidime, gentamicin and tobramycin (Lautenbach *et al.*, 2001). Two plasmids conferred resistance to amikacin, while five other plasmids conferred resistance to cotrimoxazole. Similar conjugative plasmid profiles have been isolated in Bangladesh (Haider and Huq, 1986, Hermans *et al.*, 1996) in studies on *V. cholerae*, *E. coli* and *S. typhi*. In Ethiopia investigation on the presence of transferable drug resistance found plasmid mediated transfer in 77% of the 13 strains studied (Tiruneh, 1990). Similar studies on *E. coli* in Kenya showed that plasmids were transferred quite easily from the *E. coli* resistant strains to the recipient *E. coli* K12 F- which does not normally carry any plasmid (Bebora *et al.*, 1994). Easy transfer of plasmids was also demonstrated from *Salmonella* and *Shigella* species to *E. coli* K12 F- (Bebora *et al.*, 1994).

### 2.6.3 Mechanisms of antibiotic resistance

Studies on antibiotic resistance have distinguished resistance phenotypes which are important in interpreting multidrug resistance (Vahaboglu *et al.*, 1996, Herman *et al.*, 1996). Vahaboglu *et al.*, 1996 identified four patterns well as cross transfer of resistance between different species while Herman *et al.*, 1996 identified twenty six strains which

were susceptible to all antibiotics tested and 28 were resistant to multiple antibiotics. Similar multidrug resistance by *S. typhi*, *Shigella* and *E. coli* has been reported in other parts of the world (Bhutta et al., 1991, Aggarwal et al., 1988, Xiao-Li et al., 1990 and Gebra-Yohannes and Drasar, 1988).

With the increased interest and concern regarding antimicrobial resistance, there are now several major programs in different countries to conduct surveillance for antimicrobial resistance on a national and international scale (Pfaller et al., 2001). One such program was set to evaluate the prevalence of ESBL-producing strains among species of Enterobacteriaceae in Europe (Winokur et al., 2001). The highest percentage of ESBL phenotype for ceftazidime, ceftriaxone, or aztreonam was detected among *K. pneumoniae* strains from Latin America (45%), followed by those from the western pacific region (25%), Europe (23%), the United States (8%), and Canada (5%). *P. mirabilis* and *E. coli* strains for which MICs of extended-spectrum cephalosporins or monobactams were elevated were more prominent in Latin America. ESBLs strains showed high levels of co-resistance to aminoglycosides, tetracycline, and trimethoprim-sulfamethoxazole. Ciprofloxacin and imipenem were found to be highly effective against ESBL strains. Organisms expressing an ESBL were found to be widely distributed by the study.

Although quinolones such as ciprofloxacin have been effective in treating diarrhoeal diseases (Replogle et al., 2000), resistance to other antibiotics such as trimethoprim-sulphamethaxazole, ampicillin, cefixime and nalidixic acid has been common. Aminoglycosides have also been highly active against most bacteria (Chamberland et al.,

1992). This information has helped in implementing chemotherapy for Gram-negative septicaemia and has demonstrated that several older and newer agents, alone or in combination, can be used as adequate initial therapy for Gram-negative sepsis.

Various antibiotics such as ampicillin and trimethoprim-sulphamethaxazole have been so ineffective that they are no longer recommended for treatment of diarrhoeal diseases including shigellosis in the United States and other parts of the world (Replogle *et al.*, 2000, Ashkenazi *et al.*, 1993, Bennish *et al.*, 1992). In several countries in Africa, various antibiotics have been proposed as drugs of choice for diarrhoeal diseases. Nalidixic acid is used in Rwanda and Ethiopia (Aseffa *et al.*, 1996, Mutwewingambo and Mets, 1987) while norfloxacin, ciprofloxacin, ceftriaxone and ofloxacin are used in Egypt (Mourad *et al.*, 1993) after multidrug resistance to commonly used antibiotics was noted (Frost *et al.*, 1981). However, in recent years resistance to ciprofloxacin (Patterson *et al.*, 2001) as well as other quinolones (Velasco *et al.*, 2001) has been noted. Prior use of fluoroquinolones is an important risk factor related to quinolones resistance in *E.coli* bacteremia (Cheong *et al.*, 2001).

Efforts to control antibiotics resistance should emphasize judicious use of antibiotics as well as barrier precautions to reduce spread (Lautenbach *et al.*, 2001). Control can also be achieved by a combination of contact isolation, hand hygiene, gut decontamination with orally administered fluoroquinolones (Paterson *et al.*, 2001). Molecular typing techniques can also be used to track the spread of resistance (Almuneef *et al.*, 2001). These studies

have shown that antibiotic use increases colonization of infants with resistant strains of Gram-negative rods.

In Kenya, Mutanda *et al.*, (1987) recommended routine serotyping and periodic antibiotic sensitivity testing for both enteropathogens and non-enteropathogenic *E.coli* strains as important measures in controlling multidrug resistance. This is important because some common antibiotics such as chloramphenicol have been found to be effective against *S. typhi* (Nesbitt *et al.*, 1988), while being ineffective against *E.coli* (Serenwa *et al.*, 1991) and therefore its effectiveness should be tested for each organism. In the face of rapidly changing sensitivity patterns, continuing liaison between laboratory workers and national drug purchasing bodies is important (Kariuki *et al.*, 1999).

## CHAPTER 3

### 3.0 MATERIALS AND METHODS

#### 3.1 Specimen collection

Stools from children under five years of age presenting with diarrhoea at Kenyatta National Hospital were collected in sterile disposable plastic containers. The stool specimens were labelled and carried to the laboratory where the analysis was done in the Centre for Microbiology laboratory.

#### 3.2 Physical Examination.

Stool specimens from 402 patients as determined by Fisher et al., 1998 were first observed macroscopically then microscopically. The stools were examined for colour and were recorded as formed, loose or watery. Special attention was paid to the presence of blood, mucus and pus where visible, which were dully recorded.

##### 3.2.1 Microscopic Examination

Methylene blue preparations were made according to the staining method by Cheesbrough, 1989, by mixing methylene blue stain with the specimen on the slide which was then covered using a cover slip. Examination was done for faecal leucocytes using 40x objective with the condenser iris closed sufficiently to give good contrast, and observe presence of red blood cells and pus cells.

### 3.3 Culturing of various bacteria

Various bacteria suspected to be the causative agents of diarrhoea in children were isolated on different media as outlined by Cheesbrough, (1989). The media used were prepared according to manufacturers' instructions.

#### 3.3.1 *Escherichia coli*

A loopful of the specimen was picked, streaked on MacConkey agar (Oxoid Basingstoke, UK) and incubated aerobically at 37°C overnight. To identify *E. coli* suspect lactose fermenting colonies (pink in colour) were biochemically tested using alkaline peptone water and Kovac's indole reagent. Colonies that could not be immediately identified from the plates by their morphological features were further identified by commercial biochemical tests on API 20E strips (Biomérieux). All *E. coli* isolates were stored at -70°C for further analysis.

#### 3.3.2 Identification of other Enterobacteriaceae

##### *Shigella spp* and *Salmonella spp*

Several loopfuls of stools were cultured in Selenite F broth at 37°C overnight for purposes of enrichment. A loopful of this enrichment solution was sub-cultured onto Xylose-lactose-desoxycholate (XLD) agar (Oxoid, Basingstoke, UK) plates. The plates were incubated aerobically at 37°C overnight. Suspect colonies were further tested biochemically using Simmon's citrate agar, triple sugar iron (TSI) agar and urea. Further identification of both *Shigella* and *Salmonella* was done using commercial biochemical tests on API 20E (Biomérieux).

Other bacteria that also grew on the above media were identified from the biochemical tests.

### **3.4 Biochemical tests**

All the biochemical tests were adopted from Cheesbrough, 1989.

#### **3.4.1 Indole Test**

Suspected *E.coli* colonies were picked using a sterile wire loop and inoculated into 5ml of tryptophan reagent. The inoculated medium was stoppered and incubated at 35-37°C overnight. A drop of Kovac's reagent was added to the culture. Production of red colour at the top of the reagent was a positive test indicating indole production, while lack of red colour was a negative test indicating that no indole was produced.

#### **3.4.2 Citrate utilization test.**

Colonies of the suspect organisms from morphological features were cultured in slants of simmon's citrate agar (Oxoid, Basingstoke, UK) using a straight sterile wire. The inoculated medium was incubated at 35-37°C for up to four days checking daily for growth. Blue colour was a positive result indicating citrate utilization while lack of colour change was a negative result hence lack of citrate utilization.

### 3.4.3 Triple sugar iron (TSI) test

Suspected colonies of test organisms from morphological features were inoculated in agar slants of TSI (Oxoid) using straight sterile wire. The media was incubated at 35-37°C overnight. Blackening of the medium was a positive test indicating H<sub>2</sub>S production while lack of blackening was an indication of negative test hence no production of H<sub>2</sub>S.

### 3.4.4 Urease test

A colony of the test organism was inoculated into the medium using a sterile wire loop. The tube was stoppered and incubated at 35-37°C overnight. The tube was examined for urease production by looking for a red-pink colour in the medium which was a positive test indicating urease production. The negative test was indicated by absence of red / pink colour. Positive urease control was an indication of *Proteus vulgaris* while a negative urease control was an indication of *Escherichia coli*.

### 3.5 Rota virus detection

An Enzyme-linked immunosorbent assay (ELISA) method was used to detect the rotavirus. The virus was confirmed using an electron microscope. The use of the electron microscope helped to identify characteristic particles of the corona virus. The samples for electron microscopy were sent to the Department of Medical Microbiology and Genitourinary medicine, University of Liverpool, Liverpool, United Kingdom.

### **3.5.1 Enzyme-linked immunosorbent assay (ELISA) method for detection of Rota virus.**

A 10% faecal suspension was prepared with phosphate-buffered saline and subjected to ELISA test using Rotazyme (Abbott) commercial kit (Cheesbrough, 1989). The basic test consisted of antibodies bonded to enzymes which are able to catalyse a reaction yielding a visually discernable end product while attached to the antibodies. The antibody binding sites remained free to react with their specific antigens. The ELISA test used was the solid-phase immuno-assay. The system had antibodies directed against the rotaviruses antigens firmly fixed to a solid matrix on the inside of the wells of a micro dilution tray. Approximately 0.05ml of the diluted stool was fed into each well using a micropipette. Presence of the rotaviruses antigens caused formation of antigen-antibody complexes. Unbound antigens were thoroughly removed by washing, and then a second antibody against the rotavirus antigen was added to the system. The second antibody had been bound to an alkaline phosphatase enzyme. The rotavirus antigen present in the system bonded to the second antibody, forming a sandwich with the antigen in the middle. The unbound labelled antibody was washed thoroughly followed by addition and hydrolysis of the enzyme substrate which caused the change of colour that indicated the presence of rotavirus antigen.

### **3.6 Antibiotic susceptibility testing**

Antibiotic susceptibility testing was done on bacterial isolates by the disk diffusion technique based on the Kirby Bauer technique (Oxoid, Basingstoke,

UK) (NCCLS, 2000). The disks used were Trimethoprim-sulphamethoxazole (1.25/23.75 µg), tetracycline (30 µg), streptomycin (10 µg), nalidixic acid (30 µg), gentamicin (10 µg), ciprofloxacin (30 µg), chloramphenicol (30 µg), cefuroxime sodium (30 µg), ceftazidime (30 µg), ampicillin (10 µg) and amoxicillin – clavulanic acid (10 µg). These antibiotics were chosen because they are commonly used for treatment of gastrointestinal infections in Kenya. The standard quality control strain used was *E. coli* ATCC 25922 as recommended by National Committee for Clinical Laboratory Standards (NCCLS 2000).

