ISOLATION, CHARACTERIZATION AND ANTIBIOTIC SUSCEPTIBILITY PATTERN OF

ESCHERICHIA COLI PATHOTYPES AMONG CHILDREN WITH DIARRHOEA IN ZARIA, NIGERIA.

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Abstract

Diarrhoea is one of the foremost public health problems worldwide especially among Children under five years in developing countries. Only few studies have investigated the epidemiology and virulence of *Escherichia coli* pathotypes in South-Eastern and South-Western Nigeria leaving the Northern part of the country unstudied. The isolation and biochemical identification of the isolates were performed using standard microbiological procedures. Antimicrobial Susceptibility testing was performed by disc diffusion method using modified Kirby-Bauer sensitivity testing techniques. Multiplex PCR test was performed to characterize the *E. coli* isolates into their respective pathotypes. A total of 100 isolates of *Escherichia coli* were obtained from the diarrhoeic stool of 222 paediatric patients under the age of five (5) years. The result of antimicrobial susceptibility profile of the isolates showed that, 86% of the *E. coli* isolates were resistant to Amoxicillin, 85% to Cotrimoxazole, 81% to Erythromycin, 40% to Chloramphenicol, 24% to Augmentin (Amoxicillin/Clavulanic acid), 23% to Ciprofloxacin, 23% to Ceftriaxone, and 22% to Gentamicin. The multiple antibiotic resistance index of 0.1 was obtained in 11% of the *E. coli* isolates, 0.2 in 10%, 0.3 in 19%, 0.5 in 24%, 0.6 in 15%, 0.7 in 11%, 0.8 in 6%, and 1.0 in 2%. Multiple antibiotic resistance (MAR) index of the *E. coli* isolates in this study showed that 58% have MAR index of ≥ 0.5. Following β-lactamase production test, 55.8% of the Amoxicillin resistant isolates were found to be β-lactamase positive while 44.2% were found to be β-lactamase negative. From the results obtained, only one (1) percent of the isolates was found to harbor the virulence gene out of the 100 *E. coli* isolated from the diarrhoea stools of children employed in this study. This virulence gene was found to be dispersin/antiaggregation protein (*aap*), a characteristic of Enteroaggregative *E. coli* (EAEC).

CHAPTER ONE

1.0 Introduction

1.1 *Escherichia coli*

Escherichia coli (E. coli), originally called "*Bacterium coli* commune," was first isolated from the faeces of a child in 1885 by Theodor Escherich and is nowadays the most-studied bacterium (Sousa, 2006). There are *E. coli* strains that are harmless commensals of the intestinal tract and others that are major pathogens of humans and animals. The pathogenic *E. coli* is divided into those strains causing diseases inside the intestinal tract and others capable of infection at extra intestinal sites (Kaper *et al*., 2004). *Escherichia coli* is easily cultured in the clinical laboratory, but the identification of the different pathogenic genotypes requires virulence gene detection methods. *Escherichia coli* can be found in soil and water as the result of faecal contamination. Classically, its detection has been used as an indicator of poor water and/or food quality. From biochemical, physiological and genetic perspectives, *E. coli* is one of the best understood and characterized living microorganisms, with laboratory studies on model strains such as *E. coli* K-12 taking place over the past sixty years (Sousa, 2006).

It is often the most abundant facultative anaerobe in our environment. *Escherichia coli* is a common inhabitant of the small and large intestine of mammals. The human colon maintains a microbial density approaching 10^{12} organisms per gram of faeces, representing a perfectly balanced ecosystem. The commensal microbiota consists of more than 400 species and lives in perfect harmony within the human intestine (Hooper and Gordon, 2001). They are occasionally isolated from the intestinal tract of non mammalian animals and insects. The presence of *E. coli* in the environment is usually considered to reflect faecal contamination and not the ability to

replicate freely outside the intestine. There is evidence however, to suggest that *E. coli* may freely replicate in tropical fresh water (Bermudez and Hazen, 1988).

1.2 Evolution of *E. coli* Pathotypes

Virulent strains of *E. coli* are differentiated clinically from one another on the basis of epidemiology, signs and symptoms of their respective diseases, microscopic observations of their interactions with host cells, and of biotypes and unique gene markers. *E. coli* colonizes the gastrointestinal tract of most warm-blooded animals within hours or a few days after birth. The bacterium is ingested in foods or water or obtained directly from other individuals handling the infant (Sousa, 2006). *E. coli* can adhere to the mucus overlying the large intestine. Once established, an *E. coli* strain may persist for months or years. Resident strains shift over a long period, and more rapidly after enteric infection or antimicrobial chemotherapy that perturbs the normal microbiota (Sousa, 2006). The basis for these shifts and the ecology of *Escherichia coli* in the intestine of humans are poorly understood despite the vast amount of information on almost every other aspect of the organism's existence. Infections due to pathogenic *E. coli* may be limited to colonization of a mucosal surface or can disseminate throughout the body. This dissemination may subsequently leads to urinary tract infection, sepsis, meningitis and gastrointestinal infections (Nataro and Kaper, 1998).

Several *E. coli* pathotypes have been implicated with diarrhoeal illness, and have become a major public health problem worldwide, with over two million deaths occurring each year (Kosek *et al*., 2003). The evolution of independent pathogenic types of *E. coli* is striking and to date unmatched by any other bacterial genus. How this occurred is unclear, but it is likely linked to the concomitant evolution of different mammalian hosts. Initially, isolates involved in both

intestinal and extra-intestinal human disease were thought to be concentrated mostly within the single, *Escherichia coli* reference collection group (B2 ECOR), a phylogenetic group with a smattering of isolates found in the D group. However, some studies indicated that extensive horizontal transmission of blocks of genes has occurred across the different phylogenetic clonal lines. Thus, with the possible exception of the enterohaemorrhagic *E. coli* O157:H7, many of the *E. coli* pathotypes apparently do not have unique evolutionary origins. The virulent *E. coli* strains have arisen independently on multiple occasions within clonal lines (Pupo *et al.*, 1997). On the basis of the relative number of isolates identified within the general diarrhoeagenic and extra-intestinal pathotype groupings, the former are more frequently found in the A, B1 and D phylogenetic groups, whereas the extra-intestinal *E. coli* strains are more common to the B2 lineage (Johnson, 2002).

1.3 *E. coli* Pathotypes

Based on pathogenesis, clinical manifestations and presence of specific virulence factors, there are five major DEC groups/pathotypes (Cheesbrough, 2000).

(i) Verocytotoxin-producing *E. coli* (VTEC)/Enterohaemorrhagic *E. coli* (EHEC)

(ii) Enteropathogenic *E. coli* (EPEC)

(iii) Enterotoxigenic *E. coli* (ETEC)

(iv) Enteroinvasive *E. coli* (EIEC) and

(v) Enteroaggregative *E. coli* (EAEC).

1.3.1 Verocytotoxin-producing *E. coli* (VTEC)/ Enterohaemorrhagic *E. coli* (EHEC)

Enterohaemorrhagic *E. coli* (EHEC) strains are implicated in food-borne diseases principally due to ingestion of uncooked minced meat and raw milk. These strains produce verotoxins 1 and 2

(Shiga-like-toxin 1 (stx1) and Shiga-like toxin 2 (stx2)) and variants thereof. They are involved in episodes of diarrhoea with complications. Serotype O157:H7 is the prototype of increasing importance and is associated with hemorrhagic colitis, bloody diarrhoea and the hemolytic uremic syndrome (HUS). EHEC typically causes an afebrile bloody colitis and, in about 10% of patients, this infection can be followed by HUS (Pickering *et al*., 1994). Like EPEC, EHEC elicit an attaching and effacing lesion of the intestinal mucosa, a phenotype that requires a functional *eaeA* chromosomal gene. These organisms share the ability to cause attaching and effacing(A/E) lesions with EPEC but enterohemorrhagic *E. coli* (EHEC) are set apart from EPEC by possession of Shiga-like toxins and the clinical presentation of their disease. EHEC cause disease of the large intestine that may present as simple watery diarrhoea and then progress to bloody stools with ulcerations of the bowel. In a small subset of diseased individuals, there is onset several days later of severe, life threatening hemolytic-uremic syndrome (HUS). HUS involves a triad of hemolytic anemia, thrombocytopenia and renal failure. The transmission of EHEC disease in humans is through ingestion of contaminated beef or foods contaminated with cattle faeces. In cattle, the EHEC strains are transient members of the intestinal microflora where they do not apparently cause disease. One of the remarkable features of EHEC is its low infection dose (10–100 organisms). Clearly this microorganism has special acid-tolerance ability when compared to many other enteric bacterial pathogens. Children under the age of five are the major victims of EHEC disease, although the elderly may also exhibit bloody diarrhoea and HUS. Epidemiologically in the United States, Japan, and Great Britain, a single serotype O157:H7 is the most common EHEC strain. In other parts of the world, this strain can be observed causing disease, but other serotypes (e.g., O26 and O111) cause a similar disease as well. All factors that lead to HUS are unknown except Shiga toxin (sometimes referred to as "Shiga-like toxin" or

"verotoxin"), which probably plays an important role in renal injury. Purified Stx-1 injected intravenously in baboons leads to renal disease with histopathology similar to EHEC-mediated HUS (Tailor *et al*., 1999). The Shiga toxin inhibits protein synthesis through cleavage of ribosomal RNA. Because EHEC do not cause bacteremia, Shiga toxin is thought to be released while the organism is growing in the large bowel, where it gets disseminated systemically to cause damage to renal endothelial cells and release of inflammatory mediators that eventually damage the kidney. There are two evolutionarily related forms of Shiga toxin in *E. coli* (Shiga toxin 1 and Shiga toxin 2). They share approximately 55% amino acid sequence similarity. Shiga toxin 1 is only different from the Shiga toxin of *Shigella dysenteriae* by a single amino acid substitution. There are many Shiga toxin positive *E. coli* strains (STEC) that are not associated with enterohemorrhagic colitis. It is a heterogeneous group that is occasionally associated with HUS, but their general benign nature may be due to their lack of the locus of enterocyte effacement (LEE) pathogenicity island and plasmid virulence factors. The ubiquitous dissemination of the distribution of Shiga toxin genes among *E. coli* strains is due to their transmission as part of lambdoid phages. The EHEC O157:H7 strain is likely to have originated from an O55 EPEC strain where a series of genetic events led to acquisition of shiga toxin encoding prophages and a large virulence plasmid, pO157 (Reid *et al*., 2000). The precise role of pO157 in EHEC pathogenesis is unknown but may involve some putative toxin genes and a mucin-specific zinc metalloprotease, StcE (Grys *et al*., 2005).

1.3.2 Enteropathogenic *E. coli* (EPEC)

These organisms are a significant cause of infant diarrhoea in developing nations. Enteropathogenic *E. coli* (EPEC) were historically recognized on the basis of serotypes such as O55:H6 and O127:H6. EPEC, an established etiological agent of human infantile diarrhoea, is a

pathogen that destabilises intestinal epithelial cell function to produce distinctive “attaching and effacing” (A/E) lesions. These lesions are characterized by localized destruction (effacement) of brush border microvilli, intimate bacterial attachment to the host-cell membrane and formation of an actin-rich cytoskeletal structure beneath intimately attached bacteria. In developing countries, enteropathogenic *E. coli* (EPEC) is one of the most common pathogens. In Brazil, for example, EPEC can be isolated from stools of over 40% of infants with acute diarrhoea and was associated with a mortality of 7% (Fagundes-Neto and Scaletsky, 2000). The pathogenesis of EPEC is in some way unique for enteric bacterial pathogens since it is essentially non invasive and produces no toxins. The attachment of EPEC to the epithelial cell, described as localized adherence, results in a so called attaching and effacing lesion (A/E) (Celli *et al*., 2000). EPEC disease is generally the result of growth of EPEC in the small intestine. EPEC cause a watery diarrhoea that may contain mucus but typically does not have blood in it. Vomiting, fever, malaise and dehydration are also associated. The symptoms may last for a brief period of several days, although instances of long, chronic EPEC disease have been noted (Sousa,2006). Some of the mechanisms of EPEC pathogenesis are well understood. For example, the A/E lesion is the result of a complex system of EPEC proteins that are injected into the host intestinal epithelial cell. The A/E lesion represents a dramatic rearrangement of the epithelial cytoskeleton where there is an accumulation of actin directly below the attached EPEC cell. This is described as an actin pedestal for the attached bacterial cell. There is a specific pathogenicity island, termed the "locus of enterocyte effacement" (LEE), that encodes the genes responsible for the A/E lesion. The LEE encodes a type III secretion system that provides the intimate adhesion (intimin) its receptor (which is injected into and then presented on the surface of the host cell), and the injected proteins responsible for changes in host cell signaling mechanisms, including actin

pedestal formation (Jerse *et al*., 1990). Common to most EPEC strains are plasmids EAF ("EPEC adherence factor"), which encode an adherence factor, the bundle-forming pilus (bfp) (Nataro *et al*., 1987). Results of human volunteer studies indicated that the EAF plasmid is necessary to cause disease (Levine *et al*., 1985). Although the A/E characteristic is critical for causing EPEC disease, probably through destruction of microvilli, the precise mechanism for the diarrhoea is not completely understood and may reflect the diversity of EPEC strains.

1.3.3 Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) strains are a major cause of secretory diarrhoea in both humans and animals (Bern *et al*., 1992). ETEC produce toxins which are heat-labile (LT) and/or heat-stable (STa and STb) that also cause diarrhoea. A frequent cause of diarrhoea in both humans and animals, enterotoxigenic *E. coli* (ETEC) strains are estimated to cause 600 million cases of human diarrhoea and 800,000 deaths worldwide principally in children under the age of 5 years (Sousa, 2006). Economically significant ETEC diarrhoeal disease in animals occurs in neonatal calves, pigs and lambs. ETEC cause watery diarrhoea that can be mild in nature or in some instances can be a severe, cholera-like illness where rapid dehydration can be life-threatening. In endemic areas of ETEC-mediated diarrhoea, infants and children under the age of 5 are the most commonly affected. ETEC exposure in endemic areas is one of the most common causes of traveler's diarrhoea. One of the principal virulence factors for this pathogen is the heat-labile enterotoxin (LT), which interestingly shares structural and functional similarity to the *Vibrio cholerae* cholera toxin (Spangler, 1992). LT toxin has a classic AB toxin subunit holotoxin structure. The B subunits (as a pentamer) bind to host cell surface GM1 and GD1b gangliosides and the A subunit enzymatically ADP-ribosylates the α- subunit of stimulatory G protein. This G protein regulates host cell adenylate cyclase and LT mediated modification leads to its

permanent activation and an increase in intracellular cAMP levels. This eventually leads to activation of the chloride ion channel of the intoxicated cells, increased chloride ion secretion into the intestinal lumen, and decreased sodium and chloride absorption. The overall result is to reverse the normal intestinal osmotic gradient and cause a net water loss into the gut lumen. Aside from LT, many ETEC strains also express heat-stable enterotoxins (STs), which also contribute to the watery diarrhoea. There are two structurally distinct STs, STa and STb. The STs are small polypeptides that share the common features of heat stability and multiple intramolecular disulfide bonds. The action of STa is well understood. It binds to the extracellular domain of plasma membrane embedded guanylate cyclase. The ETEC toxins are secreted in the terminal small intestine where the ETEC adhere by expression of a complex and diverse group of surface proteins commonly referred to as "colonization factors" (Gaastra and Svennerholm, 1996).

1.3.4 Enteroinvasive *E. coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) cause a broad spectrum of human's diseases. They are biochemically, genetically and pathogenetically closely related to *Shigella* spp., both characteristically cause an invasive inflammatory colitis, but either may also elicit a watery diarrhoea syndrome indistinguishable from that caused by other *E. coli* pathogens. The pathogenesis of disease caused by EIEC and *Shigella* involves cellular invasion and spread, and requires specific chromosomal and plasmid borne virulence genes (Nataro and Kaper, 1998). These organisms are pathogenetically so closely related to *Shigella* species that the nomenclature distinction is questionable. There are a few biochemical traits that can be used to distinguish enteroinvasive *E. coli* (EIEC) from *Shigella*, but the principal virulence genes are shared. The diagnostic confusion between *Shigella* and EIEC is evident in that EIEC isolates like shigella,

are nonmotile and 70% are nonlactose fermenters (Silva *et al*., 1980). In addition, EIEC share with *Shigella* the inability to decarboxylate lysine, a trait common to other *E. coli*. The traits that EIEC share with *E. coli* but not *Shigella* are the ability to produce gas from glucose and fermentation of xylose. EIEC cause invasive inflammatory colitis and dysentery with a clinical presentation (blood and mucous stools accompanied by fever and severe cramps) identical to the disease caused by *Shigella* species. Both EIEC and *Shigella* invade intestinal epithelium, principally in the large intestine. Once inside the cells, they lyse the phagocytic vesicle and replicate freely in the host cell cytoplasm. They then spread to neighboring host cells by a motility process whereby actin is nucleated on one pole of the bacillus and subsequent actin polymerization propels the bacterial cell (Goldberg and Theriot, 1995). Many of the genes necessary for cellular invasion and disease are carried on a large (>200-kb) plasmid found in both EIEC and *Shigella*. A system of type III secretion genes important for delivery of modifiers of host cell signaling and membrane lysis are found on these plasmids. In addition, the plasmid encodes an outer membrane protein (IcsA) that is localized on one pole of the bacterium and directs the actin microfilament polymerization necessary for spread of bacteria to other host cells. EIEC and *Shigella* rarely invades the bloodstream, but they do invade the lamina propria immediately under the intestinal epithelium, where interaction with macrophages causes the release of pro-inflammatory mediators and even induction of apoptosis. Interestingly, the inability to decarboxylate lysine, a trait shared by EIEC and *Shigella*, is the result of mutations and gene rearrangements at the *cadC* gene. The decarboxylation of lysine results in cadverine, which acts as an inhibitor of inflammation and migration of neutrophils into the lamina propria. The lack of this function is hypothesized to be a pathoadaptive trait that enables EIEC and *Shigella* to cause disease (Casalino *et al*., 2003).

1.3.5 Enteroaggregative *E. coli* (EAEC)

Enteroaggregative *E. coli* (EAEC) strains are defined by their distinctive adherence pattern on HEp-2 cells in culture (Nataro and Kaper, 1998). The essential element of the aggregative phenotype is the stacked brick pattern by lying side-by-side with an appreciable distinction of where one bacterium begins and another ends. The EAEC are a heterogeneous group of bacteria that display a wide array of virulence factors (Sousa and Dubreuil, 2001). EAEC are pathogens associated with persistent diarrhoea in the developing world and have been implicated recently in the developed world as causes of both outbreaks and sporadic diarrhoea among AIDS patients. These organisms are defined as *E. coli* that do not possess LT enterotoxin or Shiga toxins but adhere to cultured HEp-2 cells in self-aggregates that are classically referred to as "stacked bricks. Clearly, many *E. coli* strains can mediate the "stacked brick" adhesive phenotype, but there is a subset of these that are bona fide human diarrhoeal pathogens. Enteroaggregative *E. coli* (EAEC) disease, as described by human volunteers, is a watery diarrhoea that occurs in some cases with abdominal cramps, but no fever. There is no invasion of the bloodstream. The disease seen in natural EAEC outbreaks is often reported as a persistent, seemingly chronic watery diarrhoea. These small epidemics occur in both developing as well as industrialized countries. There are no common serotypes of EAEC to aid in their recognition in the clinical laboratory. The pathogenesis of EAEC disease is poorly understood, although several potential virulence factors are common to EAEC isolates. EAEC express a fimbrial adhesin called "aggregative adherence fimbriae" ("AAF"). EAEC isolates often produce a mucinase called "Pic" whose gene has the ability to express from its non encoding DNA strand a smaller gene that encodes an enterotoxin (*Shigella* enterotoxin [ShET1]) first described in *Shigella* strains. EAEC strains often produce a heat stable enterotoxin EAST1 that is homologous to the ST1 of ETEC.

