ISOLATION AND MOLECULAR CHARACTERIZATION OF PATHOTYPES OF *ESCHERICHIA COLI* IN DIARRHEIC PATIENTS IN KEFFI, NIGERIA

BY

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DECLARATION

I hereby declare that this dissertation has been written by me and it is a report of my research work. It has not been presented in any previous application for Master of Science degree. All quotations are indicated and sources of information specifically acknowledged by means of references.



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04/11/2016

Date

CERTIFICATION

The dissertation titled "**Isolation and Molecular Characterization of Pathotypes of *Escherichia coli* in Diarrheic Patients in Keffi, Nigeria**" meets the regulations governing the award of Master of Science in Medical Microbiology; School of Postgraduate Studies, Nasarawa State University, Keffi for it contribution to knowledge and literary presentation.

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DEDICATION

I am delighted to dedicate this piece of work to the Almighty God for His mercy and kindness towards me; and to the Chapel of Salvation (COS), Nasarawa State University, Keffi.

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ABSTRACT

Escherichia coli (*E. coli*) is among the pathogens that cause diarrhea both in developed and developing countries. The isolation and molecular characterization of pathotypes of *E. coli* causing diarrhea in Keffi, Nigeria was carried out. A total of fifty (50) stool samples were collected from patients suspected to have diarrhea attending General Hospital, Keffi and Federal Medical Centre, Keffi, Nigeria; *E. coli* was isolated from the samples using standard microbiological methods. The molecular characterization of different pathotypes of *E. coli* such as enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC) was carried out using Multiplex Polymerase Chain Reaction (PCR) method. Out of 50 stool samples from diarrheic patients, 50 (100%) of *E. coli* was isolated. The order of detection frequency of different pathotypes of *E. coli* was: EAEC (22.0%) > EIEC and ETEC (2.0%) > EHEC and EPEC were (0.0%). The order of detection frequency of EAEC in relation to age (in years) was: 6-10 (50.0%) > 11-15 (40.0%) > 16-20 (20.0%) > 0-5 (17.2%). The frequency of detection of ETEC was 3.4% in age 0-5 and EIEC was 20.0% in age 11-15. The order of detection frequency of EAEC was high in female (36.4%) than male (10.7%) respectively. The EIEC was detected only in male (2.0%) and ETEC was detected only in female (2.0%). The frequency of detection of EAEC in relation to age and gender of diarrheic patients was statistically insignificant ($P > 0.05$). The frequency of detection of EAEC was high. In addition EHEC and EPEC were not detected. Further studies on DNA sequencing for pathotypes of diarrheagenic *E. coli* in Keffi and other parts of Nasarawa State should be carried out.

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Escherichia coli (E. coli) is the predominant facultative anaerobe and commensal microbiota in the mammalian gastrointestinal gut, yet some strains can cause severe diarrheal illnesses in humans (Kaper *et al.,* 2004; Kim *et al.,* 2014). Gastrointestinal colonization occurs within hours of life and the bacteria will remain with a symbiotic relationship with the host (Nataro and Kaper, 1998; Sani *et al.,* 2015). It remains harmlessly confined to the intestinal lumen. In the debilitated or immunosuppressed hosts, or when gastrointestinal barriers are violated, even normal "non-pathogenic" strains of *E. coli* can cause infection (Koneman *et al.*, 2006).

Diarrheagenic *Escherichia coli* (DEC) are important intestinal pathogens causing a wide variety of gastrointestinal diseases, particularly among children in developing countries (Ali *et al.*, 2014). Six pathotypes of DEC strains are frequently detected worldwide; and described based on the genes mediating the virulence factors associated with the diarrheal diseases caused by them: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC) or Enterohaemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) (Onanuga *et al.*, 2014; Ali *et al.,* 2014).

Person-to-person transmission and consumption of foods and drinking water contaminated with human waste are the main modes of acquiring DEC infections (Ali *et al.*, 2014).