### **3.6.1 Inoculum preparation and inoculation of Test plates**

This method was adopted from NCCLS, 2000. Three to five well isolated colonies of the same morphological type were selected in each case from overnight agar plate culture growing the test organisms. The top of each colony was touched with a sterile wire loop and transferred to 5 ml of sterile distilled water. The distilled water was agitated by vigorous shaking to distribute the colonies evenly. The turbidity was compared with the 0.5 McFarland standard. After adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level, to remove excess inoculum from the swab. The dried surface of a Mueller-Hinton agar plate was inoculated by streaking the entire sterile agar surface. The procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. The rim of the agar plate was also swabbed.

### **3.6.2 Application of antimicrobial disks to inoculated agar plates**

The antimicrobial disks were dispensed onto the surface of the inoculated agar plates using sterilized forceps. Each disk was pressed down to ensure complete contact with the agar surface. The disks were distributed evenly from each other so that they were no closer than 24mm from centre to centre. The plates were inverted and incubated aerobically at 37°C for 16 h. Susceptibility to each antibiotic was read by measuring the zone of growth inhibition from one end of growth to the next including disc diameter (in mm) which was compared against a standard scale. The results were recorded as sensitive, intermediate or resistant, according to National Committee for Clinical Laboratory Standards (NCCLS 2000).

## **3.7 Plasmid isolation and *in-vitro* conjugation tests**

### **3.7.1 Growth of the bacterial culture**

Bacteria were grown in MacConkey agar (Oxoid Basingstoke, UK) at 37°C overnight. A single bacterial colony was picked from the agar plate and inoculated onto 2ml of Luria-Bertani (LB) broth (Difco) and grown again overnight at 37°C with vigorous shaking.

### **3.7.2 Extraction and Purification of Plasmid DNA**

The harvesting, lysis and purification of plasmid DNA was done according to Sambrook *et al.*, (1989). A portion of the culture (1.5ml) was poured into

microfuge tubes, and centrifuged at 13000rpm for 30 seconds at 4°C in a micro centrifuge. The harvesting was done by removing the medium by aspiration, leaving the bacterial pellet as dry as possible. Lysis of bacterial cells was done by resuspending the bacterial pellet in 100 µl of ice-cold solution 1 (Appendix 1) by vigorous vortexing. An amount of 200 µl freshly prepared solution 2 (Appendix 1) was added. The tube was closed tightly and the contents mixed by inverting the tube rapidly five times making sure that the entire surface of the tube came into contact with the solution. The tubes were stored in ice for five minutes.

When the tubes had been stored adequately, 150 µl of solution 3 (Appendix 1) were added. The tubes were closed and vortexed gently in an inverted position for 10 seconds to disperse solution 3 into the viscous bacterial lysate. The tubes were then stored in ice for 3-5 min. They were subsequently centrifuged at 13,000 rpm for 10 min. at 4°C in a microfuge. The supernatant was aspirated and transferred into fresh tubes using micropipettes. The double-stranded DNA was precipitated with 2 volumes of ethanol (1000 µl) at room temperature. This was mixed by inverting the tube gently five times without vortexing. Already mixed tubes were allowed to stand for 2 minutes at room temperature then centrifuged at 13,000 rpm for 5 minutes, at 4°C. The supernatant was removed again by gentle aspiration. The tubes were placed in an inverted position on a paper towel to allow all the fluid to drain away. Any drops adhering to the walls of the tubes were removed with micropipettes.

The remaining pellet of double-stranded DNA was rinsed with 1 ml of absolute ethanol followed by 70% ethanol at 4°C. The supernatant was removed by gentle aspiration, and the pellet of nucleic acid allowed to dry in air for 10 minutes. The nucleic acids were redissolved in 50µl of Tris-EDTA (TE) buffer (pH8.0) (Appendix 1) containing DNAase-free pancreatic RNAase (20µl). The DNA was stored at -20°C to wait separation of plasmid DNA by gel electrophoresis.

### 3.7.3 *In-vitro* conjugation of plasmids

Conjugation experiments were carried out using the method by Walia *et al.*, (1987) with *E. coli* K-12 which were nalidixic acid resistant and susceptible to ampicillin (Na<sup>r</sup> and F-) strains as recipient. The *E. coli* isolates from children used were ampicillin resistant and susceptible to nalidixic acid (Amp<sup>r</sup> and F+). Half litre tryptone soy broth (Difco) was prepared and 3 ml amounts dispensed into bijou bottles and labelled.

Both donors (*E. coli* isolates from children ampicillin resistant Amp<sup>r</sup> and F+) and recipient (*E. coli* K12 Nalidixic acid resistant Na<sup>r</sup> and F-) were grown in nutrient agar (Oxoid, Basingstoke, UK) plates at 37°C for 16 h. Single discreet colonies of each donor and recipient were selected and transferred into 5 ml tryptone soy broth (Difco) in the bijou bottles and incubated for 3 h with rigorous shaking at 37°C in order to allow for multiplication to the logarithmic phase. The donor and recipient cultures were diluted 1:10, that is, 0.5 ml into 4.5 ml warmed fresh tryptone soy broth (Difco), and mixed in equal proportions to make 5ml broth

culture. This was incubated at 37°C and conjugation allowed to take place overnight. The cells were then centrifuged at 13000 rpm and washed twice in sterile phosphate buffered saline with vigorous vortexing. The transconjugants were selected on MacConkey agar (Oxoid, Basingstoke, UK) supplemented with nalidixic acid (32µg/ml each) and ampicillin (32µg/mg each). Plasmid DNA was extracted from the transconjugants according to section 3.3.2 above. In order to determine the antibiotic resistance transferred to recipient *E. coli* K-12 strain, antimicrobial susceptibility tests were performed for each of the transconjugants as earlier described in section 3.7.2.

### **3.8.2 Gel electrophoresis for the extraction of plasmids**

This was done to separate plasmid DNA as described by Sambrook et al., 1989. Agarose gel (0.9%) was prepared (Appendix 1) and poured into a mould that had the required number of wells 12 or 14 depending on the tank used. The mould was submerged in the electrophoresis tank filled with electrophoresis buffer (Horizontal electrophoresis Gel (HEG)). The samples of DNA were mixed with gel loading buffer pH 8.0 and slowly loaded into wells of the submerged gel. Plasmid DNA was electrophoresed on the HEG at 100 Volts for 3 h. *E.coli* strains R39 and V517 of known sizes were electrophoresed along-side the test isolate to help determine the plasmid sizes. DNA bands were visualised with an ultraviolet Tran illuminator (UVP, San Gabriel, C.A, USA) after staining with ethidium bromide (0.05%). The gel was photographed using Polaroid film 665<sub>PN</sub> (Sigma, St. Louis, USA).

### 3.9 Data analysis

Analysis of Variance (ANOVA) was used to analyse the data using the Scientific Programme for Social Sciences (SPSS) version 11.0. This was used to determine relative significance of antibiotic resistance among the different antibiotic and the differences in the different age groups of the children. Plasmid sizes were determined by comparing with the controls *E.coli* 39R861 strain (98, 42, 24, 4.6 MDa) and V517 (35.8, 4.8, 3.7, 3.4, 2.6, 1.8, 1.4 MDa) which had known plasmid sizes.

## CHAPTER 4

## 4.0 RESULTS

## 4.1 Abundance of different genera of microorganisms in children suffering from diarrhoea

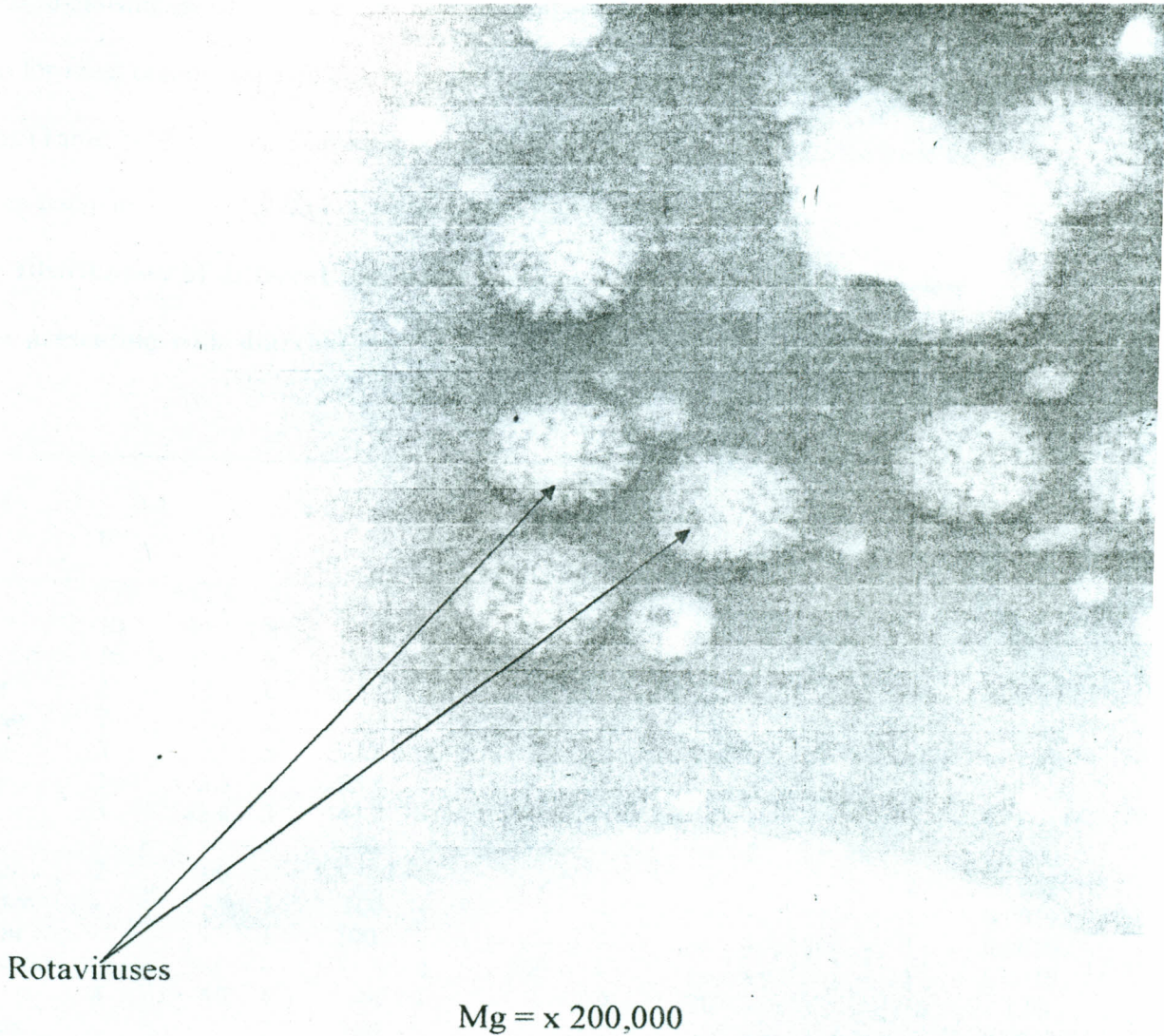
All the specimens were collected from children below five years presenting with diarrhoea at Kenyatta National Hospital. A total of thirteen different bacterial species, which consisted of a total of 504 bacteria were isolated from 402 stools cultured (Table 1). Three of the bacterial species were pathogenic, that is, *E. coli*, *Salmonella* and *Shigella*, while the rest were non-pathogenic. In addition, 12 isolates of two species of viruses were obtained from the first 100 specimens. These were rotavirus and coronavirus. Rotavirus is pathogenic causing diarrhoea especially in children (Plate 1).