1.4 Statement of Research Problem

Diarrhoea is a leading cause of morbidity and mortality among children in developing countries (Guerrant *et al*., 1994). Diarrhoeal disease continues to be one of the foremost public health problems worldwide, with over 1.5 million deaths occurring each year, mostly in children under 5 years in developing countries (Bryce *et al.*, 2005). The bacterial pathogen most commonly associated with endemic forms of childhood diarrhoea is *Escherichia coli* (Huilan *et al*., 1991). Only few studies have investigated the microbiology of acute childhood diarrhoea in Nigeria in particular and in West Africa in general. Studies conducted in Nigeria were mostly in South-Western and South-Eastetrn part of the country.

Okeke *et al*.,(2000) in a study conducted in South western part of Nigeria isolated 330 strains of *E. coli* from 187 diarrheic children and 144 apparently healthy control. In another study conducted in the south eastern part of Nigeria, Nweze (2010) found that of the 119 *E. coli* strains isolated from 520 subjects, 49.02% were EPEC, 21.57% ETEC while 7.84% were EAEC. Other studies conducted in Zaria were either limited to some parameters and did not characterize *E. coli* in to their different diarrhoeagenic groups/pathotypes, or they only focused on only one pathotype and were mostly not limited to children of 0-5 years of age. For example, Chigor *et al*. (2010) who reported a detection rate of 2.2% *E. coli* O157 in surface waters and its prevalence in children with diarrhoea was 5.4% in Zaria, Nigeria. Previous reports by Ogunsanya *et al*. (1994) and Olorunishola *et al*. (2000) in Lagos showed the prevalence of EHEC 0157:H7 in humans to be 5%.

Studies that has been performed were set predominantly in urban centers, sought only one or two diarrhoeagenic *E. coli* categories, or were not designed to detect potentially pathogenic *E. coli* (Ogunsanya *et al*., 1994). The virulence factors of some diarrhoeagenic *E. coli* have yet to be full

determined and in the meantime they remain a large and emerging problem without the availability of effective vaccines. There are different *E. coli* pathotypes and there is need to establish the presence of these pathotypes in our environment and their antibiotic sensitivity pattern.

1.5 Justification of the Study

The epidemiological significance of each *E. coli* category in childhood diarrhoea varies with the geographical area. As expected, it has become clear that there are important regional differences in the prevalence of different categories of diarrhoeagenic *E. coli* (DEC). The result of this study will establish the presence or otherwise of *E. coli* pathotypes (EIEC, EPEC, ETEC, EHEC/VTEC and EAEC) from children (0-5 years) and their antibiotic susceptibility profile within our environment. This will in turn provide a sound basis for the selection of appropriate antibiotic for the treatment of diarrhoeal diseases caused by different categories of diarrhoeagenic *E.coli.* The result of the study will add to the knowledge in the field of epidemiology and microbiology.

1.6 Aim and Specific Objectives

The aim is to characterize *E. coli* pathotypes from faecal samples obtained from children (0-5 years old), towards improved management of diarrhoea in this category of subjects.

The objectives are:

i. To isolate *E.coli* from stools of diarrhoeic children attending Ahmadu Bello University Teaching Hospital, Shika-Zaria, using standard microbiological procedures.

ii. To determine the antibiotic sensitivity pattern of the pathogenic *E. coli* by disc diffusion method using modified Kirby-Bauer sensitivity testing techniques.

iv To characterize the pathotypes of the *E. coli* isolates using Multiplex PCR.

1.7 Hypothesis

- **Null Hypothesis (H_O):**

Only one type of *E. coli* pathotype exist in stool of diarrheic children in the study area and are readily susceptible to commonly used antibiotics.

- **Alternative Hypothesis(H_A):**

Different *E. coli* pathotypes are present in the stool of children and are resistant to commonly used antibiotics in the study area.

CHAPTER TWO

2.0 Literature Review

2.1 Diarrhoea

Diarrhoea is defined as having loose or watery stools at least three times per day, or more frequently than normal for an individual. Though most episodes of childhood diarrhoea are mild, acute cases can lead to significant fluid loss and dehydration, which may result in death or other severe consequences if fluids are not replaced at the first sign of diarrhoea (UNICEF/WHO, 2009).

There are three main forms of acute childhood diarrhoea, all of which are potentially life-threatening and require different treatment courses (UNICEF/WHO, 2009):

1. Acute watery diarrhoea includes cholera and is associated with significant fluid loss and rapid dehydration in an infected individual. It usually lasts for several hours or days. The pathogens that generally cause acute watery diarrhoea include *V. cholerae* or *E. coli* bacteria, as well as rotavirus.
2. Bloody diarrhoea, often referred to as dysentery, is marked by visible blood in the stools. It is associated with intestinal damage and nutrient losses in an infected individual. The most common cause of bloody diarrhoea is *Shigella*, a bacterial agent that is also the most common cause of severe cases.
3. Persistent diarrhoea is an episode of diarrhoea, with or without blood, that lasts at least 14 days. Undernourished children and those with other illnesses, such as AIDS, are more likely to develop persistent diarrhoea. Diarrhoea, in turn, tends to worsen their condition.

2.1.1 Etiological Agents of Diarrhoea

The severity of acute diarrhoeal episodes can range from mild to moderate to severe cases that can lead to hospitalization or death. Severity is influenced by many factors, including the agent and its pathogenicity and host characteristics, such as immunodeficiency and age. Little information is available on the etiology of diarrhoea in HIV-infected or -exposed children, partially because of issues of feasibility and the costs associated with integrating HIV programmes into etiological studies. In general, studies on diarrhoea are limited by a lack of control specimens and testing of a reduced spectrum of enteric pathogens due to cost and lack of the necessary training, advanced laboratory skills, commodities and equipment (WHO, 2010).

Some examples of the etiological agents of diarrhoea are shown in Table 1

Table 1: Common Etiological Agents of Persistent and Bloody Diarrhoea

Persistent Diarrhoea	Bloody Diarrhoea
Enteropathogenic *E. coli*	Shigella
Enteroaggregative *E. coli*	Nontyphoidal *Salmonella*
Nontyphoidal *Salmonella*	*Campylobacter*
Cryptosporidium	Enteroaggregative *E. coli*
Microsporidia	Enteroinvasive *E. coli*
Giardia lamblia	Shiga toxin-producing *E. coli*
Ascaris lumbricoides	*Entamoeba histolytica*
Cytomegalovirus	
Other viruses	

Adapted from American Academy of Paediatrics (2009)

2.1.2 Epidemiology of Microbial diarrhoea

Despite global success in the reduction of all cause and diarrhoea-specific mortality in the past 30 years, diarrhoea remains the second leading cause of death due to infections among children under five years of age worldwide (Liu *et al*., 2012). It is estimated that diarrhoea accounted for 9·9% of the 6·9 million deaths among children under 5 in 2011 (Fischer-Walker *et al*., 2013).

The main examples of diarrhoeal disease associated with bacteria are cholera and diarrhoea due to enterotoxigenic *E. coli* (ETEC). Cholera remains an important cause of illness in many developing countries and has been estimated to result in more than 120 000 deaths each year. In children in developing countries, ETEC is responsible for more than 650 million diarrhoeal episodes and 800 000 deaths annually (Cravioto *et al*., 1998). However, diarrhoea as a syndrome is not limited to developing countries.

The most readily recognised bacterial agents of gastroenteritis in industrialised countries are *Salmonellae* and *Campylobacter* (Murray *et al*., 1995). Moreover, acute diarrhoea may be associated with a large number of other bacteria, such as *Yersinia enterocolitica, Staphylococcus aureus* and *Bacillus cereus*. Various Shigellae can cause diarrhoea or the syndrome known as bacillary dysentery. In particular, incompletely prepared or incompletely heated food has been reported as the source of different diarrhoeal pathogens (Murray *et al*., 1995)

Additionally, other organisms such as parasites and viruses are common causes of diarrhoea around the world. *Entamoeba histolytica* and *Giardia lamblia* are usually acquired by ingestion of food or water contaminated with faeces (Murray *et al*., 1995). Usually no symptoms result from the presence of these parasites in the colon, but some people experience abdominal pain and diarrhoea. Rotaviruses are a common cause of diarrhoeal disease in the whole world (Murray

et al., 1995). Kukkula (1998), reported a large outbreak of diarrhoea caused by calicivirus in industrialised countries. In Finland, caliciviruses have spread via drinking water and frozen berries.

2.2 Description of Microbial Causative Agents of Diarrhoea

Diarrhoea is a common symptom of gastrointestinal infections caused by a wide range of pathogens, including bacteria, viruses and protozoa. However, just a handful of organisms are responsible for most acute cases of childhood diarrhoea (UNICEF/WHO, 2009). Rotavirus is the leading cause of acute diarrhoea, and is responsible for about 40 per cent of all hospital admissions due to diarrhoea among children under five worldwide (UNICEF/WHO, 2009). Other major bacterial pathogens include *E. coli*, *Shigella, Campylobacter and Salmonella*, along with *V. cholerae* during epidemics. *Cryptosporidium* has been the most frequently isolated protozoan pathogen among children seen at health facilities and is frequently found among HIV-positive patients. Though cholera is often thought of as a major cause of child deaths due to diarrhoea, most cases occur among adults and older children.

2.2.1 Cholera

The paradigm of the enterotoxigenic diarrhoeal diseases is cholera in which stool volume can exceed 1 litre per hour with daily fecal outputs of 15 to 20 litres if the patient is kept hydrated (Gorbach, 1993). Cholera is caused by *Vibrio cholerae,* which is usually ingested in contaminated water. Vibrios that survive passage through the stomach colonize the surface of the small intestine, proliferate, and elaborate the enterotoxin. Cholera toxin acts via adenylate cyclase to stimulate secretion of water and electrolytes from the epithelial cells into the lumen of the gut. The duodenum and upper jejunum are more sensitive to the toxin than the ileum is. The

colon is relatively insensitive to the toxin and may still absorb water and electrolytes normally. Thus, cholera is an "overflow diarrhoea," in which the large volumes of fluid produced in the upper intestine overwhelm the resorptive capacity of the lower bowel (Gorbach, 1993).

Cholera stool is described as resembling rice water, a clear fluid flecked with mucus and is isotonic with plasma. Microscopy reveals no inflammatory cells in the fecal effluent; all that can be seen are small numbers of shed mucosal cells (Gorbach, 1993).

2.2.2 Enterotoxigenic *E. coli* Diarrhoea

Certain strains of *E. coli* cause diarrhoeal disease by elaborating enterotoxins. These strains produce two types of enterotoxins. One, called heat-labile toxin, is similar in structure and in its mechanism of action to cholera toxin. The other, called heat-stable toxin, appears to act via guanylate cyclase. Enterotoxigenic *E coli* strains are the most common cause of travelers' diarrhoea (Gorbach, 1993).

2.2.3 Other Diarrhoea-Causing Toxins

Many strains of *Shigella* produce an enterotoxin, called Shiga toxin, that causes secretion of fluid from the small intestine. Shiga toxin has a destructive, cytotoxic effect on the small-bowel epithelium, causing gross injury to the bowel surface. It does not activate adenylate cyclase. *E coli* 0157:H7, the organism associated with consumption of undercooked chopped meat, also produces a Shiga-like toxin; it causes bloody diarrhoea and colitis. An organism that produces a different type of cytotoxin is *Vibrio parahaemolyticus*, a bacterium associated with seafood. Food-poisoning strains of *Staphylococcus aureus* and *Clostridium perfringens* both produce

enterotoxins that are cytotoxic. The staphylococcal enterotoxin also has a direct effect on the vomiting center in the brain (Gorbach, 1993).

2.2.4 Gastrointestinal Disease Caused by Invasive Bacteria

Unlike the enterotoxigenic organisms, invasive bacteria exert their main impact on the host by causing gross destruction of the epithelial architecture; histologic findings include mucosal ulceration and an inflammatory reaction in the lamina propria. The principal pathogens in this group are *Salmonella, Shigella, Campylobacter*, invasive *E coli*, and *Yersinia*. The enteric viruses also invade intestinal epithelial cells, but the extent of mucosal destruction is considerably less than that caused by invasive bacterial pathogens (Gorbach, 1993).

2.2.4.1 Salmonella Enteritis

Salmonella species are common causes of food poisoning. The main site of attack is the lower ileum, where the salmonellae cause mucosal ulceration. They rapidly make their way through the epithelial surface into the and enter the lymphatics and bloodstream. At least two virulence factors are associated with intestinal infection: one responsible for mucosal invasion, and the other causing secretion of fluid and electrolytes into the bowel (Gorbach, 1993).

2.2.4.2 Shigella Dysentery

Shigella organisms cause bacillary dysentery, an invasive diarrhoeal disease of the lower bowel in which the stool contains an inflammatory exudate composed of polymorphonuclear leucocytes. The bacilli invade the epithelium of the colon and cause superficial ulceration. This invasive process depends on the presence of two virulence factors. The first mediates the initial penetration of the mucosal surface by destroying the brush border; the bacteria are subsequently engulfed by invagination of the plasma membrane. The second virulence factor allows the

organism to multiply within the mucosal tissue. Mucosal ulceration results, accompanied by an intense inflammatory response in the lamina propria. The infection is usually restricted to the mucosa; lymph node involvement and bacteremia are uncommon (Gorbach, 1993).

2.2.4.3 Fluid Production in Invasive Diarrhoeal Diseases

The mechanism(s) by which the fluid that causes watery diarrhoea is produced in the invasive diarrhoeal diseases is under debate. Three mechanisms have been proposed. First, *Shigella* and possibly *Salmonella* strains apparently produce an enterotoxin that stimulates the mucosa to secrete water and electrolytes. Second, there is evidence that invasive organisms stimulate prostaglandin synthesis at the site of inflammation and that the prostaglandins induce fluid secretion. In experimental animals, fluid secretion can be blocked by prostaglandin inhibitors such as indomethacin and aspirin. Third, some evidence suggests that damage to the colonic epithelium causes diarrhoea by prevention of normal resorption of fluid (Gorbach, 1993).

2.2.5 Viral Diarrhoea

Two viruses—rotavirus and Calicivirus (Norwalk virus) have been identified as major enteric pathogens in humans. The rotaviruses are a very important cause of infantile diarrhoea, which in undeveloped countries can be fatal. Adults may be infected and shed virus, but clinical disease appears almost exclusively in children younger than 2 years. Calicivirus, in contrast, can produce gastroenteritis in all age groups and is a cause of major epidemics (Gorbach, 1993).

The mechanisms responsible for fluid secretion in viral diarrhoeas have not been elucidated. It is known that infection with Calicivirus can produce steatorrhea and xylose malabsorption and causes direct damage to brush border enzymes. The activity of adenylate cyclase in the epithelial cells is not altered in the acute illness (Gorbach, 1993).

2.2.6 Parasitic Diarrhoea

Several species of protozoa and helminths can cause diarrhoeal disease. Some of these infections can be acquired worldwide, although exposure to enteric parasites is far more common in tropical and developing countries. Some of the more common causes of parasitic diarrhoea are *Entamoeba histolytica*, *Giardia lamblia*, *Strongyloides stercoralis*, and the intestinal flukes (Gorbach, 1993).

2.3 Isolation and Detection of virulence in *E. coli*

E. coli is one of the species of genus *Escherichia* which contains mostly motile Gram-negative bacilli within the family *Enterobacteriaceae* and the tribe *Escherichia*

E. coli can be recovered from clinical specimens on general or selective media at 37ºC under aerobic conditions. *E. coli* in stools are most often recovered on MacConkey or Eosin methylene-blue agar, which selectively grow members of the *Enterobacteriaceae* and permit differentiation of enteric organisms on the basis of their morphology (Murray *et al*., 1995).

The most highly conserved feature of diarrhoeagenic *E. coli* strains is their ability to colonise the intestinal mucosal surface despite peristalsis and competition for nutrients (Nataro and Kaper 1998). The presence of surface adherence fimbriae is a property of virtually all *E. coli* strains. However, diarrhoeagenic *E. coli* strains possess specific fimbrial antigens that enhance their intestinal colonising ability and allow adherence to the small bowel mucosa, a site that is not normally colonised (Levine *et al*., 1984). Three general paradigms have been described by which *E. coli* may cause diarrhoea:

- Enterotoxin production (ETEC and EAEC);

- Invasion (Enteroinvasive *E. coli* (EIEC)); and
- Intimate adherence with membrane signalling (EPEC and EHEC/VTEC). However, the interaction of the organisms with the intestinal mucosa is specific for each category (Nataro and Kaper, 1998).

The versatility of the *E. coli* genome is conferred mainly by two genetic configurations: virulence related plasmids and chromosomal pathogenicity islands. EHEC, EPEC, EAEC and EIEC strains typically harbour highly conserved plasmid families, each encoding multiple virulence factors (Nataro and Kaper, 1998). Chromosomal virulence genes of EHEC and EPEC are organised as a cluster referred to as a pathogenicity island (McDaniel *et al*., 1995).

Identification of diarrhoeagenic *E. coli* strains requires that these organisms can be differentiated from non-pathogenic members of the normal flora. Substantial progress has been made in the development of nucleic acid-based technologies. The use of DNA probes for detection of heat labile (LT) and heat-stable (ST) enterotoxins in ETEC revolutionised the study of these organisms by replacing the awkward and costly animal models of toxin detection (Mosley *et al*., 1982). Since then, gene probes have been introduced for all diarrhoeagenic *E. coli* categories.

Another widely used method is Polymerase Chain Reaction (PCR). It has been a major advance in molecular diagnostics of pathogenic microorganisms, including *E. coli*. In PCR, a pair of primers (20-40 bases) is used for selective amplification and detection of a certain DNA sequence in a target organism. PCR primers have successfully been developed for all categories of diarrhoeagenic *E. coli*. PCR can be used in both diagnosing and typing *E. coli* strains. Advantages of PCR include high sensitivity, specificity and appropriate rapidity in the detection of target DNA templates. However, substances within stools have been shown to interfere with

PCR by reducing its sensitivity (Stacy-Phipps *et al*., 1995). It has been stated that PCR detects significantly more ETEC infections than does the standard probe based hybridisation method (Caeiro *et al*. 1999). In diagnostics, PCR is commonly used for detecting different virulence associated genes of *E. coli*, such as toxin and adherence associated genes PCR is also widely used in subtyping by doing virulence gene profiles for different diarrhoeagenic *E. coli* strains.

Other methods specifically capable of detecting certain types of *E. coli* have been developed, especially for Shiga toxin producing *E. coli* (STEC). The latex agglutination test (Verotox-F assay, Denka Seyken, Tokio, Japan) for detection of toxins produced by STEC has been found 100% sensitive and 100% specific in comparison with the classical Vero cell assay (Karmali *et al*., 1999). Immunomagnetic separation with magnetic beads coated with antibody against *E. coli* O157 have been found more sensitive than direct culture of these strains (Chapman and Siddons, 1997).