Generally, acute infectious diarrhea is self-limiting and responsive to therapy by oral rehydration. However, antimicrobial therapy is recommended for severely ill patients and for those with risk factors for invasive infection after obtaining appropriate blood and faecal cultures (Ali *et al.*, 2014).

High frequency of resistance to antimicrobial agents among DEC isolated from children with diarrhea has been reported from several developing countries (Ochoa *et al.*, 2009).

The detection of DEC strains is difficult since these strains cannot be easily distinguished from the normal faecal flora using conventional phenotypic methods. Molecular identification and classification of ETEC is established by the presence or absence of one or more specific virulence genes, which are absent in the commensal *E. coli* (Kaper *et al.*, 2004).

1.2 Statement of the Problem

Escherichia coli (*E. coli*) is an emerging agent among pathogens that causes diarrhea both in developed and developing countries. In 2015, diarrhea accounted for 531,000 deaths in children under five years of age worldwide making it the second leading cause of child mortality (WHO, 2015). The highest rates of child mortality are in Sub-Saharan Africa and Southeast Asia. An estimated 1.7 billion episodes of diarrhea, equalling approximately 2.9 episodes per child per year, created health system costs of about 7 billion US dollars (Katharina *et al.*, 2014). General causative agents of diarrhea reported include: bacteria, viruses, fungi and parasites. DEC is also responsible for gastrointestinal tract diseases in developing countries (Ali *et al.*, 2014).

1.2.1 Justification

Diarrheagenic *Escherichia coli* (DEC) accounts for 70 % diarrheal cases reported globally (Bhutta *et al.*, 2013). In 2015, diarrhea accounted for 531,000 deaths in children under five years of age (Under 5) worldwide making it the second leading cause of child mortality (WHO, 2015). Diarrheagenic *Escherichia coli* pathotypes identification is necessary to ensure proper management of *E. coli* associated with diarrhea.

No information on molecular characterization of DEC is known in Keffi. However, this study was designed to isolate and characterize by molecular methods, the pathotypes of DEC in Keffi, Nigeria.

1.3 Aim and Objectives

1.3.1 Aim

This study aimed at the isolation and molecular characterization of pathotypes of *E. coli* causing diarrhea in Keffi, Nigeria.

1.3.2 Objectives

The specific objectives of the study include:

i. To isolate and identify *E. coli* from patients with cases of diarrhea in Keffi;

ii. To determine the pathotypes of the *E. coli* isolated from patients with diarrhea in Keffi using genotypic methods.

1.4 Statement of Hypotheses

1.4.1 Null Hypothesis (Ho)

i. *Escherichia coli* from diarrheal stool are non- pathogenic.

1.4.2 Alternate Hypothesis (Ha)

i. *Escherichia coli* from diarrheal stool are pathogenic.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of Diarrhea Disease

Diarrhea is a leading cause of illness and death among children in developing countries, where an estimated 1.3 thousand million episodes and 4 million deaths occur each year in under-fives. Worldwide, these children experience an average of 3.3 episodes each year, but in some areas the average exceeds nine episodes each year. Where episodes are frequent, young children may spend more than 15% of their days with diarrhea (http://www.who.int/child-adolescent-health/New Publications Accessed: November 7th, 2016).

About 80% of deaths due to diarrhoea occur in the first two years of life. The main cause of death from acute diarrhea is dehydration, which results from the loss of fluid and electrolytes in diarrhoeal stools. Other important causes of death are dysentery and undernutrition (http://www.who.int/child-adolescent-health/New Publications Accessed: November 7th, 2016).

Diarrhea is an important cause of under nutrition. This is because patients eat less during diarrhoea and their ability to absorb nutrients is reduced; moreover, nutrient requirements are increased as a result of infection. Each episode of diarrhoea contributes to undernutrition; when episodes are prolonged, their impact on growth is increased. Diarrheal disease also represents an economic burden for the developing countries. In many nations more than a third of the hospital beds for children are occupied by patients with diarrhea.

These patients are often treated with expensive intravenous fluids and ineffective drugs. Although diarrheal disease is usually less harmful to adults than to children, it

can also affect a country's economy by reducing the health of its work force. (http://www.who.int/child-adolescenthealth/New_Publications Accessed: November 7th, 2016).