**Table 1: Different genera of microorganisms isolated from children below five years presenting with diarrhoea at Kenyatta National Hospital in June 2003-Jan 2004.**

Organisms	n	% of occurrence of bacteria of different genera
<i>E.coli</i>	347	67.2
<i>Klebsiella</i>	61	11.8
<i>Proteus</i>	39	7.5
<i>Salmonella</i>	11	2.1
<i>Enterobacter</i>	10	1.9
<i>Providentia</i>	10	1.9
<i>Citrobacter</i>	9	1.7
<i>Serratia</i>	7	1.3
<i>Shigella</i>	5	0.9
<i>Hafnia alvei</i>	2	0.3
<i>Acinetobacter</i>	1	0.1
<i>Chromo violaceum</i>	1	0.1
<i>Flavii orryzihabitans</i>	1	0.1
Rota viruses	10	1.9
Corona viruses	2	0.3
<b>Total</b>	<b>516</b>	<b>100.0</b>

**KEY:** n=number of isolates, % percent occurrence of different genera of microorganisms

**Plate 1: Scanning electromicrogram of Rotaviruses isolated from children with diarrhoea at Kenyatta National Hospital.**



#### 4.2 Prevalence of organisms by age of the children

Of the 516 organisms isolated, there was higher number of the isolates within 0-2 year age group for most organisms, accounting for 63.37% which was more than all the other age groups (Table 2). There was also a significantly higher isolation rate (32.3%) from 0-1 age group compared to 12.9% from 4-5 age group ( $P < 0.05$ ) (Table 3).

**Table 2: Distribution of different genera of bacteria isolated from children below five years presenting with diarrhoea at Kenyatta National Hospital in June 2003-Jan 2004.**

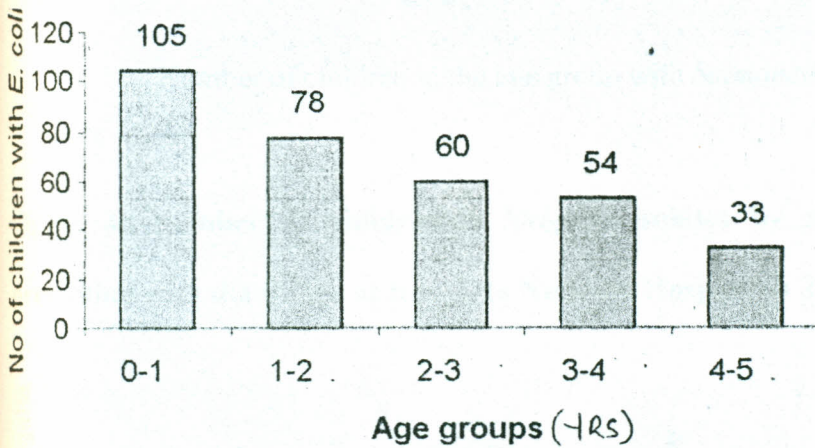
Organisms	Age groups in years										Total	
	0-1		1-2		2-3		3-4		4-5			
	n	%	n	%	n	%	n	%	n	%	n	%
<i>E. coli</i>	127	36.5	84	24.2	54	15.5	58	16.7	24	6.9	347	67.24
<i>Klebsiella</i>	30	49.1	8	13.1	11	18.0	6	9.8	6	9.8	61	11.82
<i>Proteus</i>	16	41.0	8	20.5	7	17.9	4	10.2	4	10.2	39	7.56
<i>Salmonella</i>	2	18.1	6	54.5	1	9.1	-	-	2	18.2	11	2.13
<i>Enterobacter</i>	7	70	2	20	1	10	-	-	-	-	10	1.93
<i>Providentia</i>	3	30	5	50	1	10	1	10	-	-	10	1.93
<i>Citrobacter</i>	3	3.3	5	55.5	-	-	-	-	1	11.1	9	1.74
<i>Serratia</i>	3	42.8	3	42.8	-	-	1	14.28	-	-	7	1.35
<i>Shigella</i>	-	-	3	60	-	-	-	-	2	40	5	0.009
<i>Hafnia alvei</i>	2	100	-	-	-	-	-	-	-	-	2	0.003
<i>Acinetobacter</i>	-	-	1	100	-	-	-	-	-	-	1	0.0019
<i>C. violaceum</i>	-	-	1	100	-	-	-	-	-	-	1	0.0019
<i>F. ory-habitans</i>	-	-	-	-	1	100	-	-	-	-	1	0.0019
Rotavirus	4	40	4	40	-	-	2	20	-	-	10	1.93
Coronavirus	-	-	2	100	-	-	-	-	-	-	2	0.0038
Total	197	38.17	130	25.19	76	14.72	72	13.95	40	7.75	516	100

KEY: n- Total number of organisms isolated, %- the percent of total organisms isolated, (-) no organisms isolated.

In the 0-1yr age group *E. coli* accounted for the highest number of bacterial isolations accounting for 60.8% of the total isolates (Figure 1). *Salmonella* accounted for 72.7% of

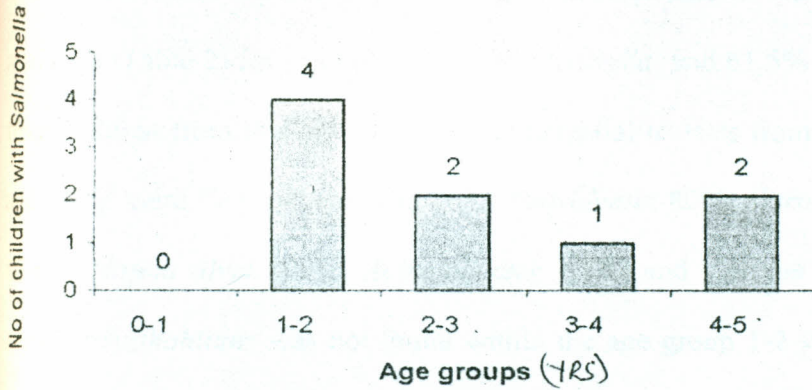
the total number isolated (Figure 2) which is proportionally more within 1-2yr age group compared to other age groups. *Shigella* had 60% of the total number isolated in the 1-2yr age group (Figure 3). Eighty percent (80%) of rotavirus isolations were from 0-2 age group while all the coronavirus isolated (100%) were in the 1-2 yr age group (Table 2).

**Figure 1: Number of children with *E. coli* isolates by age group of children presenting with diarrhoea at Kenyatta National Hospital in June 2003-Jan 2004.**



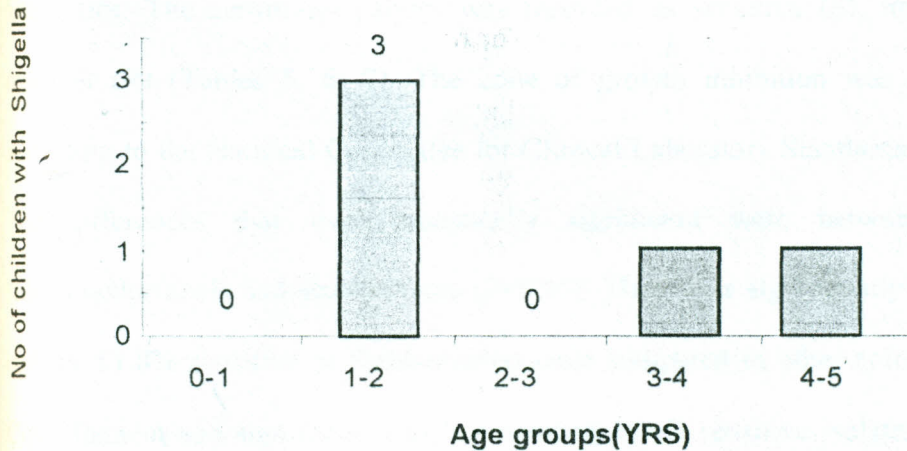
Number of children in the age group with *E. coli*

Figure 2: Number of children with *Salmonella* isolates by age group of children presenting with diarrhoea at Kenyatta National Hospital in June 2003-Jan 2004.



Number of children in the age group with *Salmonella*

Figure 3: Number of children with *Shigella* isolates by age group of children presenting with diarrhoea at Kenyatta National Hospital in June 2003-Jan 2004.



Number of children in the age group with *Shigella*

From figures 1-3 the prevalence of the pathogens isolated was significantly more in the age children below 2 yrs ( $P < 0.05$ ).

The prevalence of the non-pathogenic Enterobacteriaceae in the 1-2 yrs age groups was also high (Table 2) for example 62.2% of *Klebsiella* and 61.5% of *Proteus* isolates were from children from this age group. Other bacterial isolates from children from the 1-2yr age group were *Enterobacter* had 90%, *Providentia* 80%, *Citrobacter* 88.88%, *Serratia* 85.6%, *Hafnia alvei* 100%, *Acinetobacter* 100% and *Chromo violaceum* 100%. Only *Flavii orryzihabitans* was not found within the age group 1-2 years, but was present in the 2-3 years age group. The lowest number of the total organisms was in the age group 4-5 years having only 40 (7.75%) of the total organisms isolated (Table 2).

#### 4.3 Antibiotic sensitivity testing

All *E. coli*, *Salmonella* and *Shigella* were tested for susceptibility to a total of 12 antibiotics. The sensitivity pattern was recorded as sensitive (S), intermediate (I) or resistant (R) (Tables 5, 6, 7). The zone of growth inhibition was recorded in mm according to the National Committee for Clinical Laboratory Standards (NCCLS, 2000). The differences that were statistically significant were between trimethoprim-sulphamethaxazole and streptomycin ( $P < 0.05$ ). There was significantly lower number of isolates 11.8% sensitive to Sulphamethaxazole compared to other antibiotics ( $P < 0.05$ ). Ciprofloxacin had significantly higher numbers of all sensitive isolates than the rest of the drugs.