2.4 Serotyping of Diarrhoeagenic *E. coli*

Prior to the identification of specific virulence factors in diarrhoeagenic *E. coli* strains, serotypic analysis was the predominant means by which pathogenic strains were differentiated. In 1947, Kauffmann proposed a scheme for the serologic classification of *E. coli* (Kauffmann, 1947), which is still used in a modified form today. According to the modified Kauffmann scheme, *E. coli* are serotyped on the basis of their O (somatic), H (flagellar), and K (capsular) surface antigens (Ørskov and Ørskov, 1984). However, generally only extraintestinal *E. coli* are encapsulated (Jann and Jann, 1992), thus, among diarrhoeagenic *E. coli*, usually a specific combination of O and H antigens defines the serotype of the strain. *E. coli* of specific serogroups

can be associated with certain clinical syndromes , but it is not in general that the serologic antigens themselves confer the virulence (Ørskov and Ørskov, 1984).

2.5 Genotyping

The use of molecular "fingerprinting" methods has become standard practice in microbiology for evaluating the epidemiology of infectious diseases, investigating suspected outbreaks of bacterial infections, and typing bacteria (Mickelsen, 1997). Pulsed-field gel electrophoresis (PFGE) allows the generation of simplified chromosomal restriction fragment patterns without having to resort to probe hydridisation methods. In this method, restriction enzymes that infrequently cut DNA are used for generating large fragments of chromosomal DNA, which are then separated by special electrophoresis (Swaminatham and Matar, 1993). PFGE has been applied to subtyping of several Gram-positive and Gram-negative bacteria. It is now widely used in epidemiological surveillance and common interpretation schemes have been published (Tenover *et al*., 1997). In 1995, the Centers for Disease Control and Prevention (CDC) in the USA initiated PulseNet, a national computer network of public health laboratories that employs standard methods to subtype EHEC O157:H7 strains. Laboratories within the network can transmit PFGE patterns electronically to a databank at the CDC, where they are automatically compared with patterns of other isolates. If the patterns submitted by laboratories in different locations during a defined time period are found to match, the CDC computer will alert Pulse Net participants of a possible multistate outbreak (Centers for Disease Control, 2001).

Another technique is ribotyping, ribotyping is a method based on DNA probes that recognizes conserved RNA operon genes. Ribotyping is essentially a Southern blot analysis in which strains are characterised for restriction fragment length polymorphism of their individual ribosomal

genes. Within a species, and particularly within a strain, the DNA sequences and the restriction digest patterns of genes encoding rRNA are highly conserved and thus serve as a molecular fingerprint for that organism. All organisms have ribosomal genes. This technique is universally applicable and has also found acceptance as a clinical tool for typing *E. coli* (Tarkka *et al*., 1994).

All these new molecular typing methods have allowed highly discriminant genotyping, and are useful tools for demonstrating that isolates from different sources are identical, closely related or not related at all (Mickelsen, 1997).

2.6 Management and Control of Diarrhoea

Mortality from diarrhoea has declined over the past two decades from an estimated 5 million deaths among children under five to 1.5 million deaths in 2004, which parallels downward trends in overall under-five mortality during this period. Despite these declines, diarrhoea remains the second most common cause of death among children under five globally following closely behind pneumonia, the leading killer of young children (UNICEF/WHO, 2009).

2.6.1 Drug Therapy

Because of increasing threats from antimicrobial-resistant infections, side effects of treatment with antimicrobial agents, suprainfections when normal flora are eradicated by antimicrobial agents, and the possibility of induction of disease-producing phage by antibiotics (such as Shiga-toxin phage induced by quinolone antibiotics) (Zhang *et al*., 2000). Any consideration of antimicrobial therapy must be carefully weighed against unintended and potentially harmful consequences. New non-antimicrobial treatments to block secretory or inflammatory toxins or to enhance electrolyte absorption and intestinal repair are badly needed and are under study.

One situation in which empirical antibiotics are commonly recommended without obtaining a fecal specimen is in cases of traveler's diarrhoea, in which enterotoxigenic *E. coli* or other bacterial pathogens are likely causes, and prompt treatment with fluoroquinolone or, in children, trimethoprim-sulfamethoxazole (Cotrimoxazole) can reduce the duration of an illness from 3–5 days to <1–2 days. Some also consider empirical treatment of diarrhoea that lasts longer than 10–14 days for suspected giardiasis, if other evaluations are negative and, especially, if the patient's history of travel or water exposure is suggestive (DuPont, 1997). Otherwise, for patients with febrile diarrhoeal illnesses, especially those believed to have moderate to severe invasive disease, empirical treatment should be considered (after a fecal specimen is obtained for the performance of the studies noted above). This empirical treatment can be with an agent such as a quinolone antibiotic or, for children, Cotrimoxazole, which can reduce the duration and shedding of organisms in infections with susceptible *Shigella* species (Khan *et al*., 1997).

Salmonella infections may warrant quinolone or other antimicrobial therapy when systemic spread is considered a risk or suspected and for children <6 months of age; however, like other antibiotics, quinolones may prolong shedding of non-*typhi* species of *Salmonella* (Dupont, 1997).

Suspected or documented STEC infections should not be treated with antimotility agents (Slutsker *et al*., 1997) , and a decision to treat an illness that could be due to STEC O157 with an antimicrobial agent should be considered carefully, as it may worsen the risk of HUS developing. Treatment of STEC O157 infections with antimicrobial agents has not been shown to ameliorate illness, and several retrospective studies have noted a higher rate of HUS in treated patients (Zhang *et al*., 2000), which could be an effect of treatment or a reflection of more aggressive

treatment of patients who are more ill. In vitro data indicate that certain antimicrobial agents can increase the production of Shiga toxin, and animal studies have demonstrated harmful effects of antibiotic treatment of STEC infections (Zhang *et al*., 2000).

2.6.2 Rehydration Using Improved ORS Formulation

Over the last three decades, WHO and UNICEF have recommended a single formulation of glucose-based ORS to prevent or treat dehydration from diarrhoea regardless of cause or age group affected. The ORS recommended until recently, which provides a solution containing 90 mEq/l of sodium with a total osmolarity of 311 mOsm/l, has proven effective and without apparent adverse effects in worldwide use. It has contributed substantially to the dramatic global reduction in mortality from diarrhoeal disease during the period (WHO, 2006).

For the past two decades, numerous studies have been undertaken to develop an "improved" ORS (WHO, 2006.). The goal was a product that would be at least as safe and effective as standard ORS for preventing or treating dehydration but would also reduce stool output or have other important clinical benefits. One approach has consisted in reducing the osmolarity of ORS solution to avoid possible adverse effects of hypertonicity on net fluid absorption. This was done by reducing the solution's glucose and salt (NaCl) concentrations. Studies to evaluate this approach were reviewed at a consultative technical meeting held in New York (USA) in July 2001 (WHO, 2006). Technical recommendations were made to WHO and UNICEF on the efficacy and safety of reduced osmolarity ORS in children with acute non-cholera diarrhoea, and in adults and children with cholera. These studies showed that the efficacy of ORS solution for treating children with acute non-cholera diarrhoea is improved by reducing its sodium concentration to 75 mEq/l, its glucose concentration to 75 mmol/l and its total osmolarity to 245 mOsm/l. The need for unscheduled supplemental IV therapy in children given this solution was

reduced by 33%. In a combined analysis of this study and studies with other reduced osmolarity ORS solutions (osmolarity 210-268 mOsm/l, sodium 50-75 mEq/l), stool output was also reduced by about 20% and the incidence of vomiting by about 30%. The 245 mOsm/l solution also appeared to be as safe and at least as effective as standard ORS for use in children with cholera (WHO, 2006).

2.6.3 Zinc Supplement in the Treatment of Acute Diarrhoea

The use of zinc in treating acute diarrhoea is thought to affect immune function or intestinal structure or function, and the epithelial recovery process during diarrhoea. Zinc deficiency has been found to be widespread among children in developing countries, and occurs in most of Latin America, Africa, the Middle East and South Asia (WHO, 2006). Zinc has been identified to play a critical role in metallo-enzymes, polyribosomes, the cell membrane and cellular function, leading to the belief that it also plays a central role in cellular growth and in the function of the immune system. It has also been shown that intestinal zinc losses during diarrhoea aggravate pre-existing zinc deficiency (WHO, 2006). Convincing evidence for the clinical importance of zinc has come from randomised controlled trials evaluating the impact of zinc supplementation during acute and persistent diarrhoea. In 2001, WHO convened a meeting of experts in New Delhi, India, to review the results of all these studies. The main features of these trials include randomised placebo-controlled design, subjects aged between one month and five years, and daily elemental zinc dose ranging from 5 to 45 mg per day. In these trials, children receiving zinc had a significantly faster recovery than children receiving placebo (about 20% reduction in the duration of diarrhoea). Zinc treatment also resulted in a 20% reduction in the risk of acute episodes to last more than seven days. Some of these studies were hospital-based, and measured impact of zinc supplementation on stool output. Reduction in total stool

output ranged from 18% to 59% in the zinc-treated children when compared to the placebo group. In all these studies, the effect of zinc did not vary significantly with age or nutritional status assessed by anthropometry. The effects were not dependent upon the type of zinc salts used as zinc sulphate, zinc acetate or zinc gluconate were equally effective. Although the optimal dose is yet to be determined, there seems to be little gain in efficacy when the commonly used 20 mg daily dose of elemental zinc is increased to 30-40 mg daily. Although a majority of the studies so far were conducted in south-east Asia where zinc deficiency is common, studies conducted in other parts of the world showed similar results (WHO, 2006).

It was concluded that zinc supplementation, given at a dose of 10–20 mg per day for 10-14 days, is efficacious in significantly reducing severity of diarrhoea and duration of the episode (WHO, 2006).

2.7 Antibiotics Used in Management of Diarrhoea in Children

2.7.1 Cotrimoxazole

Co-trimoxazole is a combined antibacterial preparation with a broad spectrum of antibacterial activity. It is active against most Gram-positive and Gram negative organisms, especially the Enterobacteriaceae. Trimethoprim binds to dihydrofolate reductase and inhibits the reduction of dihydrofolic acid (DHF) to tetrahydrofolic acid (THF). THF is an essential precursor in the thymidine synthesis pathway and interference with this pathway inhibits bacterial folic acid synthesis. Trimethoprim's affinity for bacterial dihydrofolate reductase is several thousand times greater than its affinity for human dihydrofolate reductase. Sulfamethoxazole inhibits dihydrofolate synthetase (aka dihydropteroate synthetase), an enzyme involved further upstream in the same pathway. Trimethoprim and sulfamethoxazole are commonly used in combination

due to their synergistic effects. This drug combination also reduces the development of resistance that is seen when either drug is used alone (Brumfitt and Hamilton-Miller, 1993).

Resistance can develop from alterations in dihydrofolate reductase, bacterial impermeability to the drug, and by overproduction of the dihydrofolate reductase. The most important mechanism of bacterial resistance to trimethoprim clinically is the production of plasmid encoded trimethoprim-resistant forms of dihydrofolate reductase.

2.7.2 Gentamicin

Gentamicin is an aminoglycoside antibiotic composed of a mixture of related gentamicin components and fractions and is used to treat many types of bacterial infections, particularly those caused by Gram-negative organisms (Moulds and Jeyasingham, 2010). The main components of gentamicin include members of the Gentamicin C complex: Gentamicin C1, Gentamicin C1a, and Gentamicin C2 which compose approximately 80% of Gentamicin and have been found to have the highest antibacterial activity. Gentamicin A, B, X, and a few others make up the remaining 20% of Gentamicin and have lower antibiotic activity than the gentamicin C complex (Vydrin, 2003).

The antibacterial actions of Gentamicin involve two possibly synergistic effects (Craig and Stitzel, 2004). First, the positively charged aminoglycoside binds to negatively charged sites on the outer bacterial membrane, thereby disrupting membrane integrity. It is likely that the aminoglycoside-induced bacterial outer membrane degradation accounts for the rapid concentration dependent bactericidal effect of these compounds. Second, Gentamicin bind to various sites on bacterial 30S ribosomal subunits, disrupting the initiation of protein synthesis and inducing errors in the translation of messenger RNA to peptides. They also bind to sites on bacterial 50S ribosomal subunits, although the significance of this binding is uncertain. In

addition, they have a postantibiotic effect; that is, they continue to suppress bacterial regrowth even after removal of the antibiotic from the bacterial microenvironment. It is likely that ribosome disruption accounts for this postantibiotic activity. The postantibiotic effect is characterized by prolonged suppression of bacterial regrowth after the initially high aminoglycoside concentration has fallen to a subinhibitory level. Perhaps resumption of bacterial ribosomal function requires the time-consuming synthesis of new ribosomes after their disruption by aminoglycosides. Postantibiotic effect explains why aminoglycosides can be given in single daily doses despite their short half-life. Penetration of aminoglycosides through the outer bacterial membrane occurs both by outer membrane disruption and by diffusion through outer membrane porins. Penetration through the inner bacterial membrane occurs in two phases (Craig and Stitzel, 2004). The first requires that the cytosol have a negative electron potential and therefore be inhibited by the presence of a low pH. The second phase depends on aerobic bacterial metabolism and therefore will be inhibited by low oxygen tension. The latter two observations are of considerable clinical relevance, since both a low pH and a low oxygen tension frequently occur in bacterial abscesses. Administration of β-lactam antibiotics will reverse the negative effects of both low pH and low oxygen tension on the ability of aminoglycosides to penetrate into bacteria; this ability accounts in part for the synergism that occurs between aminoglycoside and β-lactam antibiotic drugs (Craig and Stitzel, 2004).

The frequency of bacterial aminoglycoside resistance encountered in clinical practice has remained nearly constant over the past two decades (Craig and Stitzel, 2004). Of the three recognized mechanisms of resistance that occur in aerobic Gram-negative bacteria, plasmid-mediated expression of enzymes that acetylate, adenylate, or phosphorylate the aminoglycosides is the most important.

2.7.3 Ciprofloxacin

Ciprofloxacin is a fluoroquinolone, a broad-spectrum antibiotic active against both Gram-positive and Gram-negative bacteria. It functions by inhibiting DNA gyrase, a type II topoisomerase, and topoisomerase IV, enzymes necessary to separate bacterial DNA, thereby inhibiting cell division (Drlica and Zhao, 1997).

There are three known mechanisms of fluoroquinolone resistance. Some types of efflux pumps can act to decrease intracellular quinolone concentration (Morita *et al*., 1998). In Gram-negative bacteria, plasmid-mediated resistance genes produce proteins that can bind to DNA gyrase, protecting it from the action of quinolones. Finally, mutations at key sites in DNA gyrase or topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drug's effectiveness (Robicsek *et al*., 2006). Research has shown that the bacterial protein LexA may play a key role in the acquisition of bacterial mutations giving resistance to quinolones and rifampicin (Cirz *et al*., 2005).

2.7.4 Amoxicillin/Clavulanic Acid Combination

Amoxicillin/Clavulanic acid is an antibacterial combination consisting of amoxicillin and the beta-lactamase inhibitor, clavulanate potassium. Amoxicillin is an analog of ampicillin, derived from the basic penicillin nucleus, 6 aminopenicillanic acid. While Clavulanic acid is produced by the fermentation of *Streptomyces clavuligerus.*

Amoxicillin has a broad spectrum of bactericidal activity against many Gram-positive and Gram-negative microorganisms. It is, however, susceptible to degradation by β-lactamases, and therefore, the spectrum of activity does not include organisms which produce these enzymes.

Clavulanic acid, a β-lactam, structurally related to the penicillins, which possesses the ability to inactivate a wide range of β-lactamase enzymes commonly found in microorganisms resistant to penicillins and cephalosporins. In particular, it has good activity against the clinically important plasmid-mediated β-lactamases frequently responsible for transferred drug resistance.

The combination of amoxicillin and clavulanic acid protects amoxicillin from degradation by β-lactamase enzymes and effectively extends the antibiotic spectrum of amoxicillin to include many bacteria normally resistant to amoxicillin and other β-lactam antibiotics. These two act by interfering with cell wall synthesis.

The final reaction in bacterial cell wall synthesis is a cross-linking of adjacent peptidoglycan (murein) strands by a transpeptidation reaction. In this reaction, bacterial transpeptidases cleave the terminal D-alanine from a pentapeptide on one peptidoglycan strand and then cross-link it with the pentapeptide of another peptidoglycan strand. The cross-linked peptidoglycan (murein) strands give structural integrity to cell walls and permit bacteria to survive environments that do not match the organism's internal osmotic pressure. The β-lactam antibiotics structurally resemble the terminal D-alanyl-D-alanine (D-Ala-D-Ala) in the pentapeptides on peptidoglycan (murein). Bacterial transpeptidases covalently bind the β-lactam antibiotics at the enzyme active site, and the resultant acyl enzyme molecule is stable and inactive. The intact β-lactam ring is required for antibiotic action. The β-lactam ring modifies the active serine site on transpeptidases and blocks further enzyme function. In addition to transpeptidases, other penicillin-binding proteins (PBPs) function as transglycosylases and carboxypeptidases. All of the PBPs are involved with assembly, maintenance, or regulation of peptidoglycan cell wall synthesis. When β-lactam antibiotics inactivate PBPs, the consequence to the bacterium is a structurally weakened cell wall, aberrant morphological form, cell lysis, and death (Craig and Stitzel, 2004).

A number of microorganisms have evolved mechanisms to overcome the inhibitory actions of the β-lactam antibiotics. There are four major mechanisms of resistance: inactivation of the β-lactam ring, alteration of PBPs, reduction of antibiotic access to PBPs, and elaboration of antibiotic efflux mechanisms. Bacterial resistance may arise from one or more than one of these mechanisms (Craig and Stitzel, 2004).

The most important mechanism of resistance is hydrolysis of the β-lactam ring by β-lactamases (penicillinases and cephalosporinases). Many bacteria (*Staphylococcus aureus, Moraxella* (*Branhamella*) *catarrhalis, Neisseria gonorrhoeae,* Enterobacteriaceae, *Haemophilus influenzae,* and *Bacteroides* spp.) produce β-lactamases that hydrolyze penicillins and cephalosporins. The β-lactamases evolved from PBPs and acquired the capacity to bind β-lactam antibiotics, form an acyl enzyme molecule, then deacylate and hydrolyze the β-lactam ring. Some bacteria have chromosomal (inducible) genes for β-lactamases. Other bacteria acquire β-lactamase genes via plasmids or transposons. Transfer of β-lactamase genes between bacterial species has contributed to the proliferation of resistant organisms resulting in the appearance of clinically important adverse consequences. Efforts to overcome the actions of the β-lactamases have led to the development of such β-lactamase inhibitors as clavulanic acid, sulbactam, and tazobactam. They are called suicide inhibitors because they permanently bind when they inactivate β-lactamases. Among the β-lactamase inhibitors, only clavulanic acid is available for oral use. Chemical inhibition of β-lactamases, however, is not a permanent solution to antibiotic resistance, since some β-lactamases are resistant to clavulanic acid, tazobactam, or sulbactam. Enzymes resistant to clavulanic acid include the cephalosporinases produced by *Citrobacter* spp., *Enterobacter* spp., and *Pseudomonas aeruginosa.* An additional mechanism of antibiotic resistance involves an alteration of PBPs. Resistant bacteria, usually Gram-positive organisms, produce PBPs with

low affinity for β-lactam antibiotics. The development of mutations of bacterial PBPs is involved in the mechanism for β-lactam resistance in *Streptococcus pneumoniae, Enterococcus faecium,* and methicillin-resistant *S. aureus* (MRSA). Some Gram-negative bacteria employ a third mechanism of resistance, namely, one that reduces antibiotic access to PBPs. Gram-positive organisms have an exposed peptidoglycan layer easily accessible to β-lactam antibiotics. In contrast, Gram-negative organisms have an outer membrane surrounding the peptidoglycan layer. The Gram-negative outer membrane hinders ingress of large molecules and helps bacteria resist the actions of antibiotics. In susceptible Gram-negative bacteria, protein channels (porins) allow β-lactam antibiotics to traverse the outer membrane and interact with PBPs in the periplasmic space. In resistant bacteria like *P. aeruginosa,* porin mutants impede the β-lactam transfer across the outer membrane. Finally, some Gram-negative organisms demonstrate a fourth mechanism of resistance. For example, strains of *P. aeruginosa* produce xenobiotic efflux pumps to eject antibiotics. Drug efflux mechanisms are associated with multiple drug resistance, including resistance to β-lactam antibiotics. Widespread use of β-lactam antibiotics exerts selective pressure on bacteria and permits the proliferation of resistant organisms. A comparison of current antibiograms with those from previous decades shows an alarming increase in bacterial resistance to β-lactam antibiotics (Craig and Stitzel, 2004).

2.7.5 Erythromycin

Erythromycin is produced from a strain of the actinomycete *Saccharopolyspora erythraea*. It is a macrolide antibiotic that has an antimicrobial spectrum similar to or slightly wider than that of penicillin, and is often prescribed for people who have an allergy to penicillins.

In structure, this macrocyclic compound contains a 14-membered lactone ring with ten asymmetric centers and two sugars (L-cladinose and D-desosamine), making it a compound very difficult to produce via synthetic methods.

Erythromycin displays bacteriostatic activity and bactericidal activity at higher concentrations, but the mechanism is not fully understood. Macrolides bind to the 50S ribosomal subunit of bacteria but not to the 80S mammalian ribosome; this accounts for its selective toxicity. Binding to the ribosome occurs at a site near peptidyltransferase, with a resultant inhibition of translocation, peptide bond formation, and release of oligopeptidyl tRNA. However, unlike chloramphenicol, the macrolides do not inhibit protein synthesis by intact mitochondria, and this suggests that the mitochondrial membrane is not permeable to erythromycin (Craig and Stitzel, 2004).

Resistance to macrolides is generally mediated by methylation of a specific adenine residue in 23S rRNA and is associated with the *erm*(B) gene, which encodes an rRNA methylase and confers resistance to macrolides, lincosamides and streptogramin B antibiotics (MLS_B phenotype), or by active macrolide-specific efflux from cells mediated by a membrane protein encoded by the *mef*(A) gene, which confers resistance to the 14- and 15-membered macrolides (Shortridge *et al*., 1996).

2.7.6 Chloramphenicol

It is an antimicrobial agent originally derived from the bacterium *Streptomyces venezuelae*.

Chloramphenicol (*Chloromycetin*) is a nitrobenzene derivative that affects protein synthesis by binding to the 50S ribosomal subunit and preventing peptide bond formation. It prevents the

attachment of the amino acid end of aminoacyl-tRNA to the A site, hence the association of peptidyltransferase with the amino acid substrate (Craig and Stitzel, 2004).

Chloramphenicol is a broad-spectrum antibiotic that is effective against Gram-positive and Gram-negative bacteria, including *Rickettsia, Mycoplasma,* and *Chlamydia* spp. Chloramphenicol is also effective against most anaerobic bacteria, including *Bacteroides fragilis.* Resistance due to changes in the ribosome binding site results in a decreased affinity for the drug, decreased permeability, and plasmids that code for enzymes that degrade the antibiotic (Craig and Stitzel, 2004).

2.7.7 Ceftriaxone

Ceftriaxone is a third-generation cephalosporin antibiotic. Like other third-generation cephalosporins, it has broad-spectrum activity against Gram-positive and Gram-negative bacteria. In most cases, it is considered to be equivalent to cefotaxime in terms of safety and efficacy.

The *syn*-configuration of the methoxyimino moiety confers resistance to β-lactamase enzymes produced by many Gram-negative bacteria. The stability of this configuration results in increased activity of ceftriaxone against otherwise-resistant Gram-negative bacteria. In place of the easily hydrolysed acetyl group of cefotaxime, ceftriaxone has a metabolically stable thiotriazinedione moiety.

Ceftriaxone inhibits bacterial cell wall synthesis by means of binding to the penicillin-binding proteins (PBPs). Inhibition of PBPs would in turn inhibit the transpeptidation step in peptidoglycan synthesis which is required for bacterial cell walls. Like other cephalosporins,

ceftriaxone is bacteriocidal and exhibits time-dependent killing (Uptodate.com/Ceftriaxone, 2013).

The bactericidal activity of ceftriaxone results from inhibition of cell wall synthesis. Ceftriaxone has a high degree of stability in the presence of beta-lactamases, both penicillinases and cephalosporinases, of Gram-negative and Gram-positive bacteria.

2.8 General Preventive Measures

In a joint publication by United Nations International Children Emergency Fund (UNICEF) and World Health Organization (WHO) (2009), the following prevention packages were recommended for the prevention of Childhood diarrhoea:

2.8.1 Water, sanitation and hygiene

Improving access to safe drinking water and adequate sanitation, as well as promoting good hygiene, are key components in preventing diarrhoea. WHO/UNICEF (2008) report indicated that, in 2006, an estimated 2.5 billion people were lacking improved sanitation facilities. Moreover, nearly 1 in 4 people in developing countries were practicing indiscriminate or open defecation (UNICEF/WHO, 2009). To further compound the problem, children's faeces are often unsafely disposed of in many developing countries. Children's stools tend to carry a higher pathogen load than adults, and many children play in areas in which stools are found (UNICEF/WHO, 2009). Safely disposing of them is therefore critical for reducing the number of diarrhoea cases. Between 1990 and 2006, the proportion of the developing world's population using an improved drinking water source rose from 71 per cent to 84 per cent (UNICEF/WHO, 2009). Still, almost 1 billion people lack access to improved drinking water sources, and many households do not treat or safely store their household water supplies. Rural access to improved

drinking water sources remains low, and many people using an improved source must still walk long distances to fetch water, thereby reducing the amount collected. While coverage is higher in urban areas, population growth presents a growing challenge in further increasing improved drinking water coverage. The lack of improved drinking water sources also tends to curtail personal hygiene practices, including handwashing (UNICEF/WHO, 2009).

2.8.2 Adequate nutrition

Undernourished children are more likely to suffer from diarrhoea and its consequences, which, in turn, increases their chances of worsening nutritional status. About 129 million children under the age of five in the developing world are underweight for their age. Together, Africa and South Asia account for more than 80 per cent of total underweight children (25 per cent and 57 per cent, respectively). About 40 per cent of children under five years of age are stunted in Africa, and nearly half in South Asia (UNICEF/WHO, 2009).

2.8.3 Breastfeeding

Over the past decade, there has been some progress in exclusive breastfeeding rates among infants in the first six months of life across the developing world, and particularly in Africa. Despite these advances, overall level remains low, and only 37 per cent of infants in developing countries are exclusively breastfed for the first six months of life (UNICEF/WHO, 2009).

2.8.4 Micronutrient supplementation

Vitamin A supplementation rates have increased significantly in recent years. Coverage of children aged 6-59 months with at least one dose of vitamin A per year has increased by nearly 50 per cent since 1999. Moreover, between 1999 and 2007, coverage of children considered fully protected by vitamin A – that is, receiving two doses per year – increased nearly fourfold in developing countries (UNICEF/WHO, 2009). Progress was made possible through innovative

strategies that included combined delivery with other high-impact interventions for health and nutrition. Reaching the poorest children and those living in rural areas, who are most at risk of vitamin A deficiency, remains the greatest challenge.

Zinc is important for normal growth and development and for reducing childhood diarrhoea cases. Yet data on improving children's zinc status as a key prevention measure are not available (UNICEF/WHO, 2009).

2.8.5 Immunization

Only a few, mostly high- and middle-income countries include rotavirus vaccine in their routine immunization schedules (UNICEF/WHO, 2009). WHO recently recommended introduction of the vaccine in all routine schedules, and data to monitor its coverage in many countries are expected to follow implementation. Deaths due to measles have declined rapidly in recent years. Between 2000 and 2007, global mortality attributed to measles was down by 74 per cent. This decrease in deaths was generally credited to increase in routine measles immunization coverage, coupled with improvements in follow-up campaigns that provide second opportunities for children to get immunized. However, more work is needed to reach the UNICEF and WHO goal of reducing measles mortality by 90 per cent by 2010 (UNICEF/WHO, 2009).

2.8.6 Mobilization and allocation of resources for diarrhoea control

Diarrhoea remains a leading killer of children, though the tools needed to address it are available and affordable. New resources for child survival must include funding for diarrhoea prevention and treatment. And global initiatives must keep the management of diarrhoea high on the list of priorities for public health resource allocation, including rotavirus vaccination, which has now been recommended for global introduction. At the same time, national and district health

planners should include diarrhoea control in programmes targeting childhood malaria, pneumonia and HIV, and ensure support to accelerate coverage of proven interventions.

2.9 Determination of Microbial Susceptibility to Antimicrobial Agents

2.9.1 Broth Dilution Tests.

One of the earliest antimicrobial susceptibility testing methods was the macrobroth or tube-dilution method (Ericsson and Sherris, 1971). This procedure involved preparing two-fold dilutions of antibiotics (eg, 1, 2, 4, 8, and 16 μg/ml) in a liquid growth medium dispensed in test tubes (Jorgensen and Turnidge, 2007). The antibiotic-containing tubes were inoculated with a standardized bacterial suspension of 1.5×10^5 cfu/ml. Following overnight incubation at 35°C, the tubes were examined for visible bacterial growth as evidenced by turbidity. The lowest concentration of antibiotic that prevented growth represented the minimal inhibitory concentration (MIC). The precision of this method was considered to be plus or minus one two-fold concentration, due in large part to the practice of manually preparing serial dilutions of the antibiotics. The advantage of this technique was the generation of a quantitative result (ie, the MIC). The principal disadvantages of the macro dilution method were the tedious, manual task of preparing the antibiotic solutions for each test, the possibility of errors in preparation of the antibiotic solutions, and the relatively large amount of reagents and space required for each test.

The miniaturization and mechanization of the test by use of small, disposable, plastic "microdilution" trays has made broth dilution testing practical and popular. Standard trays contain 96 wells, each containing a volume of 0.1 ml that allows approximately 12 antibiotics to be tested in a range of 8 two-fold dilutions in a single tray (Jorgensen and Turnidge, 2007). Microdilution panels are typically prepared using dispensing instruments that aliquot precise

volumes of preweighed and diluted antibiotics in broth into the individual wells of trays from large volume vessels. Hundreds of identical trays can be prepared from a single master set of dilutions in a relatively brief period. Few clinical microbiology laboratories prepare their own panels; instead frozen or dried microdilution panels are purchased from one of several commercial suppliers. Inoculation of panels with the standard 5×10^5 cfu/ml is accomplished using a disposable device that transfers 0.01 to 0.05 ml of standardized bacterial suspension into each well of the microdilution tray or by use of a mechanized dispenser. Following incubation, MICs are determined using a manual or automated viewing device for inspection of each of the panel wells for growth (Jorgensen and Turnidge, 2007).

The advantages of the microdilution procedure include the generation of MICs, the reproducibility and convenience of having preprepared panels, and the economy of reagents and space that occurs due to the miniaturization of the test. There is also assistance in generating computerized reports if an automated panel reader is used. The main disadvantage of the microdilution method is some inflexibility of drug selections available in standard commercial panels.

2.9.2 Antimicrobial Gradient Method

The antimicrobial gradient diffusion method uses the principle of establishment of an antimicrobial concentration gradient in an agar medium as a means of determining susceptibility. The Etest is a commercial version available in the United States. It employs thin plastic test strips that are impregnated on the underside with a dried antibiotic concentration gradient and are marked on the upper surface with a concentration scale. As many as 5 or 6 strips may be placed in a radial fashion on the surface of an appropriate 150 mm agar plate that has been inoculated

with a standardized organism suspension like that used for a disk diffusion test. After overnight incubation, the tests are read by viewing the strips from the top of the plate. The MIC is determined by the intersection of the lower part of the ellipse shaped growth inhibition area with the test strip.

The gradient diffusion method has intrinsic flexibility by being able to test the drugs the laboratory chooses. This method is best suited to situations in which an MIC for only 1 or 2 drugs is needed or when a fastidious organism requiring enriched medium or special incubation atmosphere is to be tested (eg, penicillin and ceftriaxone with pneumococci) (Jorgensen *et al*., 1994). Generally, Etest results have correlated well with MICs generated by broth or agar dilution methods (Jorgensen *et al*., 1994) However, there are some systematic biases toward higher or lower MICs determined by the Etest when testing certain organism-antimicrobial agent combinations (Jorgensen *et al*., 1994). This can represent a potential shortcoming when standard MIC interpretive criteria derived from broth dilution testing are applied to Etest MICs that may not be identical (Prakash *et al*., 2008).

2.9.3 Disc Diffusion Test

The disc diffusion susceptibility method is simple and practical and has been well-standardized (CLSI, 2009). The test is performed by applying a bacterial inoculum of approximately $1\text{-}2\times10^{8}$ cfu/ml to the surface of a large (150 mm diameter) or a small (90 mm) Mueller-Hinton agar plate. Up to 12 (in a 150 mm plate) or 6 (in a 90 mm plate) commercially-prepared, fixed concentration, paper antibiotic discs are placed on the inoculated agar surface and allowed to stand for 15-30 minutes . Plates are incubated for 16–24 h at 35°C prior to determination of results. The zones of growth inhibition around each of the antibiotic disks are measured to the

nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards or NCCLS) or those included in the US Food and Drug Administration (FDA)-approved product inserts for the disks. The results of the disk diffusion test are "qualitative," in that a category of susceptibility (ie, susceptible, intermediate, or resistant) is derived from the test rather than an MIC. However, some commercially-available zone reader systems claim to calculate an approximate MIC with some organisms and antibiotics by comparing zone sizes with standard curves of that species and drug stored in an algorithm (Nijs *et al*., 2003).

The advantages of the disc method are the test simplicity that does not require any special equipment, the provision of categorical results easily interpreted by all clinicians, and flexibility in selection of disks for testing. It is the least costly of all susceptibility methods. The disadvantages of the disc test are the lack of mechanization or automation of the test. Although not all fastidious or slow growing bacteria can be accurately tested by this method, the disk test has been standardized for testing streptococci, *Haemophilus influenzae*, and *N. meningitidis* through use of specialized media, incubation conditions, and specific zone size interpretive criteria (CLSI, 2009).

2.9.4 Automated Instrument Systems

Use of instrumentation can standardize the reading of end points and often produce susceptibility test results in a shorter period than manual readings because sensitive optical detection systems allow detection of subtle changes in bacterial growth. There are 4 automated instruments

presently cleared by the FDA for use in the United States. Three of these can generate rapid (3.5–16 h) susceptibility test results, while the fourth is an overnight system (Richter and Ferraro, 2007).

The MicroScan WalkAway (Siemens Healthcare Diagnostics) is a large self-contained incubator/reader device that can incubate and analyze 40–96 microdilution trays. The WalkAway utilizes standard size microdilution trays that are hydrated and inoculated manually and then placed in one of the incubator slots in the instrument. The instrument incubates the trays for the appropriate period, examining them periodically with either a photometer or fluorometer to determine growth development. Gram-negative susceptibility test panels containing fluorogenic substrates can be read in 3.5–7 h. Separate Gram-positive and Gram-negative panels read using turbidimetric end points are ready in 4.5–18 hours.

The BD Phoenix Automated Microbiology System (BD Diagnostics) has a large incubator reader with a capacity to process 99 test panels that contain 84 wells devoted to antibiotic doubling dilutions and are inoculated manually. The Phoenix monitors each panel every 20 min using both turbidometric and colorimetric (oxidation-reduction indicator) growth detection. Test panels for Gram-negative, Gram-positive, *S. pneumoniae*, β-hemolytic, and viridans group streptococci are available. MIC results are generated in 6–16 h.

The Vitek 2 System (bioMérieux) is highly automated and uses very compact plastic reagent cards (credit card size) that contain microliter quantities of antibiotics and test media in a 64-well format. The Vitek 2 employs repetitive turbidimetric monitoring of bacterial growth during an abbreviated incubation period. The instrument can be configured to accommodate 30–240 simultaneous tests. The susceptibility cards allow testing of common, rapidly growing Gram-

positive, and gram-negative aerobic bacteria, and *S. pneumoniae* in a period of 4–10 h. An older, less automated, Vitek 1 System is still used in some laboratories. The system is more limited with a 45-well card and does not include *S. pneumoniae* .

The Sensititre ARIS 2X (Trek Diagnostic Systems) is an automated, overnight, incubation and reading system with a 64-panel capacity. The test panels are standard 96-well microdilution plates that can be inoculated with a Sensititre Autoinculator. Growth is determined by fluorescence measurement after 18–24 h of incubation. Test panels are available for Gram-positive and Gram-negative bacteria, *S. pneumoniae*, *Haemophilus* species, and non fermentative Gram-negative bacilli.

2.9.5 Interpretation of Susceptibility Test Results

The results of a susceptibility test must be interpreted by the laboratory prior to communicating a report to a patient's physician. Optimal interpretation of MICs requires knowledge of the pharmacokinetics of the drug in humans, and information on the likely success of a particular drug in eradicating bacteria at various body sites (CLSI, 2008). This is best accomplished by referring to an expert source such as the CLSI, which publishes interpretive criteria for MICs of all relevant antibiotics for most bacterial genera. Indeed, both MIC values and disc diffusion zone diameters must be interpreted using a table of values that relate to proven clinical efficacy of each antibiotic and for various bacterial species. The CLSI zone size and MIC interpretive criteria are established by analysis of 3 kinds of data:

- Microbiologic data, including a comparison of MICs and zone sizes on a large number of bacterial strains, including those with known mechanisms of resistance that have been defined either phenotypically or genotypically;

- Pharmacokinetic and pharmacodynamic data; and
- Clinical studies results (including comparisons of MIC and zone diameter with microbiological eradication and clinical efficacy) obtained during studies prior to FDA approval and marketing of an antibiotic (CLSI, 2008).

A "susceptible" result indicates that the organism should respond to therapy with that antibiotic using the dosage recommended normally for that type of infection and species (CLSI, 2008). Conversely, an organism with a MIC or zone size interpreted as "resistant" should not be inhibited by the concentrations of the antibiotic achieved with the dosages normally used with that drug (CLSI, 2008). An "intermediate" result indicates that a microorganism falls into a range of susceptibility in which the MIC approaches or exceeds the level of antibiotic that can ordinarily be achieved and for which clinical response is likely to be less than with a susceptible strain. Exceptions can occur if the antibiotic is highly concentrated in a body fluid such as urine, or if higher than normal dosages of the antibiotic can be safely administered (eg, some penicillins and cephalosporins). At times, the "intermediate" result can also mean that certain variables in the susceptibility test may not have been properly controlled, and that the values have fallen into a "buffer zone" separating susceptible from resistant strains (CLSI, 2008). Generally, reporting of a category result of susceptible, intermediate, or resistant provides the clinician with the information necessary to select appropriate therapy. Reporting of MICs could aid a physician in selecting from among a group of similar drugs for therapy.