**Table 3: Interpretation of zones of inhibition of disk diffusion test results for *E. coli* isolated from children presenting with diarrhoea at Kenyatta National Hospital in June 2003-Jan 2004.**

Antibiotic	Disk content ( $\mu\text{g}$ )	Inhibition zone diameter to the nearest whole mm					
		R	n (%)	I	n (%)	S	n (%)
CXM	30	$\leq 19$	21 (6.5)	20-26	245(76.5)	$\geq 27$	54(16.8)
RL	23.75	$\leq 22$	275(85.9)	23-29	7(2.18)	$\geq 30$	38(11.8)
W	1.25	$\leq 22$	280(87.5)	23-29	2(0.62)	$\geq 30$	38(11.8)
C	30	$\leq 20$	98(30.6)	21-27	3(0.93)	$\geq 28$	218(68)
S	10	$\leq 11$	205(64)	12-20	30(9.3)	$\geq 21$	85(26.5)
TE	30	$\leq 17$	213(66.5)	18-25	10(3.1)	$\geq 26$	97(30.3)
Na	30	$\leq 21$	32(10)	22-28	16(5)	$\geq 29$	272(85)
CIP	5	$\leq 29$	16(5)	30-40	3(0.93)	$\geq 41$	301(94)
AMP	10	$\leq 15$	197(61.5)	16-22	5(0.15)	$\geq 23$	37(11.5)
AMC	20	$\leq 17$	138(43.1)	18-24	84(26.5)	$\geq 25$	98(30.6)
CAZ	30	$\leq 24$	15(4.68)	25-32	4(1.2)	$\geq 33$	301(94)
CN	10	$\leq 18$	24(7.5)	19-26	10(3.1)	$\geq 27$	286(89.3)

KEY: R- Resistant strains, I- Intermediate strains, S- Sensitive strains, n-number of strains, %-percent number, CXM- cefuroxime sodium, RL-sulfamethaxazole, W-trimethoprim, C- chloramphenicol, S- streptomycin, TE- tetracycline, Na- Nalidixic acid, CIP- ciprofloxacin, AMP- ampicillin, AMC- amoxicillin, CAZ- ceftazidime, CN- gentamicin.

**Table 4: Interpretation of zones of inhibition of disk diffusion tests for *Salmonella* isolated from children presenting with diarrhoea at Kenyatta National Hospital in June 2003-Jan 2004.**

Antibiotic	Disk content ( $\mu\text{g}$ )	Inhibition zone diameter to the nearest whole mm					
		R	n (%)	I	n (%)	S	n (%)
CXM	30	$\leq 19$	1(9.0)	20-26	4(36.3)	$\geq 27$	6(54.5)
RL	23.75	$\leq 22$	11(100)	23-29	-	$\geq 30$	-
W	1.25	$\leq 22$	11(100)	23-29	-	$\geq 30$	-
C	30	$\leq 20$	6(54.5)	21-27	-	$\geq 28$	5(45.45)
S	10	$\leq 11$	7(63.6)	12-20	1(9.0)	$\geq 21$	4(36.3)
TE	30	$\leq 17$	4(36.3)	18-25	-	$\geq 26$	7(63.6)
Na	30	$\leq 21$	-	22-28	1(9.0)	$\geq 29$	10(90.9)
CIP	5	$\leq 29$	-	30-40	-	$\geq 41$	11(100)
AMP	10	$\leq 15$	10(90.9)	16-22	-	$\geq 23$	1(9.0)
AMC	20	$\leq 17$	4(36.3)	18-24	1(9.0)	$\geq 25$	6(54.5)
CAZ	30	$\leq 24$	1(9.0)	25-32	-	$\geq 33$	10(90.9)
CN	10	$\leq 18$	1(9.0)	19-26	1(9.0)	$\geq 27$	9(81.8)

KEY: R- Resistant strains, I- Intermediate strains, S- Sensitive strains, n-number of strains, %-percent number, CXM- cefuroxime sodium, RL-sulfamethaxazole, W-trimethoprim, C- chloramphenicol, S- streptomycin, TE- tetracycline, Na- Nalidixic acid, CIP- ciprofloxacin, AMP- ampicillin, AMC- amoxicillin, CAZ- ceftazidime, CN- gentamicin (-) no organisms represented.

**Table 5: Interpretation of zones of inhibition of disk diffusion test for *Shigella* isolated from children presenting with diarrhoea at Kenyatta National Hospital in June 2003-Jan 2004.**

Antibiotic	Disk content ( $\mu\text{g}$ )	Inhibition zone diameter to the nearest whole mm					
		R	n (%)	I	n (%)	S	n (%)
CXM	30	$\leq 19$	-	20-26	-	$\geq 27$	5(100)
RL	23.75	$\leq 22$	5(100)	23-29	-	$\geq 30$	-
W	1.25	$\leq 22$	5(100)	23-29	-	$\geq 30$	-
C	30	$\leq 20$	4(80)	21-27	1(20)	$\geq 28$	-
S	10	$\leq 11$	5(100)	12-20	-	$\geq 21$	-
TE	30	$\leq 17$	5(100)	18-25	-	$\geq 26$	-
Na	30	$\leq 21$	-	22-28	-	$\geq 29$	5(100)
CIP	5	$\leq 29$	-	30-40	-	$\geq 41$	5(100)
AMP	10	$\leq 15$	3(60)	16-22	-	$\geq 23$	2(40)
AMC	20	$\leq 17$	2(40)	18-24	1(20)	$\geq 25$	2(40)
CAZ	30	$\leq 24$	-	25-32	-	$\geq 33$	5(100)
CN	10	$\leq 18$	-	19-26	-	$\geq 27$	5(100)

**KEY:** R- Resistant strains, I- Intermediate strains, S- Sensitive strains, n-number of strains, %-percent number, CXM- cefuroxime sodium, RL-sulfamethaxazole, W-trimethoprim, C- chloramphenicol, S- streptomycin, TE- tetracycline, Na- Nalidixic acid, CIP- ciprofloxacin, AMP- ampicillin, AMC- amoxicillin, CAZ- ceftazidime, CN-gentamicin, (-) no organisms represented.

#### 4.3.1 Antibiotic sensitivity testing on *E. coli*

There was statistically significant difference in the number of sensitive *E. coli* for all the drugs across the different age groups ( $P < 0.05$ ) (Table 6).

*E. coli* showed the highest level of resistance to trimethoprim-sulphamethaxazole (88.5%) followed by ampicillin (81%), tetracycline (66.97%), streptomycin (63.58%), amoxicillin (43.20%) and chloramphenicol (12.65%) (Table 7). The *E. coli* however showed lower resistance to nalidixic acid (9.56%), gentamicin (8.06%), cefuroxime sodium (6.48%) and ciprofloxacin (5.86%). The most effective drug on *E. coli* was ceftazidime showing 4.6% resistance ( $P < 0.05$ ) (Table 7).

**Table 6: Number of resistant and sensitive *E. coli* by age of children presenting with diarrhoea at Kenyatta National Hospital in June 2003-Jan 2004.**

Drug	Age in years										Total
	0-1		1-2		2-3		3-4		4-5		
	R	S	R	S	R	S	R	S	R	S	
CXM	11	21	5	14	4	9	1	13	1	10	<b>89</b>
TRIM	89	12	72	10	46	6	40	7	35	4	<b>321</b>
RL	92	7	75	5	47	5	43	5	33	6	<b>318</b>
C	38	61	33	49	13	37	17	31	14	26	<b>319</b>
S	68	22	54	22	30	17	27	18	21	15	<b>294</b>
TE	65	33	53	28	36	16	31	15	25	13	<b>315</b>
NA	9	86	9	71	5	44	3	42	6	35	<b>310</b>
CIP	5	96	3	87	3	51	1	49	4	40	<b>339</b>
CAZ	7	92	7	81	3	51	2	47	2	40	<b>332</b>
CN	12	84	7	78	4	45	0	46	3	40	<b>319</b>
AMP	61	9	59	13	23	10	25	10	22	8	<b>240</b>
AMC	36	33	40	22	25	16	27	14	11	19	<b>243</b>

**KEY:** R-Resistant strains, S-Sensitive strains, CXM- cefuroxime sodium, RL-sulfamethaxazole, W-trimethoprim, C- chloramphenicol, S- streptomycin, TE- tetracycline, Na- Nalidixic acid, CIP- ciprofloxacin, AMP- ampicillin, AMC- amoxicillin, CAZ- ceftazidime, CN- gentamicin.

There was statistical difference in resistance between trimethoprim-sulphamethaxazole and streptomycin ( $P < 0.05$ ). Statistically fewer isolates were sensitive to sulphamethaxazole than the rest of the drugs, while relatively more isolates were sensitive to ciprofloxacin than the rest of the drugs.

### 4.3.2 Antibiotic sensitivity testing on *Salmonella*

*Salmonella* showed the highest resistance against trimethoprim-sulphamethaxazole (90%), followed by ampicillin (81%), and then streptomycin (75%) (Table 7). However, *Salmonella* isolates were sensitive to nalidixic acid, ciprofloxacin, and ceftazidime. There was no statistically significant difference ( $P=1.0$ ) in the number of sensitive cases across the different drugs but statistically significant differences ( $P<0.05$ ) existed in the age groups. There was no statistically significant difference in the number of sensitive cases between age groups 1-2 years and 2-3 years, 3-4 years and 4-5 years. Comparisons between the rest of the age groups were statistically different ( $P<0.05$ ).

Table 7: Percent resistance of *E. coli*, *Salmonella* and *Shigella* from children presenting with diarrhoea at Kenyatta National Hospital to the antibiotics tested.

Antibiotic	% of <i>E. coli</i> showing resistance	% of <i>Salmonella</i> showing resistance	% of <i>Shigella</i> showing resistance
W-RL	88.5	90	100
AMP	81.0	81	60
TE	66.97	56	100
S	63.58	75	100
AMC	43.20	37.5	40
C	12.65	56	60
NA	9.56	0	0
CN	8.02	6.25	0
CXM	6.48	6.25	0
CIP	5.86	0	0
CAZ	4.6	0	0

KEY: W- trimethoprim, RL- sulphamethaxazole, AMP- ampicillin, TE-tetracycline, S- streptomycin, AMC- ampicillin, C- chloramphenicol, Na- nalidixic acid, CN- gentamicin, CXM- cefuroxime sodium, CIP- ciprofloxacin, CAZ- ceftazidime, % -percent number of resistant strains.

### 4.3.3 Antibiotic sensitivity testing on *Shigella*

*Shigella* isolates showed 100% resistance to three drugs namely; trimethoprim-sulphamethaxazole, tetracycline and streptomycin (Table 7). They were however sensitive to nalidixic acid, gentamicin, cefuroxime sodium, ciprofloxacin and ceftazidime. There was no statistically significant difference in sensitivity among the different drugs ( $P=0.7$ ). There was however statistically significant difference among the different age group that is between 1-2 and 2-3 years ( $P<0.05$ ).

### 4.3.4 Multi drug resistance

Some isolates of *E. coli*, *Salmonella*, and *Shigella* tested showed multidrug resistance (Table 8). For *E. coli*, only 3.4% of the 320 isolates tested were sensitive to the 12 antibiotics tested, the rest (92.18%) were multidrug resistant, showing resistance to 2 or more antibiotics. Four of the *E. coli* isolates tested showed resistance to a total of 11 antibiotics. None of the *Salmonella* isolates tested were sensitive to the antibiotics tested, 9% were resistant to only one antibiotic while the rest (91%) were multidrug resistant. All the *Shigella* isolates (100%) were multidrug resistant, showing resistance to 3 or more antibiotics.