2.9.6 Bacterial Resistance to Antimicrobial Agents

Antibiotic resistance is the ability of a bacterium or other microorganism to survive and reproduce in the presence of antibiotic doses that were previously thought effective against them.

Pathogens resistant to multiple antibiotics are considered multidrug resistant (MDR) or, more colloquially, superbugs. Microbes, rather than people, develop resistance to antibiotics (CDC, 2009).

Antibiotic resistance is a serious and growing phenomenon in contemporary medicine and has emerged as one of the pre-eminent public health concerns of the 21st Century, in particular as it pertains to pathogenic organisms (the term is especially relevant to organisms that cause disease in humans). In the simplest cases, drug-resistant organisms may have acquired resistance to first-line antibiotics, thereby necessitating the use of second-line agents. Typically, a first-line agent is selected on the basis of several factors including safety, availability, and cost; a second-line agent is usually broader in spectrum, has a less favourable risk-benefit profile, and is more expensive or, in dire circumstances, may be locally unavailable. In the case of some MDR pathogens, resistance to second- and even third-line antibiotics is, thus, sequentially acquired, a case quintessentially illustrated by *Staphylococcus aureus* in some nosocomial settings. Some pathogens, such as *Pseudomonas aeruginosa*, also possess a high level of intrinsic resistance (Zainuddin and Dale,1990). It may take the form of a spontaneous or induced genetic mutation, or the acquisition of resistance genes from other bacterial species by horizontal gene transfer via conjugation, transduction, or transformation. Many antibiotic resistance genes reside on transmissible plasmids, facilitating their transfer. Exposure to an antibiotic naturally selects for the survival of the organisms with the genes for resistance. In this way, a gene for antibiotic resistance may readily spread through an ecosystem of bacteria. Antibiotic-resistance plasmids frequently contain genes conferring resistance to several different antibiotics. This is not the case for *Mycobacterium tuberculosis*, the bacteria that causes tuberculosis, since evidence is lacking

for whether these bacteria have plasmids. Also *M. tuberculosis* lack the opportunity to interact with other bacteria in order to share plasmids (Zainuddin and Dale,1990).

Genes for resistance to antibiotics, like the antibiotics themselves, are ancient (D'Costa *et al*., 2011). However, the increasing prevalence of antibiotic-resistant bacterial infections seen in clinical practice stems from antibiotic use both within human medicine and veterinary medicine. Any use of antibiotics can increase selective pressure in a population of bacteria to allow the resistant bacteria to thrive and the susceptible bacteria to die off. As resistance towards antibiotics becomes more common, a greater need for alternative treatments arises. However, despite a push for new antibiotic therapies, there has been a continued decline in the number of newly approved drugs (Donadio *et al*., 2010). Antibiotic resistance therefore poses a significant problem.

The growing prevalence and incidence of infections due to MDR pathogens is epitomised by the increasing number of familiar acronyms used to describe the causative agent and sometimes the infection; of these, MRSA is probably the most well-known, but others including Vancomycin-intermediate *S. aureus*(VISA), Vancomycin-resistant *S. aureus*(VRSA), Extended spectrum beta-lactamase(ESBL), Vancomycin-resistant *Enterococcus* (VRE), and Multidrug-resistant *A. baumannii* (MRAB) are prominent examples.

2.9.7 Mechanisms of Antibiotics Resistance

Antibiotic resistance can be a result of horizontal gene transfer (Ochiai *et al*., 1959) and also of unlinked point mutations in the pathogen genome at a rate of about 1 in 10^8 per chromosomal replication. The antibiotic action against the pathogen can be seen as an environmental pressure.

Those bacteria with a mutation that allows them to survive live to reproduce. They then pass this trait to their offspring, which leads to the evolution of a fully resistant colony.

The four main mechanisms by which microorganisms exhibit resistance to antimicrobials are:

1. Drug inactivation or modification: for example, enzymatic deactivation of penicillin G in some penicillin-resistant bacteria through the production of β-lactamases.
2. Alteration of target site: for example, alteration of PBP—the binding target site of penicillins—in MRSA and other penicillin-resistant bacteria
3. Alteration of metabolic pathway: for example, some sulfonamide-resistant bacteria do not require para-aminobenzoic acid (PABA), an important precursor for the synthesis of folic acid and nucleic acids in bacteria inhibited by sulfonamides, instead, like mammalian cells, they turn to using preformed folic acid.
4. Reduced drug accumulation: by decreasing drug permeability and/or increasing active efflux (pumping out) of the drugs across the cell surface (Li and Nikadio, 2009).

Antibiotic resistance can also be introduced artificially into a microorganism through laboratory protocols, sometimes used as a selectable marker to examine the mechanisms of gene transfer or to identify individuals that absorbed a piece of DNA that included the resistance gene and another gene of interest. A recent study demonstrated that the extent of horizontal gene transfer among *Staphylococcus* is much greater than previously expected—and encompasses genes with functions beyond antibiotic resistance and virulence, and beyond genes residing within the mobile genetic elements (Chan *et al*., 2011).

CHAPTER THREE

3.0 Materials and Methods

3.1 Materials

3.1.1 Culture Media

MacConkey Agar (Becton Dickson Microbiology Systems Cockeyville, USA), Eosin-Methylene Blue Agar (Micro Master, India), Nutrient Agar (Titan Biotech Limited, India), Mueller-Hinton Agar (Micro Master, India), Simmon's Citrate Agar, Peptone water MR-VP broth, Lactose broth, Nutrient broth (Oxoid Ltd, Basingstoke, England).

3.1.2 Reagents and Diagnostics

Kovac's Reagent, Methyl Red, α naphthol, Potassium hydroxide, Concentrated Sulphuric acid, Dihydrate barium chloride, Ethidium bromide, Diarrhoeagenic *E.coli* Primer (Inqaba Biotech, Pretoria, South Africa) Agarose (Cleaver Scientific Ltd.) etc.

3.1.3 Equipment

Incubator (Gallenkamp), Electrophoresis tank (BioRad), Autoclave (Gallenkamp), Molecular Imager (BioRad), Thermocycler/PCR machine (Applied Biosystems) Centrifuge (Sigma), Electrophoresis Machine (BioRad) etc.

3.1.4 Antibiotic Discs

Cotrimoxazole (25 µg), Gentamicin (10 µg), Ciprofloxacin (5 µg), Amoxicillin/Clavulanic acid (30 µg), Erythromycin (15 µg), Chloramphenicol(30 µg), Amoxicillin (10 µg) and Ceftriaxone (30 µg) (Oxoid Ltd, Basingstoke, England).

3.2 Methods

3.2.1 Study area and population

a. Study Population

The samples for this study were collected from Ahmadu Bello University Teaching Hospital, Zaria (including Institute of Child Health, Banzazzau) , after ethical clearance. The samples were collected for a period of six months (November, 2013 to April, 2014) from children (both male and female) with incidents of diarrhoea under the age of five (5) years, after obtaining informed consent from their parents or their guardians/attendants.

b. Inclusion Criteria

- The study was limited to children of 0-5 years of age in Zaria within the study period
- Only diarrhoea stool samples were analysed in the study.
- Only samples from patients attending Ahmadu Bello University Teaching Hospital, Shika-Zaria were used.

c. Exclusion Criteria

- Samples outside Ahmadu Bello University Teaching Hospital, Shika-Zaria were not used.
- Non diarrheic stool samples were not analysed.
- Diarrhoeic patients above five (5) years were not included.

3.2.2 Sample Collection and Treatment

The stool samples were cultured within 4 hours of collection. About one gram of stool sample was suspended in sterile nutrient broth and incubated at 37^{o}C for 24 hours. The concentrated isolates from the nutrient broth were then used to inoculate McConkey agar plate and incubated at 37^{o}C for 24 hours. Colonies that showed typical characteristics of *E. coli* morphology were

subcultured into Eosin-Methylene blue agar and incubated at 37^{o}C for 24 hours. The colonies that showed green metallic sheen, were further subjected to biochemical tests for confirmation.

3.2.3 Biochemical Tests

a. Indole Test

Testing for indole production is important in the identification of Enterobacteria especially *E. coli* which is indole positive. The test is to determine the ability of an organism to break down the amino acid tryptophan with the release of indole, pyruvic acid and ammonia.

The test organism was suspended in a bijou bottle containing 5 ml of sterile peptone water and incubated at 37^{0}C for 48 hours.

Five drops of Kovac's reagent were added to the bijou bottle and shaken gently. A positive reaction was indicated by the development of a red colour in the surface layer within one minute. In the negative reaction, the indole reagent retained its yellow colour.

b. Methyl Red - Voges-Proskauer (MR-VP) Test

The MR-VP is used to distinguish between mixed acid fermenters and is useful in classifying members of the Enterobacteriaceae.

The MR-VP broth contains glucose. The amount of acid that results from fermentative breakdown of glucose can vary widely depending on the pathway employed. If a pathway chosen is such that a large quantity of acid is produced, the pH of the medium would fall below 4.2 and when methyl red indicator is added, the medium would turn red. If a pathway is chosen which results in the accumulation of small quantities of acid, though the medium may still be acidic, at the end of 48 hours incubation, the pH is not low enough, (i.e. below 4.2), to change

methyl red from yellow to red. Among the Enterobacteriaceae, the VP test is most useful in separating members of the Klebsielleae from all the other tribes in the Family. With few exceptions, members of enterobacteria other than members of Klebsielleae are negative.

Five (5) ml of MR-VP broth were inoculated and incubated at 37^0C for 48 hours. After incubation, 2.5 ml of the culture were transferred to a small serological tube. To this small quantity, 2-3 drops of methyl red were added, and the colour change was noted. To the rest of the culture (2.5 ml) in the original tube, 0.6 ml of 5% α naphthol was added and shaken followed by addition of 0.2 ml of 40% potassium hydroxide and shaken, and the colour change was noted within 5 minutes. A no colour change indicates a VP negative test result.

c. Citrate Utilization Test

The Simmon's citrate agar medium is used to identify organisms that are able to utilize citrate as its only source of carbon.

The test organism was inoculated onto citrate agar slant in a bijou bottle and incubated at 37^0C for 24-48 hours. The development of a bright blue colour indicate a positive reaction. No change in colour indicate a negative citrate test.

d. Eijkmann Test

The organism was further confirmed using Eijkmann test. This is the fermentation of lactose and gas production at 44.5^{o}C. The organism was inoculated in to a sterile lactose broth containing inverted Durham tube and incubated at 44.5^{o}C for 24 to 48 hours. The development of turbidity and gas production inside the Durham tube confirmed the presence of *E. coli.*

3.2.4 Preparation of Barium Sulphate Standard (McFarland 0.5)

One percent v/v solution of sulphuric acid was prepared by adding 1 ml of concentrated sulphuric acid to 99 ml of water. One percent w/v solution of barium chloride was then prepared by dissolving 0.5 g of dihydrate barium chloride ($Bacl_2.2H_20$) in 50 ml of distilled water. A 0.6 ml of the barium chloride solution was added to 99.4 ml of the sulphuric acid solution and mixed. Small volume of the turbid solution was then transferred to a screw cap bottle of the same types as used for preparing the test and control inocula (Cheesbrough, 2000).

3.2.5 Inoculum Preparation

The inoculum was prepared by picking 3-5 parts of colonies of the test organism with a sterile wire loop. This was suspended in a sterile peptone water and incubated up to two hours to allow organisms reach their log phase in growth. This was then diluted to match the turbidity standard (McFarland 0.5) which contains approximately $1.5x10^8$ cfu/ml (McFarland, 1907).

3.2.6 Antibacterial Susceptibility Testing

Susceptibility testing was performed by disc diffusion method using modified Kirby-Bauer sensitivity testing techniques (Cheesbrough, 2000). A sterile swab was dipped into the standardized bacterial cell suspension and used to evenly inoculate the entire surface of sterile Mueller-Hinton agar plate. After the agar surface has dried for about five minutes, the appropriate antibiotic discs were placed on it with a pair of sterilized forceps. The plates were allowed to stand at room temperature for 30 minutes before they were placed in a 37^0C incubator for 16-18 hours (Barry, 1986). The diameters of the zones of inhibition were measured to the nearest millimeters according to CLSI (Cheesbrough, 2000). The antibiotic agents that were used

include Co-trimoxazole, Gentamicin, Ceftriaxone, Augmentin (Amoxicillin + Clavulanic acid), Chloramphenicol, Ciprofloxacin, Amoxicillin and Erythromycin.

3.2.7 Determination of Multiple Antibiotic Resistance (MAR) Index

The multiple antibiotic resistance (MAR) index was determined for each isolate as shown in the equation below. MAR index is the number of antibiotic(s) to which the organism is resistant divided by the total number of antibiotics tested (Paul *et al*., 1997).

$$\text{MARI} = \frac{\text{Number of Antibiotic (s) to which isolate was resistant}}{\text{Total number of antibiotics tested}}$$

3.2.8 Test for detection of β-lactamases

The tube iodometric method as described by Catlin, (1975) was adopted.

Hydrolysis of penicillin yields penicilloic acid, which reduces iodine, decolourising starch-iodine complex. This reaction can be exploited to detect β-lactamase activity in tubes.

Benzylpenicillin, 6 g/l in 0.1 M phosphate buffer pH 6.0, was distributed in 0.1 ml quantities in tubes. Bacterial colonies from agar (not broth) were suspended in these solutions until they were heavily turbid (c. 10^9 cfu/ml). The suspensions were held at room temperature for 30-60 min, and 20 µl of 1% (w/v) soluble starch in distilled water were added, followed by 20 µl of 2% (w/v) iodine in 53% (w/v) aqueous potassium iodide. β-lactamase activity was indicated by decolourisation of the iodine within 5 minutes (Catlin, 1975).

3.2.9 Molecular Characterization of the *E. coli* Pathotypes

This experiment was carried out at Molecular Diagnostic Laboratory, Veterinary Teaching Hospital, Ahmadu Bello University, Zaria, Nigeria.

3.2.9.1 Genomic DNA Extraction

All isolates were inoculated into 5 ml Luria-Bertani liquid medium and incubated for 24 hrs at 37^{o}C. DNA extraction was carried out using the ZR Fungal/Bacterial DNA MiniPrep™ ZRD6005 (Zymo research, CA, USA) as follows:

1. About 1.5ml of the bacterial cell suspension was added into a microcentrifuge tube and centrifuged at 10,000xg for 1 minute. It was then decanted and 400µl of lysis buffer was added to the pellets. This was incubated at 70^{0}c for 30mins and then centrifuged at 10,000 xg for 1 minute.

2. The pellets were then transferred into a Zymo-Spin™ IIC Column in a new collection tube and centrifuged at 10,000 x g for 1 minute.

3. Then 200 µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC Column in a Collection Tube and centrifuge at 10,000 x *g* for 1 minute.

4. This was followed by adding 500 µl Fungal/Bacterial DNA Wash Buffer to the Zymo-Spin™ IIC Column and centrifuge at 10,000 x *g* for 1 minute.

5. The Zymo-Spin™ IIC Column was transfered to a clean 1.5 ml microcentrifuge tube and added 100 µl DNA Elution Buffer directly to the column matrix, and centrifuged at 10,000 x *g* for 30 seconds to elute the DNA. This was then used as template for the PCR

3.2.9.2 Identification of the E. coli Pathotypes

A multiplex Polymerase Chain Reaction (PCR) technique was used to differentiate the diarrhoeagenic *Escherichia coli* pathotypes as described by Persson *et al*., 2007. PCRs were performed in a total reaction volume of 25 µL containing 1× PCR buffer (50 mM Tris-HCl, 10

mM KCl, 5 mM $(NH4)_2SO4$, pH 8.3), 2.6 mM $MgCl_2$, 260 lM each of dATP, dCTP and dGTP, 520 lM dUTP, 0.15 U of UNG (Applied Biosystems, Foster City, CA, USA), 1.25 U of Taq polymerase (FastStart; Roche Diagnostics), and the 18 primers (Inqaba biotec, Pretoria, South Africa). These included a primer pair for the 16S rDNA gene as a positive internal PCR control. (Table 2). Template volumes were 7µL when PCRs were performed with extracted DNA from the *E. coli.*

Amplification conditions were comprised of 94^0C for 6 min (initial template denaturation), followed by 35 cycles at 94^0C for 50s (final denaturation), primer annealing at 57^0C for 40s, primer extension at 72^0C for 50s, and finally at 72^0C for 3 minutes (final extension).

3.2.9.3 Agarose Gel Electrophoresis

Amplicons were analysed by electrophoresis on agarose 1.5% w⁄v gels prepared under standard conditions, followed by staining with 20 µl of ethidium bromide. The gel was then poured onto a mini horizontal gel electrophoresis tank and the casting combs were inserted and allowed to solidify. The casting combs were then carefully removed after the gel had completely solidified, TAE electrophoresis buffer was then added to the reservoir until the buffer just covered the agarose gel. 25 µl of the sample (Amplicons) was then loaded onto the wells of the gel, the electrophoresis gel set up was then covered and the electrodes connected. This was allowed to run for 30 minutes at 120 v. At the completion of the electrophoresis, the gel was removed from the buffer and observed under a Trans-illuminator UV light of wavelength 302 nm. This was then photographed with a Polaroid camera and documented using gel electrophoresis documentation system.

Table 2: Gene targets, primer sequences for the multiplex PCR

Primer	Gene target	Virulence factor/gene	Sequence (5'-)	Pathotypes	Amplicon size
StFh	Human estA	STIh	TTTCGCTCAGGATGCTAAACCAG	ETEC	151 bp
StRh			CAGGATTACAACACAATTCACAGCAGTA		
StFp	Porcine estA	STIp	CTTTCCCCTCTTTTAGTCAGTCAACTG	ETEC	160 bp
StRp			CAGGATTACAACAAAGTTCACAGCAG		
PS1	eltA	LTI	AAACCGGCTTTGTCAGATATGATGA		
PS2			TGTGCTCAGATTCTGGGTCTCCT	ETEC	479 bp
PS3	vtx1	VT1	GTTTGCAGTTGATGTCAGAGGGA	VTEC	260 bp
PS4			CAACGAATGGCGATTTATCTGC		
PS5	vtx2	VT2	GCCTGTCGCCAGTTATCTGACA		
PS6			GGAATGCAAATCAGTCGTCACTC	VTEC	420 bp
PS7	eae	Intimin	GGYCAGCGTTTTTTCCTTCCTG		
PS8			TCGTCACCARAGGAATCGGAG	EPEC	377 bp
PS9	ipaH	IpaH	TTGACCGCCTTTCCGATACC		
PS10			ATCCGCATCACCGCTCAGAC	EIEC	647 bp
PS11	aap	antiaggregation protein	CTTTTCTGGCATCTTGGGT		
PS12		(dispersin)	GTAACAACCCCTTTGGAAGT	EAEC	232 bp
PS13	16S rDNA	16S rDNA	GGAGGCAGCAGTGGGGAATA		
PS14			TGACGGGCGGTGTGTACAAG	CTRL	1062 bp

These Primer sequences identifies only five (5) *E. coli* Pathotypes.

R = A or G, Y = C or T, CTRL=CONTROL

CHAPTER FOUR

4.0 Results

4.1 Isolation and Identification of *E. coli*

A total of 100 isolates of *E. coli* were obtained from 222 paediatric patients during the period of the study. Isolates that showed pink red on MacConkey agar, single rod-like shapes on microscopy, greenish metallic sheen on Eosin-Methylene blue agar (EMB (+)), Red ring on indole test (+), Red colour in Methyl Red (+), Colourless in Voges-Proskauer test (-), Green colour (no growth) on Simmon's citrate agar (-), Turbidity and gas production in Eijkmann (+) were considered as *Escherichia coli*.

4.2 Susceptibility of the Isolates to Some Antibiotics

The summary of the Antibiotic Susceptibility Tests is shown in Table 3 Using the CLSI (2008) Interpretative Chart.

Table 3: Summary of Antibiotic Susceptibility Tests

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
1	1	R	S	S	S	R	S	R	S
2	2	R	S	S	S	R	R	R	S
3	3	S	S	S	S	R	S	S	S
4	5	R	R	R	I	R	R	R	R
5	7	I	S	I	S	R	I	S	S
6	8	R	S	R	S	R	S	R	S
7	10	R	S	R	S	R	S	R	S
8	12	R	S	S	I	R	S	R	S
9	13	S	S	S	R	R	S	R	S
10	17*	R	R	S	S	R	R	R	S
11	17**	S	S	S	S	I	S	S	S
12	20	R	S	I	I	R	R	R	R
13	22	S	S	S	S	R	S	S	S
14	23	R	S	S	I	R	R	R	R
15	26	R	R	R	I	R	R	R	R
16	28*	R	S	R	S	R	S	R	S
17	28**	S	S	S	S	I	S	S	S
18	31	S	S	R	S	R	S	S	S
19	33	R	S	S	R	R	R	R	S
20	34	R	S	R	R	R	R	R	S
21	35	R	R	I	R	R	S	R	R
22	36	R	S	R	S	R	S	R	S
23	37	R	S	S	R	R	R	R	S
24	40	R	S	R	S	R	R	R	S
25	41	R	S	S	S	I	S	R	R
26	42	R	S	S	R	R	S	R	S

Table 3 cont'd.

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
27	44	S	S	S	S	R	S	S	S
28	45	R	R	S	S	I	R	R	R
29	46	R	I	I	S	R	S	R	R
30	50	R	S	S	S	R	R	R	S
31	51	R	S	S	S	R	S	R	S
32	52	R	S	S	R	R	S	R	S
33	53	R	S	S	S	I	R	R	S
34	58	S	S	S	S	R	S	S	S
35	59	R	S	S	S	I	S	R	S
36	64	R	S	S	S	I	S	R	S
37	66	R	I	I	S	R	S	R	R
38	69	R	S	S	S	I	S	R	S
39	73	R	R	S	R	R	S	R	R
40	78	R	S	S	S	R	S	R	S
41	83	R	R	S	R	R	S	R	R
42	84	R	S	S	S	I	S	R	S
43	85	R	S	S	R	R	S	R	S
44	87	R	S	R	R	R	I	R	R
45	93	R	R	R	S	R	R	R	R
46	95	S	S	S	R	R	S	R	S
47	100	R	S	S	S	R	S	R	S
48	101	R	S	S	S	R	S	R	S
49	102	R	S	S	S	I	S	R	S

Table 3 cont'd.

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
50	105	R	R	S	S	R	S	R	S
51	106	R	S	S	I	R	R	R	S
52	107	S	S	S	S	R	S	S	S
53	111	R	I	S	S	R	S	S	I
54	115	R	R	I	I	R	R	R	I
55	116	R	R	S	I	R	R	R	S
56	120	R	S	S	I	R	R	R	R
57	124	R	S	S	S	R	S	R	S
58	128	R	R	I	R	R	S	R	I
59	132	R	S	S	S	I	R	R	S
60	133	R	S	S	S	I	R	R	S
61	136	S	S	S	R	R	S	R	S
62	137	R	S	I	S	R	S	R	S
63	138	S	S	S	S	R	S	S	S
64	139	R	S	S	R	R	S	R	S
65	140	R	S	S	I	R	S	R	S
66	145	R	S	S	I	R	R	R	S
67	148	R	R	R	R	R	R	R	R
68	151	R	S	S	S	R	S	R	S
69	153	R	S	S	R	R	S	R	S
70	154	R	S	I	S	R	R	R	S
71	160	R	S	S	S	R	S	R	S
72	161	R	R	R	I	R	R	R	R

Table 3 cont'd.

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
73	163	R	S	S	I	R	S	R	S
74	164	R	S	R	R	R	R	R	S
75	167*	S	S	S	S	I	S	R	S
76	167**	S	S	S	S	R	S	S	S
77	168	R	R	S	I	R	R	R	S
78	169	R	R	R	I	R	R	R	S
79	174	R	I	S	S	S	S	S	I
80	175	R	S	S	S	R	R	R	S
81	177	R	S	S	I	R	R	R	S
82	178	R	R	R	R	R	R	R	R
83	180*	R	S	R	S	R	S	R	S
84	180**	R	S	R	S	R	S	R	S
85	181	R	S	S	S	R	R	R	S
86	184	R	S	R	R	R	R	R	S
87	191	R	S	S	S	R	S	R	S
88	192	R	S	S	S	I	S	S	S
89	196	R	R	R	I	R	R	R	R
90	197	R	S	R	S	R	S	R	S
91	198*	R	S	S	S	R	R	R	S
92	198**	R	R	S	S	R	R	R	S
93	201	R	S	S	S	I	S	R	S
94	203	R	S	S	S	I	S	R	S
95	212	R	I	S	R	R	R	R	R

Table 3 cont'd.

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
96	214	R	R	I	R	R	R	R	R
97	215	R	I	S	R	R	R	R	R
98	216	R	S	R	S	I	R	R	R
99	218	R	R	S	S	I	R	R	R
100	222	R	R	R	R	R	S	R	S

Key: SXT= COTRIMOXAZOLE, CN= GENTAMICIN, CIP= CIPROFLOXACIN, AMC= AMOXICILLIN/CLAVULANIC ACID, E= ERYTHROMYCIN, C= CHLORAMPHENICOL, AML= AMOXICILLIN, CRO= CEFTRIAXONE

R= RESISTANT, S= SENSITIVE, I= INTERMEDIATE.

*/**= HETERORESISTANT ISOLATES

Some examples of the susceptible and resistant isolates are as shown in Plate I, II, and III.



Plate I: Multidrug Resistant *E. coli* showing intermediate susceptibility only to Amoxicillin/Clavulanic acid (AMC) left.

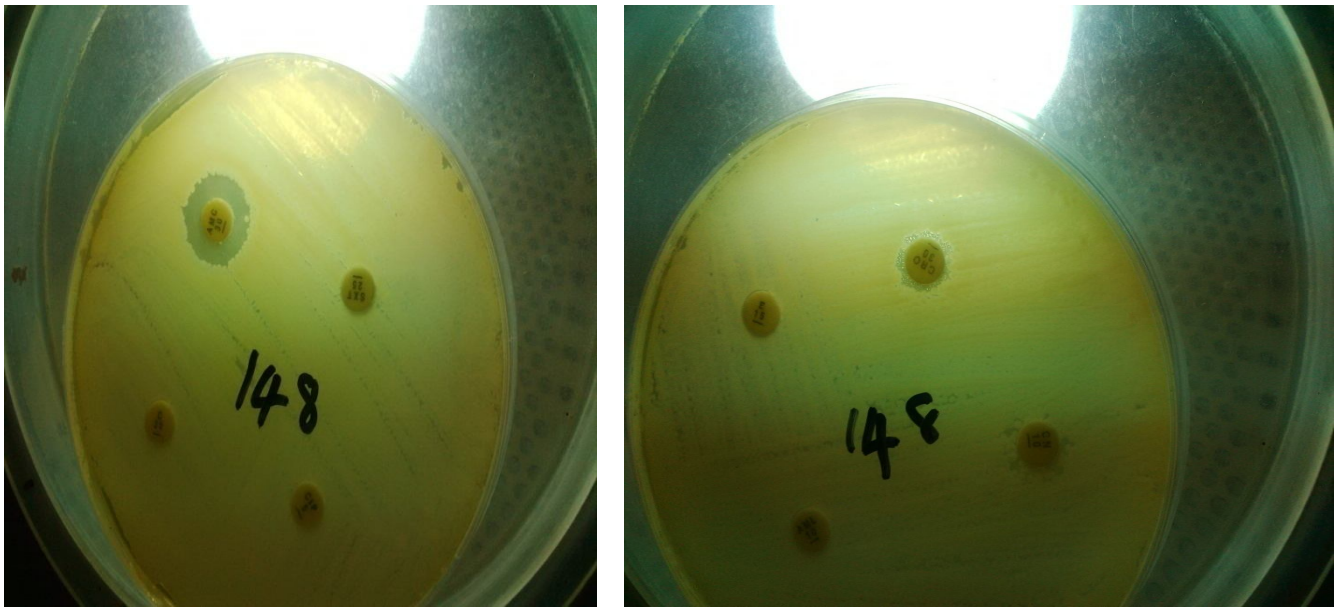
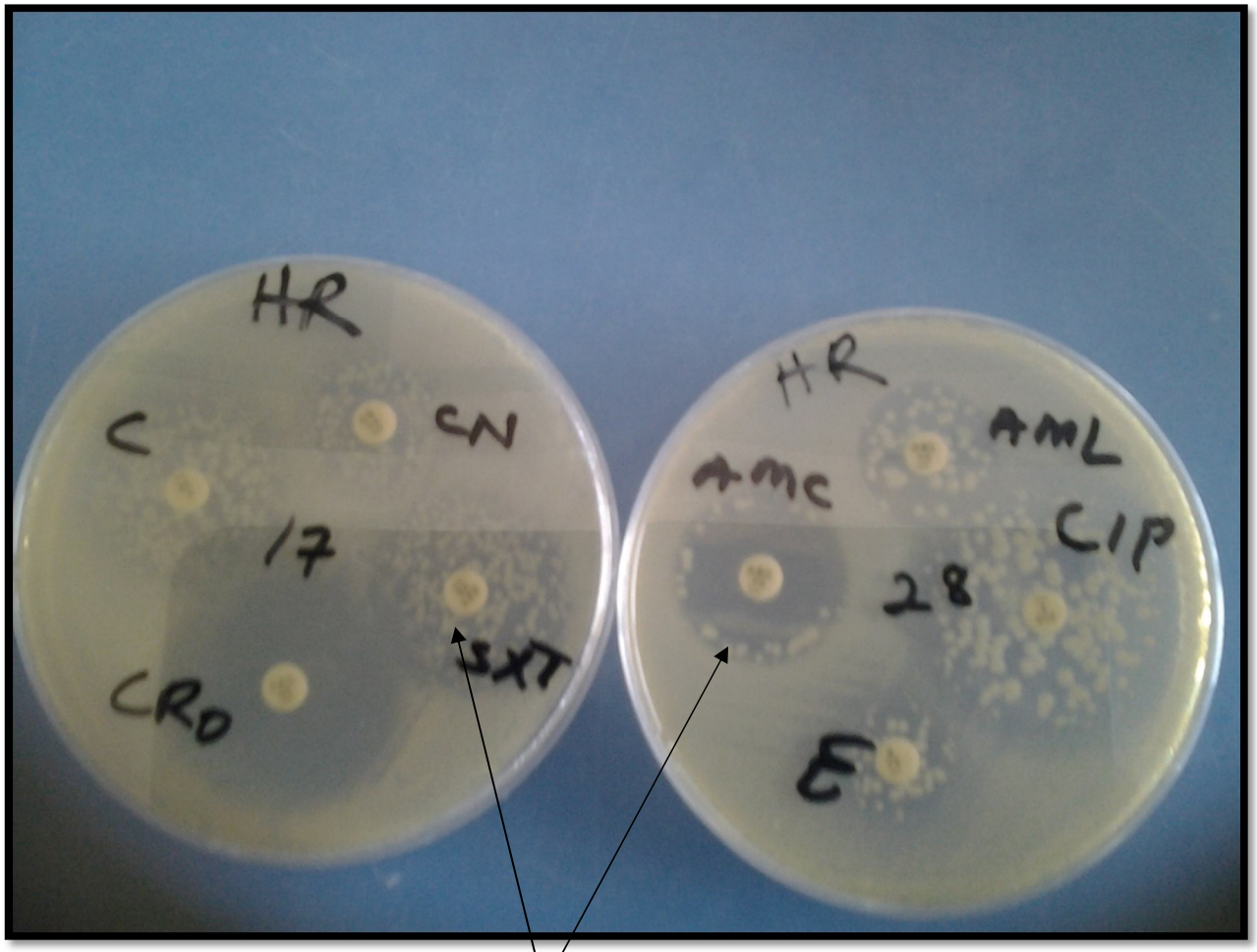


Plate II: Multidrug Resistant *E. coli* showing resistance to all the antibiotics used



Heteroresistant *E. coli* isolates

Plate III: Heteroresistant (HR) *E. coli* isolates showing resistance to Chloramphenicol(C), Gentamicin (CN), Cotrimoxazole (SXT) (Left) and Amoxicillin/Clavulanic acid(AMC), Amoxicillin (AML), Ciprofloxacin (CIP), Erythromycin (E) Right

The summary of the susceptibility test results is as shown in Table 4

Table 4: Summary of Percentage Antibiotic Susceptibility of *E.coli* Isolates From Diarrheic Children in Zaria, Nigeria to 8 Commonly Used Antibiotics.

S/No.	Antibiotics/ Disc potency	Resistant(%)	Intermediate(%)	Sensitive(%)
1	Cotrimoxazole (25µg)	85	1	14
2	Gentamicin (10µg)	22	6	72
3	Ciprofloxacin (5µg)	23	10	67
4	Amoxicillin-Clavulanic acid (30µg)	24	17	59
5	Erythromycin (15µg)	81	18	1
6	Chloramphenicol (30µg)	40	2	58
7	Amoxicillin (10µg)	86	-	14
8	Ceftriaxone (30µg)	23	4	73

4.3 Multiple Antibiotic Resistance Index (MARI)

The pattern of resistance and the multiple antibiotic resistance index is as shown in Table 5

Table 5: Resistance Pattern and Multiple Antibiotic Resistance(MAR) Index of the *E. coli* isolates

S/No.	Isolate No.	Resistance Pattern	MAR Index
1	1	SXT, E, AML	0.3
2	2	SXT, E, C, AML	0.5
3	3	E	0.1
4	5	SXT, CN, CIP, E, C, AML, CRO	0.8
5	7	E	0.1
6	8	SXT, CIP, E, AML	0.5
7	10	SXT, CIP, E, AML	0.5
8	12	SXT, E, AML	0.3
9	13	AMC, E, AML	0.3
10	17*	SXT, CN, E, C, AML	0.6
11	17**	-	0

Table 5 cont'd.

S/No.	Isolate No.	Resistance Pattern	MAR Index
12	20	SXT, E, C, AML, CRO	0.6
13	22	E	0.1
14	23	SXT, E, C, AML, CRO	0.6
15	26	SXT, CN, CIP, E, C, AML, CRO	0.8
16	28*	SXT, CIP, E, AML	0.5
17	28**	-	0
18	31	CIP, E	0.2
19	33	SXT, AMC, E, C, AML	0.6
20	34	SXT, CIP, AMC, E, C, AML	0.7
21	35	SXT, CN, AMC, E, AML, CRO	0.7
22	36	SXT, CIP, E, AML	0.5
23	37	SXT, AMC, E, C, AML	0.6

Table 5 cont'd.

S/No.	Isolate No.	Resistance Pattern	MAR Index
24	40	SXT, CIP, E, C, AML	0.6
25	41	SXT, AML, CRO	0.3
26	42	SXT, AMC, E, AML	0.5
27	44	E	0.1
28	45	SXT, CN, C, AML, CRO	0.6
29	46	SXT, E, AML, CRO	0.5
30	50	SXT, E, C, AML	0.5
31	51	SXT, E, AML	0.3
32	52	SXT, AMC, E, AML	0.5
33	53	SXT, C, AML	0.3
34	58	E	0.1
35	59	SXT, AML	0.2

Table 5 cont'd.

S/No.	Isolate No.	Resistance Pattern	MAR Index
36	64	SXT, AML	0.2
37	66	SXT, E, AML, CRO	0.5
38	69	SXT, AML	0.2
39	73	SXT, CN, AMC, E, AML, CRO	0.7
40	78	SXT, E, AML	0.3
41	83	SXT, CN, AMC, E, AML, CRO	0.7
42	84	SXT, AML	0.2
43	85	SXT, AMC, E, AML	0.5
44	87	SXT, CIP, AMC, E, AML, CRO	0.7
45	93	SXT, CN, CIP, E, C, AML, CRO	0.8
46	95	AMC, E, AML	0.3
47	100	SXT, E, AML	0.3

Table 5 cont'd.

S/No.	Isolate No.	Resistance Pattern	MAR Index
48	101	SXT, E, AML	0.3
49	102	SXT, AML	0.2
50	105	SXT, CN, E, AML	0.5
51	106	SXT, E, C, AML	0.5
52	107	E	0.1
53	111	SXT, E	0.2
54	115	SXT, CN, E, C, AML	0.6
55	116	SXT, CN, E, C, AML	0.6
56	120	SXT, E, C, AML, CRO	0.6
57	124	SXT, E, AML	0.3
58	128	SXT, CN, AMC, E, AML	0.6
59	132	SXT, C, AML	0.3

Table 5 cont'd.

S/No.	Isolate No.	Resistance Pattern	MAR Index
60	133	SXT, C, AML	0.3
61	136	AMC, E, AML	0.3
62	137	SXT, E, AML	0.3
63	138	E	0.1
64	139	SXT, AMC, E, AML	0.5
65	140	SXT, E	0.2
66	145	SXT, E, C, AML	0.5
67	148	SXT, CN, CIP, AMC, E, C, AML, CRO	1
68	151	SXT, E, AML	0.3
69	153	SXT, AMC, E, AML	0.5
70	154	SXT, E, C, AML	0.5
71	160	SXT, E, AML	0.3

Table 5 cont'd.

S/No.	Isolate No.	Resistance Pattern	MAR Index
72	161	SXT, CN, CIP, E, C, AML, CRO	0.8
73	163	SXT, E, AML	0.3
74	164	SXT, CIP, AMC, E, C, AML	0.7
75	167*	AML	0.1
76	167**	E	0.1
77	168	SXT, CN, E, C, AML	0.6
78	169	SXT, CN, CIP, E, C, AML	0.7
79	174	SXT	0.1
80	175	SXT, E, C, AML	0.5
81	177	SXT, E, C, AML	0.5
82	178	SXT, CN, CIP, AMC, E, C, AML, CRO	1
83	180*	SXT, CIP, E, AML	0.5

Table 5 cont'd.

S/No.	Isolate No.	Resistance Pattern	MAR Index
84	180**	SXT, CIP, E, AML	0.5
85	181	SXT, E, C, AML	0.5
86	184	SXT, CIP, AMC, E, C, AML	0.7
87	191	SXT, E, AML	0.3
88	192	SXT	0.1
89	196	SXT, CN, CIP, E, C, AML, CRO	0.8
90	197	SXT, CIP, E, AML	0.5
91	198*	SXT, E, C, AML	0.5
92	198**	SXT, CN, E, C, AML	0.6
93	201	SXT, AML	0.2
94	203	SXT, AML	0.2
95	212	SXT, AMC, E, C, AML, CRO	0.7

Table 5 cont'd.