**Table 8: Multidrug resistance patterns for *E.coli*, *Salmonella* and *Shigella* from children with diarrhoea at Kenyatta National Hospital tested with various antibiotics**

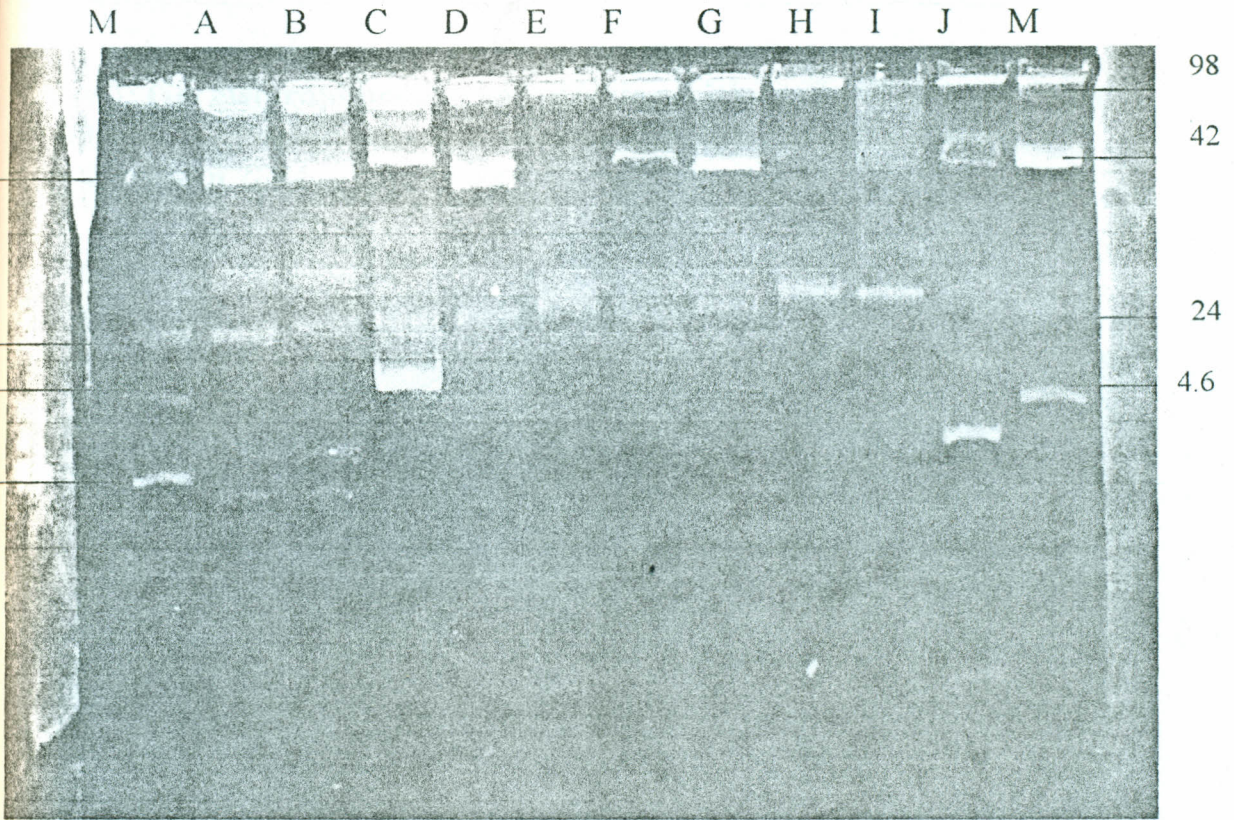
Resistance pattern	<i>E. coli</i> %	<i>Salmonella</i> %	<i>Shigella</i> %	Total %
Fully sensitive	93.4	0	0	3.3
Resistance to 1	4.3	9	0	4.5
Resistance to $\geq 2$	92.18	91	100	92.2
Total	100	100	100	100

KEY: %- percent number of isolates representing each pattern.

#### 4.4 Plasmid characterization

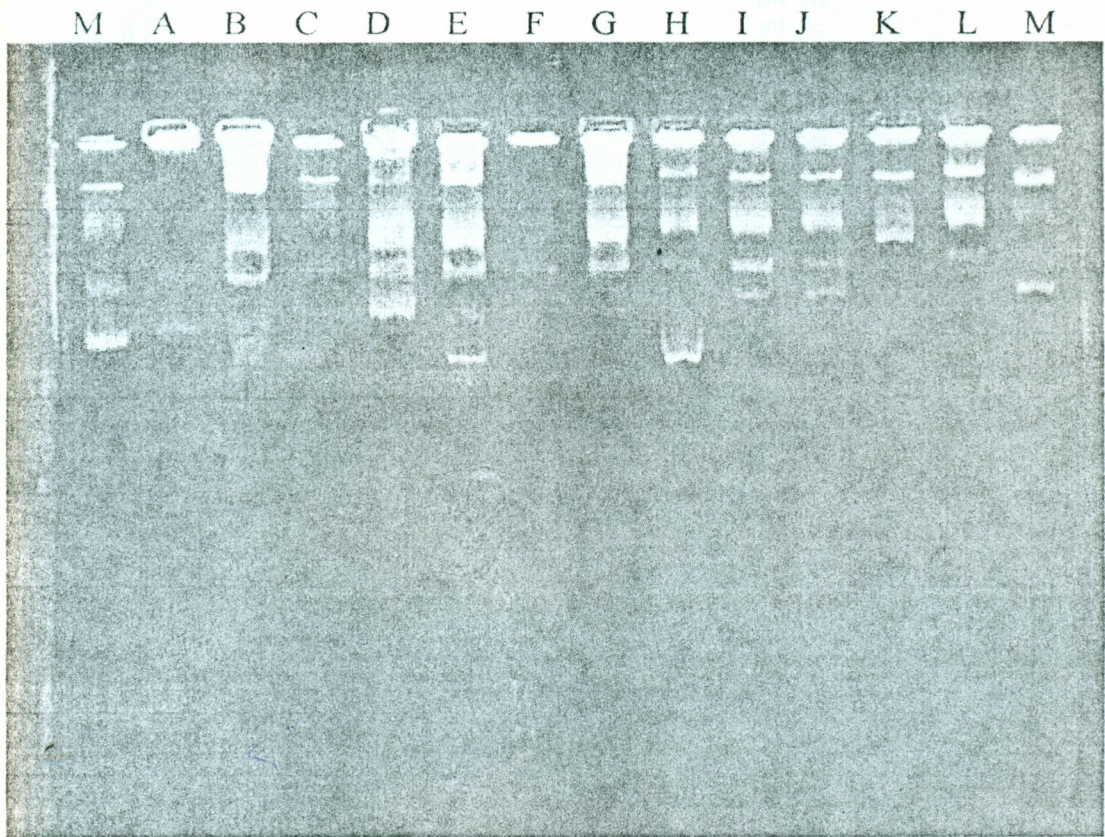
Plasmids were isolated and analyzed from 312 (97.5%) of the 320 *E. coli* isolates tested for sensitivity. Plasmid analysis was carried out on horizontal agarose gel electrophoresis (HGE). The sensitive and resistant isolates were analyzed separately and there were no differences noted with respect to the plasmid contents between these isolates. Plate 1 shows plasmids isolated from the sensitive strains, and Plate 2 shows plasmids isolated from the resistant strains. Thirty eight of these the resistant strains (12%) did not have any plasmids. A larger plasmid of 98MDa was present in 198 (63.46%) of the isolates, occurring separately or together with smaller plasmids while 31.7% of the isolates had plasmids smaller than 35.5MDa (Table 9).

Plate 2: Gel electrophoresis of the antibiotic sensitive strains of *E. coli* isolated from children with presenting with diarrhoea at Kenyatta National Hospital in June 2003 –Jan 2004



M-Size marker plasmids from *E. coli* 39R861 strain (98, 42, 28, 4.6 MDa), V517 strain (35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4 MDa), A-J-plasmids from sensitive strains isolated from children.

Plate 3: Gel electrophoresis of antibiotic resistant *E.coli* isolated from children presenting with diarrhoea at Kenyatta National Hospital in Jan 2003-Jan 2004



M-Size marker plasmids from *E. coli* 39R861 strain (98, 42, 28, 4.6 MDa), V517 strain (35.8, 4.8, 3.7, 3.4, 2.6, 2.0, 1.8, 1.4 MDa), A-L plasmids from *E. coli* resistant strains isolated from children.

**Table 9: Frequency of occurrence of plasmids of different sizes analyzed in the *E. coli* isolates from children presenting with diarrhoea at Kenyatta National Hospital in June 2003-Jan 2004.**

Plasmid size in MDa	Frequency	%
No plasmid	38	12
98	198	68.46
98, 40, 24, 4.6	87	27.88
98, 35.8, < 4.8	99	31.78
98, <3.7	51	16.34
35.8	52	16.66
<3.7	51	16.34
<1.8	27	8.6

#### 4.5 Transfer of resistance through *in-vitro* conjugation

The 40 isolates that were multidrug resistant were conjugated with *E. coli* strain K-12, F- and Na<sup>r</sup>. Of the 40 isolates, where 34 (85%) successfully transferred a resistance phenotype. In 6 (15%) of the isolates no plasmids were detected. Several resistant phenotypes were successfully transferred, with the 98 MDa plasmid, being most commonly transferred in 28 (70%) of the isolates (Table 10).

**Table 10: Percentage of occurrence of plasmid sizes analyzed from *E. coli* K-12 F-Na<sup>r</sup> transconjugants.**

Transferred plasmid(MDa)	Frequency	Percentage
No plasmids detected	6	15
98	28	70
98, 35.8	24	60
98, 35.8, < 4.8	7	17.5
<4.8	5	12.5

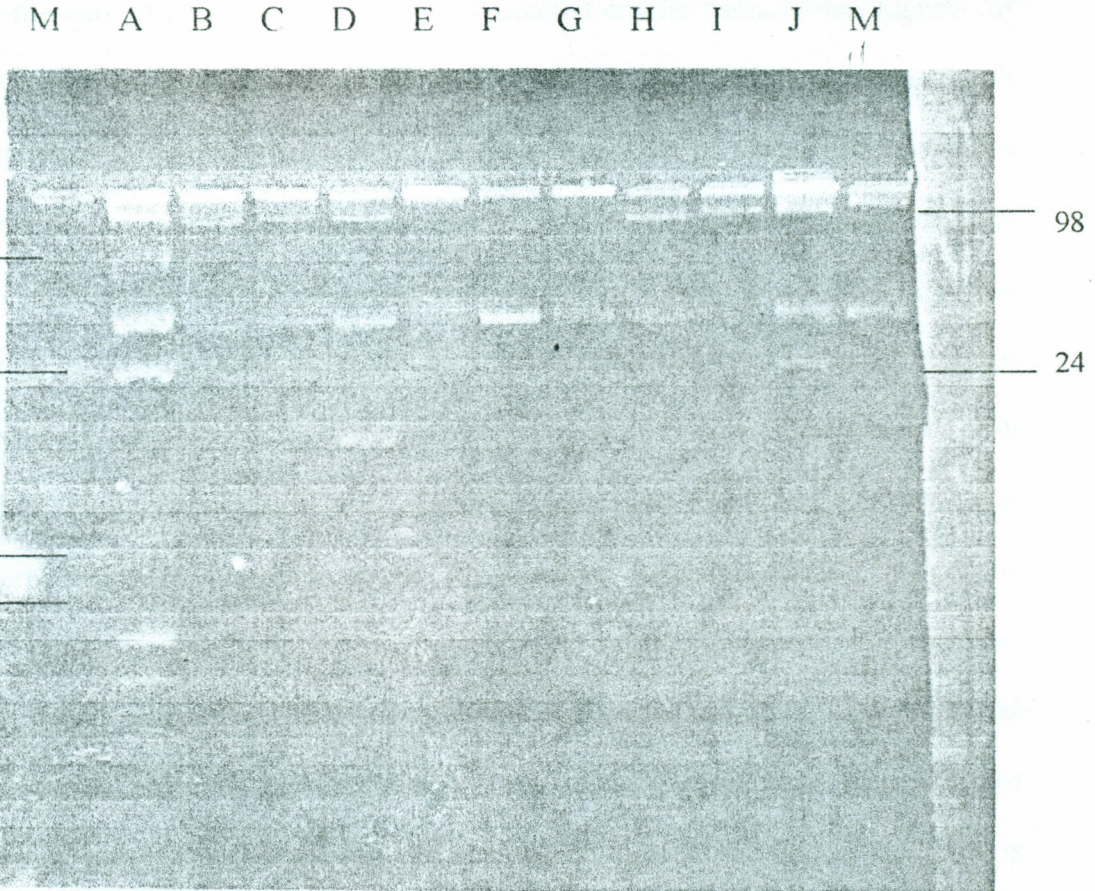
Five plasmids (12.5%) with less than 4.8 MDa were also transferred alongside the 98MDa. The plasmid's commonest resistant pattern transferred was trimethoprim-sulphamethaxazole, tetracycline, and ampicillin (Table 11). Other phenotypes successfully transferred were streptomycin and chloramphenicol. Plate 4 shows gel electrophoresis of the transconjugants.

**Table 11: Resistance transfer phenotypes from resistant isolates of *E.coli* to transconjugants**

Resistance pattern of donor	Successful transfers	Phenotype transferred
W,RL,TE,AMP	5	W,RL,AMP
W,RL,S,TE,AMP	8	W,RL,TE,AMP
W,RL,S,C,TE,AMP	2	C,TE,AMP
W,RL,S,TE,AMP,AMC	3	TE,AMP
W,RL,S,TE,Na,AMP,AMC	2	W,RL,TE
W,RL,S,TE,AMP,CN	2	S,TE,AMP
W,TE,AMP	2	W,TE,AMP

**KEY:** C-chloramphenicol, Na-nalidixic acid, CN- gentamicin, W- trimethoprim, RL- sulphamethaxazole, TE- tetracycline, AMP-ampicillin, AMC-amoxicillin, S- streptomycin.

Plate 4: Gel electrophoresis of the transconjugants from conjugation with *E. coli* K 12 from children presenting with diarrhoea at Kenyatta National Hospital in June 2003- Jan 2004



M-Size marker plasmids from *E. coli* 39R861 strain (98, 42, 24, 4.6 MDa), V517 strain (35.8, 4.8, 3.7, 3.4, 2.6, 1.8, 1.4MDa), A-J plasmids from *E. coli* transconjugants obtained from conjugation.

## CHAPTER 5

### 5.0 DISCUSSION

#### 5.1 Prevalence of isolated organism

In the current study, the prevalence of isolated enteric bacteria was highest for *E.coli* (67.2%) and *Klebsiella* (11.8%) (Table 2). This was in agreement with studies by Malonza *et al.*, (1997). In their study, out of all the Gram-negative isolates, *E.coli* and *Klebsiella* represented over 70% of isolations. Prevalence of *E.coli* from our study also agrees with data from Laving *et al.*, (2003), whose study showed *E.coli* as the most common etiopathological agent in neonatal bacterial meningitis forming 46.7% of the isolates followed by *Klebsiella pneumoniae* with 13.3%. Similarly English *et al.*, (2003) showed that *E.coli* and *Klebsiella spp* were important causative agents for invasive bacterial disease. *E. coli* is considered a predominant cause of diarrhoea in developing countries (Guerrant *et al.*, 1990). *E. coli* forms part of the normal intestinal flora, and a general commensal that does not pose a risk to the host. However, some *E. coli* strains have acquired genetic materials that allow them to become pathogenic. *E. coli* is therefore the most common cause of bacterial diarrhoea in diverse populations (Adachi *et al.*, 2001). It requires constant investigation and whenever possible, typing in order to determine its role in diarrhoeal incidences

In most studies on causes of diarrhoea other enteric organisms are also isolated since most diarrhoeal diseases have one or multiple causes (Shaheen *et al.*, 2003). Of the pathogenic organisms isolated in the current study *E. coli* formed the

highest percentage (67.2%), followed by *Salmonella* (2.1%) rotavirus (1.9%) and *Shigella* (0.9%). The highest number of organisms isolated in a single patient was three. This agrees with studies by Mutanda *et al.*, (1990) where *E. coli* isolates were 54%, followed by *Salmonella* (16%), rotavirus (6%) while only 2 *Shigella* strains were isolated from neonates with diarrhoea at the Kenyatta National Hospital. Although the rotavirus was lower in this study they were only isolated from the first 100 out of 402 samples. The multiple isolation in our study also agrees with studies by Richie *et al.*, (1997), where other bacteria were isolated including *Aeromonas* (32%), *Salmonella* spp. (1.8%) and *Shigella* spp. (0.7%). Cheng-Rong *et al.*, (1990), Simwa *et al.*, (1987) and Geyer *et al.*, (1993) also isolated up to four organisms in a single patient.

In our study, several enteric non-pathogenic organisms were isolated which included *Proteus* (7.5%), *Enterobacter* (1.9%), *Providencia* spp (1.9%) and *Citrobacter* (1.7%). Isolates whose prevalence was less than 1% were *Hafnia alvei*, *Acinetobacter*, *Chromo violaceum* and *Flavii orryzihabitans*. The presence and transmission of non-pathogenic bacteria is an important aspect in the epidemiology of infection because they serve as reservoirs of antibiotic resistant genes that may be transferred to pathogens (Ewing and Edward, 1989). Others such as *Hafnia alvei* have been found to be potentially pathogenic even causing gastroenteritis in certain cases (Kalashnikova *et al.*, 1975, Albert *et al.*, 1991). They have also been found to be resistant to various antibiotics, such as cephalothin and tetracycline (Westblom and Milligan, 1992). Their presence

should therefore not be ignored but should be closely monitored in future studies to establish if they are an important etiology for diarrhoea in Kenya.

The prevalence of organisms such as *E. coli*, *Salmonella* and *Shigella* in children below the age of 2 years in the current study was consistent with a study by Nesbitt *et al.*, (1988) where children below 1 year were reported to be most vulnerable to infection. Geyer *et al.*, (1993) also found children below 6 months to be most susceptible to severe diarrhoea requiring hospitalization. The most likely reason why this age group is vulnerable to infection could be attributed to having an immune system that is not completely developed. There are also some cases of immune suppression, malnutrition, poor sanitation and lack of clean drinking water which is a problem in many communities in developing countries.

## 5.2 Isolation of rotavirus

In the current study, (Table 1), 1.9% rotaviruses were isolated from the first 100 samples. This result is similar to Coker *et al.*, (1987) where 2.3% stools analyzed were positive for rotavirus. However Saidi *et al.*, (1997) reported that rotavirus was more abundant (16.1%) than in our study (6%). The reason for this is probably due to the fact that Saidi *et al.*, (1997) screened more cases (428) compared to the current study which screened only 100 cases for the rotavirus. In our study there was multiple isolation, with *Salmonella*, *Shigella*, *E. coli* and rotavirus being isolated. Several other researches including Esser *et al.*, (2000), Black *et al.*, (1982) and Aggarwal *et al.*, (1988) also isolated rotavirus and several

bacteria which included *E. coli*, *Salmonella*, *Shigella*, *Vibrio cholerae* and *Campylobacter* which agree with our study.

Children aged 6-24 months seem most susceptible to clinical illness following rotavirus infection with peak incidence at below 12 months (Leung and Pai, 1988). In our study the prevalence of rotavirus was high in the 0-1 year age group where 40% rotaviruses were isolated. This agrees with Panigrahi *et al.*, (1985) and Candeias *et al.*, (1989) where 38.5% and 34% of children respectively below 1 year with diarrhoea were positive for rotavirus. Olusanya and Taiwo, (1989) found prevalence of rotavirus in children below 12 months, where isolation was 57.27%.

### 5.3 Antibiotic resistance

In the current study high levels of antibiotic resistance among *E.coli* were recorded trimethoprim-sulphamethaxazole (88.5%), ampicillin (81%), tetracycline (66.97%), streptomycin (63.58%), amoxicillin (43.20%) and chloramphenicol (12.65%) (Table 7). Resistance of less than 10% was shown towards nalidixic acid 9.56%, gentamicin 8.02%, cefuroxime sodium 6.48%, ciprofloxacin 5.6% and ceftazidime 4.6%. 92.18% *E. coli* isolates were multidrug resistant (resistant to 2 or more antibiotics). This means that treatment of diarrhoea caused by *E.coli* can be difficult especially when the drugs they were resistant to are the 'first line' drugs. These drugs are cheaper and easily available and resistance shown by *E.*

*E. coli* to them means that only the more expensive drugs such as fluoroquinolones can now be used to treat *E. coli* caused diarrhoeal infections effectively.

The findings of our study agree with Shaheen *et al.*, (2003) where one third of the *E. coli* strains they isolated were resistant to sulphamethaxazole-trimethoprim or tetracycline, while only six strains were resistant to nalidixic acid. Vila *et al.*, (1999) and Sang *et al.*, (1997) also isolated multiresistant *E. coli* resistant to tetracycline, ampicillin, erythromycin, trimethoprim-sulphamethaxazole and amoxicillin/clavulanic acid. However the *E. coli* sensitive to chloramphenicol, nalidixic acid and cefuroxime was also isolated by Sang *et al.*, (1997) and Vila *et al.*, (1999), which is not the case in the current study. The possible reason is that the resistance to these antibiotics could have increased in the years between their study and the current one. Our study also concurs with Gakuya *et al.*, (2001) and Abimbiola *et al.*, (1993) where resistance to ampicillin, sulphamethaxazole and streptomycin was high. However, *E. coli* isolates studies by Gakuya *et al.*, (2001) did not show resistance to gentamicin, nalidixic acid, ceftazidime and ciprofloxacin as shown in our study. Similar to our results, *E. coli* resistant to several antibiotics including tetracycline, ampicillin, sulphonamide, streptomycin and cotrimoxazole (a combination of trimethoprim-sulphamethaxazole) were isolated by Ambibiola *et al.*, (1993) and Mutanda *et al.*, (1987).

The risk of transmission of antibiotic resistant (AR) bacteria between country borders continues to increase as the 'global village' shrinks (Okeke and Edelman,

2001). Strains can be imported into a country and become disseminated before their presence is recognized. The emergence of an antibiotic resistant strain in one location quickly becomes a global problem.

Although fluoroquinolones have been effective for most bacterial isolates, our study recorded resistance to ciprofloxacin in 16 (5.6%) *E. coli*. This agrees with studies by Carratala *et al.*, (1995) whose study found that resistance rates of *E. coli* towards fluoroquinolones increased after introduction of norfloxacin from zero to 1,000 hospital admissions probably because the bacteria developed resistance mechanisms to the antibiotics. Turnidge *et al.*, (2003) in a five year study on norfloxacin and ciprofloxacin however revealed that the fluoroquinolones resistance by *E. coli* had not yet emerged as a significant problem in Australia. Our study where 5.6% of *E. coli* isolates were resistant to ciprofloxacin, also disagree with with results from other workers such as Shaheen *et al.*, (2003), where none of the *E. coli* strains they isolated were resistant to ciprofloxacin. This could be attributed to different environments where the two studies were carried out. Another possibility could be attributed to the fact that resistance to ciprofloxacin could have increased in the duration between their study and the current one.