S/No.	Isolate No.	Resistance Pattern	MAR Index
96	214	SXT, CN, AMC, E, C, AML, CRO	0.8
97	215	SXT, AMC, E, C, AML, CRO	0.7
98	216	SXT, CIP, C, AML, CRO	0.6
99	218	SXT, CN, C, AML, CRO	0.6
100	222	SXT, CN, CIP, AMC, E, AML	0.7

KEY: SXT= COTRIMOXAZOLE, CN= GENTAMICIN, CIP= CIPROFLOXACIN, AMC= AMOXICILLIN-CLAVULANIC ACID, E= ERYTHROMYCIN, C= CHLORAMPHENICOL, AML= AMOXICILLIN, CRO= CEFTRIAXONE, */**= HETERORESISTANT ISOLATES, NO. OF ANTIBIOTICS USED= EIGHT (8)

The MAR index, the number of *E. coli* isolates and the percentage occurrence is as shown in Table 6

Table 6: Multiple Antibiotic Resistance Index and the Percentage Occurrence of the *E. coli* Isolates from Diarrheic Children in Zaria, Nigeria

MAR Index	No. of *E. coli* Isolates	Percentage Occurrence(%)
0	2	2
0.1	11	11
0.2	10	10
0.3	19	19
0.5	24	24
0.6	15	15
0.7	11	11
0.8	6	6
1.0	2	2

4.4 β-Lactamase Production

The summary of the β-lactamase production is as shown in Table 7

Table 7: Percentage of β-lactamase production by Amoxicillin Resistant *E.coli* Isolates

	No. of Isolates	Percentage(%)
β-lactamase Positive	48	55.8
β-lactamase Negative	38	44.2

4.5 Genomic DNA Extraction and PCR analysis

The Results of the Genomic DNA extraction and the PCR analysis are as shown in Plate IV and V respectively.

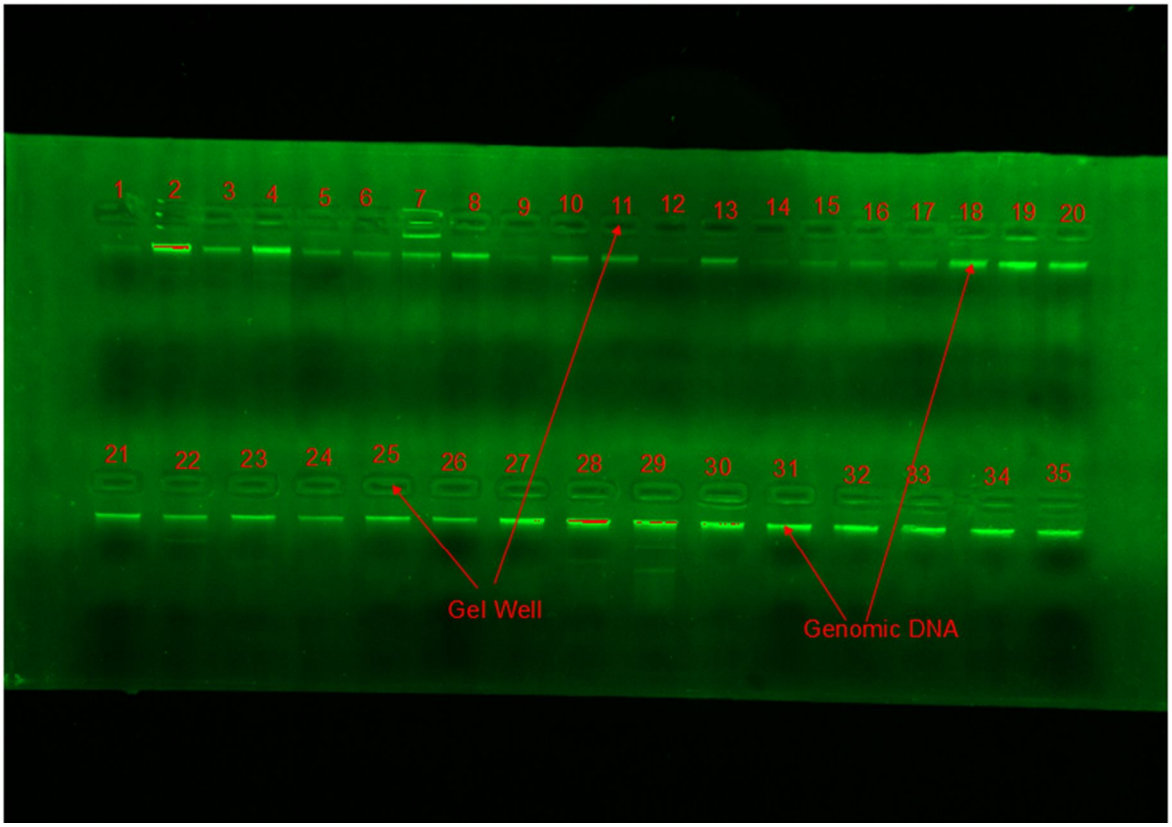


Plate IV: Genomic DNA on Agarose Gel (1.5%) Electrophoresis

Key:

Lane 1 : Isolate 1	Lane 10: Isolate 17*	Lane 19: Isolate 33	Lane 28: Isolate 45
Lane 2: Isolate 2	Lane 11: Isolate 17**	Lane 20: Isolate 34	Lane 29: Isolate 46
Lane 3: Isolate 3	Lane 12: Isolate 20	Lane 21: Isolate 35	Lane 30: Isolate 50
Lane 4: Isolate 5	Lane 13: Isolate 22	Lane 22: Isolate 36	Lane 31: Isolate 51
Lane 5: Isolate 7	Lane 14: Isolate 23	Lane 23: Isolate 37	Lane 32: Isolate 52
Lane 6: Isolate 8	Lane 15: Isolate 26	Lane 24: Isolate 40	Lane 33: Isolate 53
Lane 7: Isolate 10	Lane 16: Isolate 28*	Lane 25: Isolate 41	Lane 34: Isolate 58
Lane 8: Isolate 12	Lane 17: Isolate 28**	Lane 26: Isolate 42	Lane 35: Isolate 59
Lane 9: Isolate 13	Lane 18: Isolate 31	Lane 27: Isolate 44.	

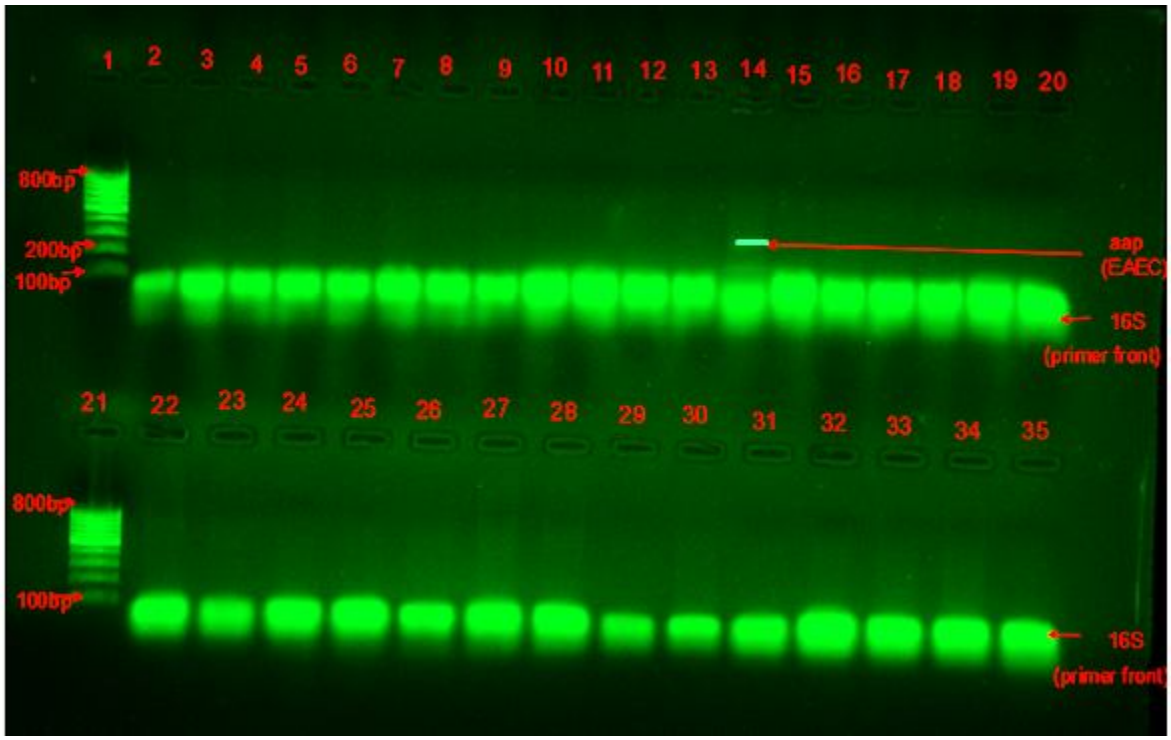


Plate V: Multiplex PCR on Agarose Gel (1.5%) Electrophoresis.

Key:

Lane 1: DNA ladder	Lane 10: Isolate 13	Lane 19: Isolate 31	Lane 28: Isolate 42
Lane 2: Isolate 1	Lane 11: Isolate 17*	Lane 20: Isolate 33	Lane 29: Isolate 44
Lane 3: Isolate 2	Lane 12: Isolate 17**	Lane 21: DNA ladder	Lane 30: Isolate 45
Lane 4: Isolate 3	Lane 13: Isolate 20	Lane 22: Isolate 34	Lane 31: Isolate 46
Lane 5: Isolate 5	Lane 14: Isolate 22	Lane 23: Isolate 35	Lane 32: Isolate 50
Lane 6: Isolate 7	Lane 15: Isolate 23	Lane 24: Isolate 36	Lane 33: Isolate 51
Lane 7: Isolate 8	Lane 16: Isolate 26	Lane 25: Isolate 37	Lane 34: Isolate 52
Lane 8: Isolate 10	Lane 17: Isolate 28*	Lane 26: Isolate 40	Lane 35: Isolate 53
Lane 9: Isolate 12	Lane 18: Isolate 28**	Lane 27: Isolate 41	

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CHAPTER FIVE

5.0 Discussion

In this study, a total of 100 isolates of *E. coli* (45%) were obtained from the stool of 222 diarrhoea patients who were children below five (5) years attending Ahmadu Bello University Teaching Hospital, Shika, and Institute of Child Health , Banzazzau, an annex of Ahmadu Bello University Teaching Hospital, Shika-Zaria, Nigeria. The isolation and biochemical identification were performed using standard microbiological procedures. The low percentage of *E. coli* obtained (45%) from children with diarrhoea may be due to the fact that antimicrobial therapy previous to sample collection might have been given in some cases and it is a known fact that this can reduce the percentage of bacterial enteropathogens isolation (Rohner *et al*., 1997).

The role of antibiotics in the treatment of childhood diarrhoea is limited because of their limited benefits and potential side effects (Alam and Ashraf, 2003). Yet, knowledge about antibiotic sensitivity pattern of these strains may be beneficial for hospital treatment in severe and complicated cases (Pickering and Snyder, 2004). The antimicrobial susceptibility testing was performed using modified Kirby-Bauer disc diffusion technique (Cheesbrough, 2000). Eight (8) commonly used antibiotics in the treatment of diarrhoeal diseases were selected which include Cotrimoxazole, Gentamicin, Ciprofloxacin, Amoxicillin/Clavulanic acid, Erythromycin, Chloramphenicol, Amoxicillin, and Ceftriaxone. The results of antimicrobial susceptibility profile of the 8 commonly prescribed antibiotics showed that, 86% of the *E. coli* isolates were resistant to Amoxicillin, 85% to Cotrimoxazole, 81% to Erythromycin, 40% to Chloramphenicol, 24% to Amoxicillin/Clavulanic acid, 23% to Ciprofloxacin, 23% to Ceftriaxone and 22% to Gentamicin. The high level of resistance of *E. coli* to Amoxicillin (86%), Cotrimoxazole (85%), Erythromycin (81%) and Chloramphenicol (40%) may be due to the fact that these antibiotics

have been in use for much longer time and also their oral route of administration is known to affect their rate of absorption into the blood stream (Seppala *et al*., 1992), their resistance could also be due to the presence of factors such as plasmids (Elmer *et al*., 1997). The resistance observed could also be attributed to the low cost and irrational use of antibiotics for conditions that may not clinically indicate their use, over-the-counter sale of antibiotics in Pharmacies without prescription by authorized practitioners. The resistance rate of 86% to Amoxicillin and 23% to Ceftriaxone is also supported by the work of Chijioke and Christian (2012) in Enugu, Nigeria; using *E. coli* isolates from livestock, they found resistance rates of 85% to Ampicillin and 22.5% to Ceftriaxone. *E. coli* isolates resistant to Amoxicillin showed high susceptibility to Amoxicillin-Clavulanic acid. Resistance to Amoxicillin–Clavulanic acid could be due to presence of enzymes such as AmpC (Denton, 2007). In order of susceptibility, 73% of the *E. coli* isolates were susceptible to Ceftriaxone, 72% to Gentamicin, 67% to Ciprofloxacin, 59% to Amoxicillin/Clavulanic acid, 58% to Chloramphenicol, 14% to Cotrimoxazole and Amoxicillin, and only 1% to Erythromycin. The *E. coli* isolates showed 18% intermediate susceptibility to Erythromycin, 17% to Amoxicillin/Clavulanic acid, 10% to Ciprofloxacin, 6% to Gentamicin, 4% to Ceftriaxone, 2% to Chloramphenicol, 1% to Cotrimoxazole and no intermediate susceptibility was observed to Amoxicillin (0%). Ciprofloxacin, Ceftriaxone and Amoxicillin-Clavulanic acid are relatively expensive, due to their high cost of procurement means these antibiotics have not been misused and hence are more effective compared to those that have been in use for quite a long time. It is worthy of note that Ceftriaxone is probably less abused than other antibiotics because of its mode of administration (solely by injection) and its high cost of procurement. Gentamicin showed better activity than newer antibiotics with 72% of the *E. coli* isolates showing susceptibility to Gentamicin, this may be due to its limited use (Olatoye, 2010).

Five (5) of the *E. coli* isolates exhibit heteroresistance to different antibiotics that include Cotrimoxazole, Gentamicin, Erythromycin, Chloramphenicol, Ciprofloxacin and Amoxicillin. Heteroresistance refers to phenotypic heterogeneity of microbial clonal populations under antibiotic stress, and it has been thought to be an allocation of a subset of "resistant" cells for surviving in higher concentrations of antibiotic. The exploration of possible mechanisms of such resistance has led to intensive investigations of heteroresistance: phenotypic heterogeneous resistance to an antibiotic treatment within a clonal population (Hung *et al*., 2012). Heteroresistance is observed in a wide range of microbes (Morand and Mühlemann, 2007) and often in cases of treatment failures (Moore *et al*., 2003); it has been thought to be a prerequisite of resistant subpopulations that lead to full resistance due to their selective advantages upon antibiotic stress (Libby and Rainey, 2011). In addition, the genetic basis of heteroresistance remains obscure, although attempts to discover it have been made (Holmes *et al*., 2012). According to the exclusion rule (Libby and Rainey, 2011), heteroresistance can be a result of heterogeneous expression of antibiotic hydrolase genes and provides a heterogeneous selective advantage.

Multiple antibiotic resistance (MAR) indexing of the *E. coli* isolates in this study showed that 58% have MAR index of ≥ 0.5. At MARI ≥ 0.5, there is a significant level of misuse of these antibiotics. It should be noted that an antibiotics is said to be abused if the MAR Index is ≥ 1 in a particular environment, this increase bacterial drug resistance worldwide that exacerbate the diminishing number of new antimicrobial drugs in the pharmaceutical pipeline (Spellberg *et al.,* 2004; Talbot *et al.,* 2006); this will affect the worldwide escalation in both community-and hospital-acquired antimicrobial-resistant bacteria and hence, threatening the ability to effectively treat patients. Therefore, there is need to emphasize more on continued surveillance; which is

indispensable for empirically treating infections, implementing resistance control measures and preventing the spread of antimicrobial-resistant microorganisms (WHOCCDSM; 1999), more appropriate antimicrobial prescription, prudent infection control and new treatment alternatives (Weshnoweski *et al.,* 2008).

Among the 86 (86%) *E. coli* isolates resistant to Amoxicillin, 48 (55.8%) were found to be β-lactamase positive while 38 (44.2%) were found to be β-lactamase negative. One of the main mechanism of resistance to antibacterial agents is the production of β-lactamase enzymes. In most cases, β-lactamase causes bacteria to get resistant to a broad spectrum of antibiotics like fluoroquinolones, aminoglycosides and trimethoprim (Poole, 2004). According to Ambler classification, these enzymes, most of which are called ESBLs, are divided into four main groups from A to D (Jacoby and Munoz-Price, 2005). ESBL enzymes of CTX-M, TEM and SHV, from group A, have been widely reported to be produced by *E. coli*. These enzymes can hydrolyze ampicillin, carbenicillin, oxacillin and an extended spectrum of cephalosporins like ceftazidime and cefotaxime; and are usually encoded by plasmids (Paterson and Bonomo, 2005). AmpC β-lactamases are able to hydrolyse monobactams, cephamycins and cephalosporins and are not inhibited by ESBL inhibitors such as clavulanic acid (Sundin, 2009).

Multiplex PCR was performed to characterize the *E. coli* isolates into their respective pathotypes/ diarrhoeagenic groups as described by Persson *et al*. (2007). From the result obtained, only one (1) percent of the isolates was found to harbor the virulence gene out of the 100 *E. coli* isolated from the diarrhoea stools of children employed in this study. Plate v, lane 14 showed a band which is interpreted to be antiaggregation protein (*aap*), a characteristic of Enteroaggregative *E. coli* (EAEC) at a base pair of 232 though not well resolved. Interestingly, this *E. coli* pathotype (EAEC) is susceptible to almost all the antibiotics used in the susceptibility

testing, except Erythromycin which shows no activity on the pathotype. The very low percentage (1%) incidence of diarrhoeagenic *E. coli* obtained in this study is in close agreement with the study conducted and reported by Ogunsanya *et al*. (1994) and Olorunishola *et al*. (2000) in Lagos. They showed that the prevalence of EHEC 0157:H7 in humans was 5%. But it is in contrast with the studies conducted by Okeke *et al*. (2000), which showed an incidence of 40.4% of diarrhoeagenic *E. coli* in children, Nweze (2009), reported 19.6% prevalence of Diarrhoeagenic *E. coli* in a study conducted in Southeastern Nigeria, an incidence higher than 40% has been reported in Bangladesh (Albert *et al*., 1999). A lower incidence of about 30% has been reported in Jordan (Shehabi *et al*., 2003). The very low percentage (1%) incidence of diarrhoeagenic *E. coli* obtained in this study might be attributed to encouragement of breast feeding and educating the mothers on proper personal hygiene by the dieticians both in the main Teaching Hospital and the Institute of Child Health. The subjects employed in this study may be infected by other pathogens other than diarrhoeagenic *E. coli* since there are many different pathogens that can cause diarrhoea in children, including other bacteria, viruses, and parasites. The most common isolated diarrhoeagenic pathogens include *Escherichia coli*, Rotavirus, *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni*, *Entamoeba histolytica,* and *Giardia lamblia* (Vargas *et al*., 2004).

A typical example was obtained by some researchers in Saudi Arabia, which reported *Shigella* as having the highest incidence among other bacterial pathogens causing diarrhoea disease in that country (Al-Jurayyan *et al*., 1994). Various factors may account for such a difference. Antimicrobial therapy previous to sample collection might have been given in some cases where it was recommended, and it is known that this can reduce the percentage of bacterial enteropathogens isolation (Rohner *et al*., 1997) Finally, the direct effect of other pathologies,

such as malaria, cannot be discarded. It has been previously described that between 5% and 38% of malaria cases present diarrhoea as one of the main symptoms. Thus, even taking into consideration the aforementioned study limitations, including the possible presence in the area of other enteropathogens that we did not test for, such as some diarrhoeagenic viruses, we must not underestimate the impact of malaria, which may account for an important percentage of the diarrhoea among children.