In order to design new effective agents, it is necessary to understand the mechanisms responsible for resistance in older antibiotics. Virtually all quinolone resistance encountered in clinical isolates had evolved mechanisms that result

from mutations in chromosomal genes of the isolates (Sanders and Sanders, 1995; Drlica and Zhao, 1997; Acar and Goldstein, 1997). These include the drug targets; cellular mechanisms involved in drug entry into the cell. The exact steps vary greatly depending on the organisms involved and the quinolones used to select each mutant (Fukuda *et al.*, 1998, Pan and Fisher, 1998). Among these mechanisms, two are important to quinolones. For example, altered target and modifications affecting cellular concentrations of the drug.

Although resistance of *E.coli* to the fluoroquinolones has been rare, the situation is rapidly evolving and quinolones resistant *E.coli* strains are increasingly being isolated (Aguar *et al.*, 1992, Perez-Trallero *et al.*, 1993). This increase in resistance could be related to the fact that *E. coli* has become increasingly resistant to 'first line' drug such as tetracycline, chloramphenicol and ampicillin as shown in this study. As a result the use of fluoroquinolones has increased resulting in the increase in resistance shown in this study. Further studies therefore need to be carried out to assess the current use of fluoroquinolones. The resistance recorded by *E. coli* to cephalosporins in this study (ceftazidime 4.6%) contrary to Laving *et al.*, (2003) where isolated *E.coli* were sensitive to the third generation cephalosporins (ceftriaxone, ceftazidime, and cefotazidime). The possible reason for the difference could be that resistance has emerged between the time of their study and the current one.

All the *Salmonella* isolated from the current study were multidrug resistant. These results agree with data obtained by Nesbitt *et al.*, (1988) where *Salmonella* studied over a 30-month period were multidrug resistant to ampicillin (95%), streptomycin (68%), tetracycline (63%), chloramphenicol (42%), and were 100% sensitive to ceftazidime which concur with the current study. Studies by Kariuki *et al.*, (1994) showed multiple resistances of *Salmonella* to tetracycline, rifampicin, streptomycin, amoxicillin, cotrimoxazole augmentin and chloramphenicol, a trend also noted in the current study. Multidrug resistant *Salmonella* is therefore a common occurrence in Nigeria, Algeria, Kenya and Rwanda (Okubedejo *et al.*, 1971,; Rocha, 1978,; Wamola and Mirza, 1981).

In our study, resistance of *Salmonella* to chloramphenicol (Table 7) was 56%, compared to 30% in 1980 and 42% in 1986 (Wamola and Mirza, 1981). This study confirms that resistance to various antibiotics increases every year. Indeed in Kigali, Rwanda all strains of *Salmonella* were reported as chloramphenicol resistant (Lepage *et al.*, 1984). This implies that chloramphenicol may soon become ineffective in treating infection caused by *Salmonella*. This is worrying and requires constant monitoring and research in more novel antibiotics.

The genus *Shigella* which causes dysentery is a potentially fatal disease and therefore the resistance to commonly used antibiotics is critical. All the *Shigella* isolates in our study were resistant to trimethoprim-sulphamethaxazole, tetracycline and streptomycin, similar to results obtained by Kariuki *et al.*, (1994)

where resistance to tetracycline, streptomycin and cotrimoxazole was more than 80% in each case. Multidrug resistance by *Shigella* to ampicillin trimethoprim-sulphamethaxazole has also been reported in different parts of the world (Hanson *et al.*, (1981); Bennish *et al.*, (1992); Bennish *et al.*, (1985); Frost *et al.*, 1985; Bravota and John, (1989); and Habte-Gabr, (1984). The Multidrug resistance shown by *Shigella* to chloramphenicol, ampicillin, tetracycline and trimethoprim-sulphamethaxazole which are first line drugs in Kenya mean that their use should be reviewed.

All the *Shigella* isolates in our study were susceptible to nalidixic acid, although Bennish *et al.*, (1992) reported high resistance to nalidixic acid by *Shigella*. The possible reason for this difference is that nalidixic acid was introduced as a first line drug in 1986 to replace chloramphenicol after resistance to the latter had spread widely. The use of nalidixic acid over all these years could have caused emergence of resistance by *Shigella* towards it. The sensitivity of the organisms to nalidixic acid is comforting but may not be dependable for long because resistance to antibiotics is very dynamic. Nalidixic acid should therefore be monitored closely.

#### **5.4 Plasmid isolation**

In the current study, plasmids of 98 MDal were isolated in 68.46% of the multidrug resistant isolates while those ranging from 4.6 to 98MDal were present in 27.88% of the isolates. Gakuya *et al.*, (2001) also recorded similar plasmids in

72.7% *E. coli* isolates resistant to one or more antibiotics. Kariuki *et al.*, (1994) implicated plasmids as the main genetic elements of antibiotic resistance transmission, while Kariuki *et al.*, (1999) reported association of a plasmid of 90 to 100 MDal with antimicrobial resistant *E. coli* from chicken and children who lived in close proximity. Isolated plasmids with molecular weights ranging from 0.62 to 60 kb were also common in other studies Abiombiola *et al.*, (1993). The majority of these plasmids were greater than 2.8kb which agrees with the current study. In both this study and others, all the strains possessing plasmids showed resistance to tetracycline and ampicillin.

### 5.5 Transfer of resistance through *in-vitro* conjugation

The results of this study show that plasmids of 98MDal were transferred to the *E. coli* K 12 F- Na<sup>r</sup> recipient in 70% of the transconjugants and were responsible for transfer of resistance to various antibiotics, the most common being trimethoprim-sulphamethaxazole, streptomycin, chloramphenicol, tetracycline and ampicillin.

Gakuya *et al.*, (2001) recorded similar results whereby five (22.7%) of the 22 antimicrobial resistant *E. coli* isolates transferred ampicillin resistance to *E. coli* K 12. However there is no evidence from our study that resistance to other antibiotics tested was not transferred which does not agree with the current study where resistance to other antibiotics was transferred. This could be attributed to the fact that the isolates conjugated were isolated from rats and therefore transfer of resistance may be different from that of children.

The ability of multidrug resistant *E. coli* isolates to transfer resistance to *E. coli* K 12 has been reported by Niljesten *et al.*, (1996) to range from 26% to 50% in isolates from humans and 50% to 70% in isolates from pigs. Similarly O'Brein *et al.*, (1993) reported transfer of plasmids in 24% isolates from poultry. The current study does not agree with these two studies where the only resistance transferable was for ampicillin. The reason for this could be because they performed their studies on animals whose *E. coli* isolates could have different plasmids that could not transfer resistance for other antibiotics.

In the current study, large plasmids of 90-110 MDal were transferable similar to observations by Gakuya *et al.*, (2001) where plasmids of 90-100 MDal and 55-65MDal were transferred in 60% of the five isolates conjugated. Kariuki *et al.*, (1997) also reported a plasmid of about 100-110 kb to have been present in all the antimicrobial resistant *E. coli* isolates in both children and poultry studied and the plasmid was able to transfer five different resistance patterns to recipient *E. coli* strains.

In conjugation studies done to monitor transmission of plasmids coding for antibiotic resistance plasmids were transferred quite easily from the *E. coli* K12 F- which does not carry any plasmids (Bebora, 1997). Such transfer was also demonstrated from *Salmonella* and *Shigella* species to *E. coli* K 12 F-. This simplicity of plasmid transfer has also been reported by Ozeki and Smith, (1962) and Stocker *et al.*, (1963). Yamamoto *et al.*, (1984) reported transfer of virulence

plasmid coding for colonization factor antigen (CFA-I) fimbriae and heat stable enterotoxin (ST) from *E. coli* to several species of the family enterobacteriaceae including the enteropathogens *Shigella* and *Salmonella* species. The transfer was also recorded to have been transferred to the opportunistic pathogens *Klebsiella*, *Enterobacter*, *Citrobacter*, *Edwardsiella*, *Serratia* and *Proteus* species. This ease of plasmid transfer means that the factors they code for including antibiotic resistance, when acquired by one species are easily transferred to other species, and this is what makes resistance to spread. Interaction of resistant strains with non-resistant ones therefore almost certainly means that the resistance will spread. The interaction of different species of bacteria as shown in the current study where multiple infections were recorded is bound to occur in enteric diarrhoeal infections. It is therefore important to try and limit acquisition of resistance by advocating for better use of antibiotics because once acquired, chances of its spread from one species to another are high.

## CHAPTER 6

### 6.0 CONCLUSION AND RECOMMENDATIONS

#### 6.1 Conclusions

Our study has shown that childhood diarrhoeal illness is caused by different organisms including *Salmonella spp.*, *Shigella spp.*, and *E. coli* which supports the first hypothesis that most diarrhoea in children have a bacterium or several bacteria as the causative agent(s). Diarrhoea an important cause of both morbidity and mortality especially in children under the age of 5 years.

The presence of opportunistic pathogens such as *Klebsiella*, *Enterobacter*, *Citrobacter*, *Serratia* and *Proteus* as shown in our study should be examined further. This is because other studies (Yamamoto *et al.*, 1984) have reported transfer of virulence plasmids from *E. coli* to these enterobacteriaceae. *Hafnia alvei* also isolated in our study have been reported to cause infections including diarrhoea (Albert *et al.*, 1991) and should be investigated further.

Antimicrobial drug resistance as observed in our study is becoming an increasing cause of concern. This does not support the second hypothesis that the antibiotics being used are effective in treating bacterial diarrhoea. With the rising cost of drug development, newer drugs are expensive and used sparingly, in developing countries. The resistance shown to chloramphenicol, ampicillin, trimethoprim-sulphamethaxazole, tetracycline and streptomycin in our study therefore present a serious problem because these are first line drugs used for treatment of bacterial

infections in Kenya. In addition they are cheaper and more readily available than fluoroquinolones. The emergence of fluoroquinolones resistance as shown by *E. coli* towards ciprofloxacin in our study, presents evidence that their usefulness may not be prolonged as may have been anticipated.

*E. coli* is an important cause of bacteraemia and a significant public health problem, and antibiotics resistance has been noticed in both pathogenic and non-pathogenic *E. coli* strains. Constant antibiotic testing should be done against all the antibiotics, both the prescription drugs and the reserve. This will help to show any emerging resistance especially to those antibiotics that are not in constant use.

The problem of resistance has further been complicated by the use of antibiotics in veterinary practice and in animal feeds. The animals and their products eventually end up being consumed by humans and thus pass on the resistance. Use of antibiotics in veterinary practice and animal feed should therefore be regulated to prevent its transfer to humans.

Future research should also focus more on alternative methods of treatment including the use of herbal medicine. Certain herbal extracts have been shown to be effective against Gram-negative bacteria such as *Salmonella* (Okemo and Mwatha, 2002). If research in this line is fully exploited, it can provide an important relieve from antibiotics whose usefulness is slowly diminishing.

## 6.2 Recommendations

- Introduction of good sanitation facilities with treated piped, water supply, basic hygiene measures such as washing of hands before eating as well as health education may help to reduce the prevalence of diarrhoea.
- The presence of non-pathogenic bacteria in stools should be investigated to determine their role in diarrhoeal infections in children in Kenya especially *Hafnia alvei* which has been associated with diarrhoea.
- The use of chloramphenicol, ampicillin, trimethoprim-sulphamethaxazole, tetracycline and streptomycin for treatment of diarrhoea in Kenya should be reviewed. In addition constant surveillance on quinolones resistance for example ciprofloxacin should be designed to target all quinolones in use at a given time in order to detect any cross-resistance.
- Antibiotics should not be given until sensitivity tests have been done to prevent accumulation of resistance pools that eventually spread in the population.
- Strict regulations should be put in place to limit the purchase of antibiotics by prescription only, to reduce misuse that has resulted in emergence and spread of resistance.
- Future research should also focus more on alternative methods of treatment including the use of herbal medicine.

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**APPENDIX 1****ISOLATION OF PLASMID DNA FROM BACTERIA****Harvesting**

1. Transfer a single bacterial colony into 2 ml of LB medium containing the appropriate antibiotic in a loosely capped 15 ml tube. Incubate the culture overnight at 37<sup>0</sup>C with vigorous shaking.
2. Pour 1.5 ml of the culture into a microfuge tube. Centrifuge at 12,000g for 30 seconds at 4<sup>0</sup>C in a microfuge. Store the remainder of the culture at 4<sup>0</sup>C.
3. Remove the medium by aspiration, leaving the bacterial pellet as dry as possible.

**Lysis by alkali solution**

1. Resuspend the bacterial pellet (obtained in step 3 above) in 100µl of ice cold solution 1 by vigorous vortexing.

**Solution 1**

50 Mm glucose

25 Mm Tris. Cl (pH 8.0)

10Mm EDTA (pH 8.0)

2. Add 200  $\mu$ l of freshly prepared Solution 2

#### Solution 2

0.2 N NaOH (freshly diluted from a 10 N stock)

1% SDS

Close the tube tightly, and mix the contents by inverting the tube rapidly five times.

Make sure that the entire surface of the tube comes in contact with Solution 2. Do not vortex. Store the tube in ice.

3. Add 150  $\mu$ l of ice cold solution 3

#### Solution 3

5 M potassium acetate 60ml

glacial acetic acid 11.5ml

H<sub>2</sub>O 28.5ml

The resulting solution is 3M with respect to potassium and 5M with respect to acetate.

Close the tube and vortex it gently in an inverted position for 10 seconds to disperse Solution 3 through the viscous bacterial lysate. Store the tube in ice for 3-5 minutes.

4. Centrifuge at 12,000g for 5 minutes at 4<sup>0</sup>C in a microfuge. Transfer supernatant to a fresh tube.

5. Precipitate the double stranded DNA with 2 volumes of ethanol at room temperature. Mix by vortexing. Allow the mixture to stand for 2 minutes at room temperature.
6. Centrifuge at 12,000g for 5 minutes at 4<sup>0</sup>C in a microfuge.
7. Remove the supernatant by gentle aspiration. Stand the tube in an inverted position on a paper towel to allow all of the fluid to drain away. Remove any drops of fluid adhering to the walls of the tube.
8. Rinse the pellet of double stranded DNA with 1ml of 70% ethanol at 4<sup>0</sup>C. Remove the supernatant as described in step 7, and allow the pellet of nucleic acid to dry in the air for 10 minutes.
9. Redissolve the nucleic acid in 50  $\mu$ l of TE buffer (pH 8.0) containing DNAase-free pancreatic RNAase (20  $\mu$ l/ml). Store the DNA at -20<sup>0</sup>C.

**APPENDIX 2****BIOCHEMICAL IDENTIFICATION TESTS****a) Citrate utilization test****Principle:**

The test is based on the ability of an organism to use citrate as its only source of carbon and ammonia as its only source of nitrogen. The test isolates is cultured on simmon's citrate agar, which contains sodium citrate, an ammonium salt, and the indicator bromothymol blue. Growth in the medium is shown by turbidity and change in colour of the indicator from light green to blue, due to the alkaline reaction, following citrate utilization.

**Procedure:**

Suspected colonies were inoculated on simmon's citrate agar (Oxoid) using a sterile straight wire. The inoculated medium was the incubated at 35-37<sup>0</sup>C overnight.

**Expected results:**

Turbidity and blue colour -----positive test (citrate utilization).

No growth-----negative test (citrate not utilized)

**Controls:**

Positive citrate control: *Klebsiella pneumoniae*

Negative: *Escherichia coli*.

### b) Triple sugar iron test (TSI)

#### Principle:

This medium is suitable for detecting H<sub>2</sub>S production by *Salmonella* species. Hydrogen sulphide is detected by the ferric citrate contained in the medium. The citrate reacts with H<sub>2</sub>S produced when sulphur containing amino-acids are decomposed. This produces FeS which is recognized as a black precipitate in the media.

#### Procedure:

The TSI agar slants in a tube were inoculated with test organisms. The slants were first streaked before being stabbed.

#### Expected results:

Blackening of medium-----positive test (H<sub>2</sub>S produced).

No blackening-----negative test (no H<sub>2</sub>S produced).

#### Controls:

Positive hydrogen sulphide control: *Proteus vulgaris*.

Negative hydrogen sulphide control: *Shigella* species.

### c) Indole production test

#### Principle:

The test isolate is cultured in a medium, which contain tryptophan. Indole production is detected by Kovac's or Erlich's reagent which contains 4(p)-dimethylaminobenzaldehyde. This reacts with indole to produce a red coloured compound.

#### Procedure:

Motility indole urea (MIU) medium (Oxoid), a semi solid medium was used in this test. Using a sterile wire, 5ml of sterile MIU medium was inoculated with a smooth colony of the test isolate. An indole paper strip was placed in the neck of the MIU tube above the medium and the tube stopped before being inoculated at 35-37°C overnight. The tube was examined for indole production by looking for a reddening of the lower part of the strip.

Expected results:

Reddening of the strip: Positive test (indole production).

No red colour: Negative test (no indole production).

Weak reactions were confirmed by adding 1ml of Kovac's reagent to the culture and examining for a red colouring of the surface layer within 10 minutes.

Controls:

Positive indole control: *Escherichia coli*

Negative indole control: *Enterobacter aerogenes*.

#### d) Urea test

Principle:

The test isolate is cultured in a medium, which contains urea and the indicator phenol red. If the stain is urease producing, the enzyme will break down the urea (by hydrolysis) to give ammonia, the medium becomes alkaline as by a change in colour of the indicator to red-pink.

Procedure:

Motility indole urea (MIU) medium (Oxoid), was used for the test. Using a sterile straight wire, a tube of sterile MIU medium was inoculated with a smooth colony of the test

isolate. An indole paper strip was placed in the neck of the MIU tube above the medium. The tube was stoppered before using incubated at 35-37°C overnight. The tube was examined for urease production by looking for a red-pink colour in the medium.

Expected results:

Red-pink medium: Positive test (urease production).

No red colour: Negative test (no urease production).

Controls:

Positive urease control: *Proteus vulgaris*.

Negative urease control: *Escherichia coli*.

## APPENDIX 3

## Reaction Interpretation Table for API 20E

Tests	Substrates	Reaction/ Enzymes	Results	
			Negative	Positive
ONPG	Ortho-Nitro-Pheny-Galactoside	Beta-galactosidae	Colourless	Yellow(1)
ADH	Arginine	Arginine dyhydrolase	Yellow	Red/orange(2)
LDC	Lysine	Lysine decarboxylase	Yellow	Orange
ODC	Ornithine	Ornithine decarboxylase	Yellow	Red/Orange(2)
CIT	Sodium citrate	Citrate utilization	Pale Green/yellow	Blue-green/green (3)
H <sub>2</sub> S	Sodium thiosulphate	H <sub>2</sub> S production	Colourless/Grayish	Black deposit/thin line
URE	Urea	Urease	Yellow	Red/Orange
TDA	Tryptophane	Tryptophane determinase	TDA/immediate Yellow	Dark brown
IND	Tryptophane	Indole production	JAMES Reagent/immediate or IND/2 min James Pale green-yellow IND Yellow ring	James Pink IND Red ring
VP	Sodium pyruvate	Acetoin production	VP + VP2/10 min Colourless	Pink red
GEL	Kohn's gelatin	Gelatinase	No diffusion of Black pigment	Diffusion of Black pigment
GLU	Glucose	Fermentation/Oxidation(4)	Blue/blue-green	Yellow
MAN	Mannitol	Fermentation/Oxidation(4)	Blue/blue-green	Yellow
INO	Inositol	Fermentation/Oxidation(4)	Blue/blue-green	Yellow
SOR	Sorbitol	Fermentation/Oxidation(4)	Blue/blue-green	Yellow
RHA	Rhamnose	Fermentation/Oxidation(4)	Blue/blue-green	Yellow
SAC	Sucrose	Fermentation/Oxidation(4)	Blue/blue-green	Yellow
MEL	Melibiose	Fermentation/Oxidation(4)	Blue/blue-green	Yellow
AMY	Amygdalin	Fermentation/Oxidation(4)	Blue/blue-green	Yellow
ARA	Arabinose	Fermentation/Oxidation(4)	Blue/blue-green	Yellow
OX	On filter paper	Cytochrome oxidase	OX/1-2 Colourless	Violet

(1) A very pale yellow should also be considered positive.

(2) An orange colour after 24 hours incubated must be considered negative.

(3) Reading made in cupule (aerobic).

(4) Fermentation begin in the lower portion of the tubes, oxidation begins in the cupule.

## APPENDIX 4

The features used in the biochemical identification of Enterobacteria and other enteric organisms

Species							MIU Medium			KIA Medium			
	Lact	Man	Glu	Suc	Ox	Cit	Mot	Ind	Urea	Slope	Butt	H <sub>2</sub> S	Gas
<i>Escherichia coli</i>	+	+	+	d	-	-	+ <sup>5</sup>	+ <sup>2</sup>	-	Y <sup>6</sup>	Y	-	+ <sup>2</sup>
<i>Shigella species</i>	-	d	+	- <sup>1</sup>	-	-	-	d	-	R	Y	-	- <sup>3</sup>
<i>Salmonella typhi</i>	-	+	+	-	-	-	+	-	-	R	Y	+	-
<i>Salmonella paratyphi</i> A	-	+	+	-	-	-	+	-	-	R	Y	-	+
Other <i>Salmonella</i>	-	+	+	-	-	d	+	-	-	R	Y	-	+
<i>Citrobacter freundii</i>	+	+	+	d	-	+	+	- <sup>2</sup>	d	R/Y	Y	d	+
<i>Klebsiella spp</i>	+	+	+	+	-	+	-	- <sup>3</sup>	+	Y	Y	-	+
<i>Enterobacter spp</i>	+	+	+	d	-	+ <sup>2</sup>	+	-	-	Y	Y	-	+
<i>Serratia marcescens</i>	d	+	+	+	-	+	+	-	d	R/Y	Y	-	d
<i>Proteus vulgaris</i>	-	-	+	+	-	d	+	+	+	R	Y	+	d
<i>Proteus mirabilis</i>	-	-	+	d	-	+ <sup>2</sup>	+	-	+	R	Y	+	+
<i>Providentia spp</i>	-	d	+	d	-	+	+	+	d	R	Y	-	-
<i>Yersinia enterocolitica</i>	-	-	-	-	+	d	+	-	-	R	R	-	-

KEY: Lact=Lactose, Man=Manitol, Glu= Glucose, Suc=Sucrose, Ox=Oxidase test, Cit=Citrate test, Mot=Motility, Ind= Indole test, H<sub>2</sub>S=Hydrogen sulphide (blackening), R=Red-pink (alkaline reaction), Y=Yellow (acid reaction), d=different strains give different results.

1. *S. sonnei* ferments sucrose slowly.
2. A minority of strains give negative result.
3. A minority of strains give a positive result.
4. Tests should be incubated at 20-28°C.
5. A few strains are non-motile.
6. A few strains produce a Red-pink slope (alkaline reaction).