CHAPTER SIX

6.0 Summary, Conclusion and Recommendations

6.1 Summary

This study was designed to isolate, characterize *E. coli* from diarrheic children into their pathotypes and to carry out their antibiotic susceptibility pattern studies.

- One hundred (100) isolates of *E. coli* were recovered from the stool of 222 diarrheic children, in Zaria, Nigeria, using standard microbiological procedures

- The result of antimicrobial susceptibility profile to the 8 commonly prescribed antibiotics showed that, 85% of the *E. coli* isolates were resistant to Cotrimoxazole, 22% to Gentamicin, 23% to Ciprofloxacin, 24% to Augmentin (Amoxicillin/Clavulanic acid), 81% to Erythromycin, 40% to Chloramphenicol, 86% to Amoxicillin and 23% to Ceftriaxone. In order of susceptibility, 73% of the *E. coli* isolates were susceptible to Ceftriaxone, 72% to Gentamicin, 67% to Ciprofloxacin, 59% to Augmentin (Amoxicillin/Clavulanic acid), 58% to Chloramphenicol, 14% to Cotrimoxazole and Amoxicillin, and only 1% to Erythromycin.

- Multiple Antibiotic Resistance (MAR) index of the *E. coli* isolates in this study showed that 58% have MAR index of ≥ 0.5, this indicate a significant level of misuse of these antibiotics.

- Among the 86 (86%) *E. coli* isolates resistant to Amoxicillin, 48 (55.8%) of the Amoxicillin resistant isolates were found to be β-lactamase positive while 38 (44.2%) were found to be β-lactamase negative.

- From the results obtained, only one (1) percent of the isolates was found to harbor the virulence gene out of the 100 *E. coli* isolated from the diarrhoea stools of children employed in this study. The isolate carried an antiaggregation protein (*aap*) at a base pair of 232, a characteristic of Enteroaggregative *E. coli*.

6.2 Conclusion

It can be concluded that, only 100 (45%) *E. coli* isolates were recovered from 222 diarrrheic stool samples of Children. The *E. coli* isolates were generally resistant to β-lactam antibiotics except Ceftriaxone. Molecular characterization of the isolates showed that, only 1% of the isolates carried a virulence gene (antiaggregation protein/dispersin), a characteristic of Enteroaggregative *E. coli*.

6.3 Recommendations

- Although the etiologic causes of diarrhoea in children younger than 5 years of age remain unknown for most of the country, this report opens the door to further studies addressed to analyze the specific epidemiology, resistance patterns, and virulence of diarrhoea-causing pathogens in Zaria, and the whole country in general.

- It is recommended that Ciprofloxacin, Gentamicin, Amoxicillin-Clavulanic acid and Ceftriaxone be used since they are more effective against the *E. coli* isolates in preference to Co-trimoxazole, Erythromycin, Amoxicillin and Chloramphenicol.

- The prevention package should focus on five main elements to reduce diarrhoea in the medium to long term: Rotavirus and measles vaccinations, behaviour change interventions, such as face-to-face counselling, to encourage exclusive breastfeeding and

vitamin A supplementation, Promotion of handwashing with soap, Improved water supply quantity and quality, including treatment and safe storage of household water and Community-wide sanitation promotion.

6.4 Contribution to Knowledge

- Antiaggregation protein, a virulence factor, was found in 1% of the *E. coli* isolates obtained from diarrheic children in Zaria, a characteristic of Enteroaggregative *E. coli*.
- Multiple antibiotic resistance index were found to be ≥ 0.5 in 58% of the *E. coli* isolated from Children in Zaria.
- Not all *E. coli* isolated from diarrheoic Children are pathogenic.

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APPENDICES

Appendix I: Biochemical Identification of *E. coli* isolates

S/No.	Isolate No.	Indole	MR	VP	Citrate	Eijkmann	Inference
1	1	+	+	-	-	+	*E. coli*
2	2	+	+	-	-	+	*E. coli*
3	3	+	+	-	-	+	*E. coli*
4	5	+	+	-	-	+	*E. coli*
5	7	+	+	-	-	+	*E. coli*
6	8	+	+	-	-	+	*E. coli*
7	10	+	+	-	-	+	*E. coli*
8	12	+	+	-	-	+	*E. coli*
9	13	+	+	-	-	+	*E. coli*
10	17*	+	+	-	-	+	*E. coli*
11	17**	+	+	-	-	+	*E. coli*

Appendix I cont'd.

S/No.	Isolate No.	Indole	MR	VP	Citrate	Eijkmann	Inference
12	20	+	+	-	-	+	*E. coli*
13	22	+	+	-	-	+	*E. coli*
14	23	+	+	-	-	+	*E. coli*
15	26	+	+	-	-	+	*E. coli*
16	28*	+	+	-	-	+	*E. coli*
17	28**	+	+	-	-	+	*E. coli*
18	31	+	+	-	-	+	*E. coli*
19	33	+	+	-	-	+	*E. coli*
20	34	+	+	-	-	+	*E. coli*
21	35	+	+	-	-	+	*E. coli*
22	36	+	+	-	-	+	*E. coli*

Appendix I cont'd.

S/No.	Isolate No.	Indole	MR	VP	Citrate	Eijkmann	Inference
23	37	+	+	-	-	+	*E. coli*
24	40	+	+	-	-	+	*E. coli*
25	41	+	+	-	-	+	*E. coli*
26	42	+	+	-	-	+	*E. coli*
27	44	+	+	-	-	+	*E. coli*
28	45	+	+	-	-	+	*E. coli*
29	46	+	+	-	-	+	*E. coli*
30	50	+	+	-	-	+	*E. coli*
31	51	+	+	-	-	+	*E. coli*
32	52	+	+	-	-	+	*E. coli*
33	53	+	+	-	-	+	*E. coli*

Appendix I cont'd.

S/No.	Isolate No.	Indole	MR	VP	Citrate	Eijkmann	Inference
34	58	+	+	-	-	+	*E. coli*
35	59	+	+	-	-	+	*E. coli*
36	64	+	+	-	-	+	*E. coli*
37	66	+	+	-	-	+	*E. coli*
38	69	+	+	-	-	+	*E. coli*
39	73	+	+	-	-	+	*E. coli*
40	78	+	+	-	-	+	*E. coli*
41	83	+	+	-	-	+	*E. coli*
42	84	+	+	-	-	+	*E. coli*
43	85	+	+	-	-	+	*E. coli*
44	87	+	+	-	-	+	*E. coli*

Appendix I cont'd.

S/No.	Isolate No.	Indole	MR	VP	Citrate	Eijkmann	Inference
45	93	+	+	-	-	+	*E. coli*
46	95	+	+	-	-	+	*E. coli*
47	100	+	+	-	-	+	*E. coli*
48	101	+	+	-	-	+	*E. coli*
49	102	+	+	-	-	+	*E. coli*
50	105	+	+	-	-	+	*E. coli*
51	106	+	+	-	-	+	*E. coli*
52	107	+	+	-	-	+	*E. coli*
53	111	+	+	-	-	+	*E. coli*
54	115	+	+	-	-	+	*E. coli*
55	116	+	+	-	-	+	*E. coli*

Appendix I cont'd.

S/No.	Isolate No.	Indole	MR	VP	Citrate	Eijkmann	Inference
56	120	+	+	-	-	+	*E. coli*
57	124	+	+	-	-	+	*E. coli*
58	128	+	+	-	-	+	*E. coli*
59	132	+	+	-	-	+	*E. coli*
60	133	+	+	-	-	+	*E. coli*
61	136	+	+	-	-	+	*E. coli*
62	137	+	+	-	-	+	*E. coli*
63	138	+	+	-	-	+	*E. coli*
64	139	+	+	-	-	+	*E. coli*
65	140	+	+	-	-	+	*E. coli*
66	145	+	+	-	-	+	*E. coli*

Appendix I cont'd.

S/No.	Isolate No.	Indole	MR	VP	Citrate	Eijkmann	Inference
67	148	+	+	-	-	+	*E. coli*
68	151	+	+	-	-	+	*E. coli*
69	153	+	+	-	-	+	*E. coli*
70	154	+	+	-	-	+	*E. coli*
71	160	+	+	-	-	+	*E. coli*
72	161	+	+	-	-	+	*E. coli*
73	163	+	+	-	-	+	*E. coli*
74	164	+	+	-	-	+	*E. coli*
75	167*	+	+	-	-	+	*E. coli*
76	167**	+	+	-	-	+	*E. coli*
77	168	+	+	-	-	+	*E. coli*
78	169	+	+	-	-	+	*E. coli*

Appendix I cont'd.

S/No.	Isolate No.	Indole	MR	VP	Citrate	Eijkmann	Inference
79	174	+	+	-	-	+	*E. coli*
80	175	+	+	-	-	+	*E. coli*
81	177	+	+	-	-	+	*E. coli*
82	178	+	+	-	-	+	*E. coli*
83	180*	+	+	-	-	+	*E. coli*
84	180**	+	+	-	-	+	*E. coli*
85	181	+	+	-	-	+	*E. coli*
86	184	+	+	-	-	+	*E. coli*
87	191	+	+	-	-	+	*E. coli*
88	192	+	+	-	-	+	*E. coli*
89	196	+	+	-	-	+	*E. coli*

Appendix I cont'd.

S/No.	Isolate No.	Indole	MR	VP	Citrate	Eijkmann	Inference
90	197	+	+	-	-	+	*E. coli*
91	198*	+	+	-	-	+	*E. coli*
92	198**	+	+	-	-	+	*E. coli*
93	201	+	+	-	-	+	*E. coli*
94	203	+	+	-	-	+	*E. coli*
95	212	+	+	-	-	+	*E. coli*
96	214	+	+	-	-	+	*E. coli*
97	215	+	+	-	-	+	*E. coli*
98	216	+	+	-	-	+	*E. coli*
99	218	+	+	-	-	+	*E. coli*
100	222	+	+	-	-	+	*E. coli*

KEY: MR= METHYL RED, VP= VOGES-PROSKAUER,+ =POSITIVE REACTION, - =NEGATIVEREACTION

Appendix II: Zone Diameter Interpretative Standard Using Clinical and Laboratory Standard Institute Guidelines (CLSI, 2008.)

S/No.	Antibiotics	Zone Diameter(mm)		
		Resistance	Intermediate	Sensitive
1	Cotrimoxazole (25µg)	≤10	11-15	≥16
2	Gentamicin (10µg)	≤12	13-14	≥15
3	Ciprofloxacin (5µg)	≤15	16-20	≥21
4	Amoxicillin+ Clavulanic acid (30µg)	≤13	14-17	≥18
5	Erythromycin (15µg)	≤13	14-22	≥23
6	Chloramphenicol (30µg)	≤12	13-17	≥18
7	Amoxicillin (10µg)	≤13	14-16	≥17
8	Ceftriaxone (30µg)	≤13	14-20	≥2

Appendix III: Zones of Inhibition(mm) for Antibiotic Susceptibility Tests of Different *E.coli* Isolates

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
1	1	0	22	26	19	0	27	0	33
2	2	0	20	22	23	0	0	0	34
3	3	28	20	30	24	11	26	21	34
4	5	0	0	0	15	0	0	0	10
5	7	15	16	20	18	10	17	0	28
6	8	0	21	0	20	0	28	0	31
7	10	0	18	0	19	0	26	0	36
8	12	0	17	25	17	10	20	0	35
9	13	20	16	22	9	10	21	0	27
10	17*	0	10	22	24	0	0	0	30
11	17**	28	18	24	25	14	24	21	30
12	20	0	16	17	15	0	0	0	6
13	22	31	20	35	24	13	30	21	35
14	23	0	17	21	16	10	0	0	10
15	26	0	11	0	16	0	0	0	12
16	28*	0	21	0	24	0	29	0	33
17	28**	30	20	33	25	19	28	22	32
18	31	27	18	0	20	12	26	17	31
19	33	0	18	30	13	10	0	0	31
20	34	0	17	0	8	10	0	0	30

Appendix III: Zones of Inhibition(mm) for Antibiotic Susceptibility Tests of Different *E.coli* Isolates cont'd.

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
21	35	0	8	17	12	9	23	0	8
22	36	0	17	0	18	12	26	0	30
23	37	0	16	26	10	0	0	0	2
24	40	0	18	0	20	11	11	0	30
25	41	0	18	25	25	15	27	0	12
26	42	0	19	25	10	0	18	0	31
27	44	26	20	30	26	10	27	23	31
28	45	0	12	30	22	14	0	0	10
29	46	0	13	20	24	0	27	0	9
30	50	0	19	27	18	12	0	0	32
31	51	0	18	31	19	12	27	0	31
32	52	0	19	25	10	0	19	0	34
33	53	0	20	33	19	16	0	0	28
34	58	26	19	30	25	12	24	22	31
35	59	0	20	30	26	15	25	0	31
36	64	0	19	27	23	16	27	0	30
37	66	0	14	19	22	0	25	0	10
38	69	0	18	31	24	16	25	0	10

Appendix III: Zones of Inhibition(mm) for Antibiotic Susceptibility Tests of Different *E.coli* Isolates cont'd.

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
39	73	0	10	21	11	12	25	0	12
40	78	0	16	26	21	10	27	0	31
41	83	0	0	26	12	8	25	0	10
42	84	0	18	26	20	16	27	0	29
43	85	0	18	21	0	0	21	0	33
44	87	0	17	0	11	0	15	0	9
45	93	0	0	0	18	0	0	0	12
46	95	23	20	32	9	10	26	0	35
47	100	0	16	25	20	0	25	0	29
48	101	0	18	30	20	12	27	0	28
49	102	0	19	33	27	20	30	0	32
50	105	0	0	27	19	0	24	0	32
51	106	0	17	27	14	13	0	0	26
52	107	20	15	22	25	8	20	25	22
53	111	0	13	21	30	11	26	26	20
54	115	0	8	20	14	0	0	0	18
55	116	0	9	28	15	0	0	0	29

Appendix III: Zones of Inhibition(mm) for Antibiotic Susceptibility Tests of Different *E.coli* Isolates cont'd.

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
56	120	0	18	25	14	0	0	0	12
57	124	0	18	29	18	10	24	0	30
58	128	0	0	20	0	0	21	0	17
59	132	0	23	30	18	17	0	0	33
60	133	0	18	22	25	19	8	0	27
61	136	27	18	30	8	9	30	0	31
62	137	0	15	20	19	0	18	0	25
63	138	22	16	22	21	9	22	17	27
64	139	0	16	23	7	0	20	0	26
65	140	0	18	25	14	11	22	0	30
66	145	0	18	25	14	10	0	0	28
67	148	0	0	0	13	0	0	0	8
68	151	0	18	26	20	8	25	0	32
69	153	0	16	21	8	8	25	0	29
70	154	0	17	20	20	0	0	0	32
71	160	0	19	29	20	11	25	0	32
72	161	0	8	0	14	0	0	0	10
73	163	0	19	25	17	0	25	0	32
74	164	0	19	0	12	0	0	0	28

Appendix III: Zones of Inhibition(mm) for Antibiotic Susceptibility Tests of Different *E.coli* Isolates cont'd.

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
75	167*	21	20	32	22	17	28	0	30
76	167**	25	18	27	23	9	23	21	28
77	168	0	10	30	16	0	0	0	30
78	169	0	10	0	14	0	0	0	29
79	174	0	13	22	30	29	28	28	20
80	175	0	19	29	18	0	0	0	31
81	177	0	19	27	16	13	0	0	30
82	178	0	0	0	11	0	0	0	9
83	180*	0	18	0	22	12	26	0	30
84	180**	0	18	0	25	11	22	0	30
85	181	0	21	32	21	12	0	0	30
86	184	0	21	0	11	0	0	0	30
87	191	0	19	24	21	10	26	0	30
88	192	0	17	23	33	20	31	30	25
89	196	0	0	0	17	0	0	0	8
90	197	0	20	0	20	0	27	0	30
91	198*	0	22	33	22	12	0	0	33
92	198**	0	8	23	22	0	0	0	35
93	201	0	21	28	22	15	29	0	36
94	203	0	23	38	24	17	31	0	36

Appendix III: Zones of Inhibition(mm) for Antibiotic Susceptibility Tests of Different *E.coli* Isolates cont'd.

S/No.	Isolate No.	SXT	CN	CIP	AMC	E	C	AML	CRO
95	212	0	14	27	11	9	11	0	8
96	214	0	8	17	13	0	0	0	10
97	215	0	13	31	13	13	9	0	10
98	216	0	23	0	20	14	0	0	9
99	218	0	12	26	22	15	0	0	9
100	222	0	10	11	12	10	23	0	28

Key: SXT= COTRIMOXAZOLE, CN= GENTAMICIN, CIP= CIPROFLOXACIN, AMC= AMOXICILLIN-CLAVULANIC ACID, E= ERYTHROMYCIN, C= CHLORAMPHENICOL, AML= AMOXICILLIN, CRO= CEFTRIAXONE.

Appendix IV: β- Lactamase(s) production Test on Amoxicillin Resistant *E.coli*

S/No.	Isolate No.	Reaction
1	1	-
2	2	-
3	5	-
4	8	-
5	10	-
6	12	-
7	13	-
8	17*	-
9	20	+
10	23	+
11	26	+
12	28*	-
13	33	+
14	34	-
15	35	-

Appendix V: β- Lactamase(s) production Test on Amoxicillin Resistant *E.coli* cont'd.

S/No.	Isolate No.	Reaction
16	36	+
17	37	+
18	40	-
19	41	+
20	42	+
21	45	-
22	46	-
23	50	-
24	51	+
25	52	+
26	53	+
27	59	+
28	64	+
29	69	+
30	73	-

Appendix V: β- Lactamase(s) production Test on Amoxicillin Resistant *E.coli* cont'd.

S/No.	Isolate No.	Reaction
31	78	+
32	84	+
33	85	+
34	87	+
35	93	+
36	95	-
37	100	+
38	101	-
39	102	+
40	105	+
41	106	-
42	115	-
43	116	-
44	120	-
45	124	-

Appendix IV: β- Lactamase(s) production Test on Amoxicillin Resistant *E.coli* cont'd.

S/No.	Isolate No.	Reaction
46	128	-
47	132	-
48	133	-
49	136	+
50	137	+
51	139	+
52	140	+
53	148	-
54	151	+
55	153	-
56	154	-
57	160	-
58	163	+
59	164	+
60	168	+

Appendix IV: β- Lactamase(s) production Test on Amoxicillin Resistant *E.coli* cont'd.

S/No.	Isolate No.	Reaction
61	169	+
62	175	+
63	177	+
64	178	-
65	180*	-
66	180**	-
67	181	-
68	184	+
69	191	-
70	197	-
71	198*	+
72	198**	+
73	196	+
74	161	+
75	201	+

Appendix IV: β- Lactamase(s) production Test on Amoxicillin Resistant *E.coli* cont'd.

S/No.	Isolate No.	Reaction
76	203	+
77	212	-
78	214	+
79	215	-
80	216	+
81	218	+
82	222	+
83	83	+
84	145	+
85	167*	+
86	66	+

KEY: + = β-LACTAMASE POSITIVE, - = β-LACTAMASE NEGATIVE

**/* = HETERORESISTANT ISOLATES