Fortunately, simple and effective treatment measures are available that can markedly reduce diarrhea deaths, make hospitalization unnecessary in most cases, and prevent the adverse effect of diarrhoea on nutritional status. Practical preventive measures can also be taken that substantially reduce the incidence and severity of diarrhoeal episodes. This unit provides information on the epidemiology and aetiology of diarrhea that is essential for an understanding of the principles of treatment and prevention. The treatment and prevention of diarrhoea are considered in later units (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

2.1.1 Types of Diarrhea

Diarrhea is usually defined in epidemiological studies as the passage of three or more loose or watery stools in a 24-hour period, a loose stool being one that would take the shape of a container. However, mothers may use a variety of terms to describe diarrhoea, depending, for example, upon whether the stool is loose, watery, bloody or mucoid, or there is vomiting. It is important to be familiar with these terms when asking whether a child has diarrhoea. Exclusively breast-fed infants normally pass several soft, semi-liquid stools each day; for them, it is practical to define diarrhoea as an increase in stool frequency or liquidity that is considered abnormal by the mother.

Acute Watery Diarrhea

This term refers to diarrhea that begins acutely, lasts less than 14 days (most episodes last less than seven days), and involves the passage of frequent loose or watery stools without visible blood. Vomiting may occur and fever may be present. Acute watery diarrhoea causes dehydration; when food intake is reduced, it also contributes to undernutrition. When death occurs, it is usually by acute dehydration.

The most important causes of acute watery diarrhea in young children in developing countries are rotavirus, enterotoxigenic *Escherichia coli, Shigella, Campylobacter jejuni,* and *Cryptosporidia.* In some areas, *Vibrio cholerae* 01, *Salmonella* and enteropathogenic *E. coli* are also important causes (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

Dysentery

The term dysentery refers to diarrhoea with visible blood in the faeces. Important effects of dysentery include anorexia, rapid weight loss, and damage to the intestinal mucosa by the invasive bacteria. A number of other complications may also occur. The most important cause of acute dysentery is *Shigella*; other causes are *Campylobacter jejuni* and, infrequently, enteroinvasive *E. coli* or *Salmonella*. *Entamoeba histolytica* can cause serious dysentery in young adults but is rarely a cause of dysentery in young children (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

Persistent Diarrhea

This term refers to diarrhoea that begins acutely but is of unusually long duration (at least 14 days). The episode may begin either as watery diarrhoea or as dysentery. Marked weight loss is frequent. Diarrheal stool volume may also be great, with a risk of dehydration. There is no single microbial cause for persistent diarrhoea; enteroadherent *E. coli* and *Cryptosporadia* may play a greater role than other agents. Persistent diarrhoea should not be confused with chronic diarrhoea, which refers to recurrent or long-lasting diarrhoea due to non-infectious causes, such as sensitivity to gluten or inherited metabolic disorders.

2.1.2 Diarrhea Epidemiology

Transmission of agents that cause diarrhea Routes of transmission

The infectious agents that cause diarrhoea are usually spread by the faecal-oral route, which includes the ingestion of faecally contaminated water or food, person-to-person transmission, and direct contact with infected faeces. Examples of behaviours that help enteric pathogens to spread are: preparing food with hands that have been soiled during defecation and not washed; or allowing an infant to crawl, or a child to play in an area where human or animal faeces are present (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

Behaviours that increase the risk of diarrhea

A number of specific behaviours help enteric pathogens to spread and thus increase the risk of diarrhoea. These include:

- Failing to breast-feed exclusively for the first 4-6 months of life. The risk of developing severe diarrhoea is many times greater in non-breast-fed infants than in infants who are exclusively breast-fed; the risk of death from diarrhoea is also substantially greater.
- Failing to continue breast-feeding until at least one year of age. Prolonged breast-feeding reduces the incidence or severity of certain types of diarrhoeal disease, such as shigellosis and cholera.
- Using infant feeding bottles. These easily become contaminated with faecal bacteria and are difficult to clean. When milk is added to an unclean bottle it becomes contaminated; if it is not consumed immediately, further bacterial growth occurs.
- Storing cooked food at room temperature. When food is cooked and then saved to be used later, it may easily be contaminated, for example, by contact with contaminated surfaces or containers. If food is kept for several hours at room temperature, bacteria in it can multiply many times.
- Drinking water that is contaminated with faecal bacteria. Water may be contaminated at its source or during storage in the home. Contamination in the home may occur when the storage container is not covered, or when a contaminated hand comes into contact with the water while collecting it from the container.
- Failing to wash hands before handling food, after defecation, or after handling faeces.

Failing to dispose of faeces (including infant faeces) hygienically. It is often believed that infant faeces are harmless, whereas they may actually contain large numbers of infectious viruses or bacteria; animal faeces also can transmit enteric infections to man (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

2.1.3 Risk factors that increase susceptibility to diarrhea

Several host factors are associated with increased incidence, severity, or duration of diarrhoea. They include:

- Undernutrition. The frequency, severity, duration, and risk of death from diarrhoea are increased in undernourished children, especially those with severe undernutrition.
- Current or recent measles. Diarrhoea and dysentery are more frequent or severe in children with measles or who have had measles in the previous four weeks. This presumably results from immunological impairment caused by measles.

Immunodeficiency or immunosuppression. This may be a temporary effect of certain viral infections (e.g., measles), or it may be prolonged, as in persons with the acquired immunodeficiency syndrome (AIDS). When immunosuppression is severe diarrhoea can be caused by unusual pathogens and may also be prolonged (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

Age

Most diarrhoeal episodes occur during the first two years of life. Incidence is highest in the age group 6-11 months, when weaning often occurs. This pattern reflects the combined effects of declining levels of maternally-acquired antibodies, the lack of active immunity in the infant, the introduction of food that may be contaminated with faecal bacteria and direct contact with human or animal faeces when the infant starts to crawl. Most enteric pathogens stimulate at least partial immunity against repeated infection or illness, which helps to explain the declining incidence of disease in older

children and adults (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

Seasonality

Distinct seasonal patterns of diarrhea occur in many geographical areas. In temperate climates, bacterial diarrhoeas tend to occur more frequently during the warm season, whereas viral diarrheas, particularly disease caused by rotavirus, peak during the winter. In tropical areas, rotavirus diarrhoea tends to occur throughout the year, increasing in frequency during the drier, cool months, whereas bacterial diarrhoeas tend to peak during the warmer, rainy season. The incidence of persistent diarrhea follows the same seasonal pattern as that of acute watery diarrhea (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

Asymptomatic infections

Most enteric infections are asymptomatic, and the proportion that is asymptomatic increases beyond 2 years of age owing to the development of active immunity. During asymptomatic infections, which may last for several days or weeks, stools contain infectious viruses, bacteria, or protozoal cysts. Persons with asymptomatic infections play an important role in the spread of many enteric pathogens, especially as they are unaware of their infection, take no special hygienic precautions and move normally from place to place.

2.1.4 Aetiologic Agent of Diarrhea

Until a few years ago, pathogenic organisms could be identified in the faeces of only about 25% of patients with acute diarrhoea. Today, using new techniques, experienced laboratories can identify pathogens in about 75% of cases seen at a treatment facility and up to 50% of milder cases detected in the community (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

Several pathogens are important causes of acute diarrhea in all developing countries. They are:

- Rotavirus
- Enterotoxogenic *Escherichia coli*
- *Shigella*
- *Campylobacter jejuni*
- *Cryptosporidium*

Others may be of local importance; these include *V. cholerae* 01 (in endemic areas and during epidemics), non-typhoid *Salmonella* (in areas where commercially processed foods are widely used), and enteropathogenic *E. coli* (in infants in hospitals). Mixed infections involving two or more enteropathogens occur in 5-20% of cases seen at health facilities. In general, their importance as causes of acute diarrhoea in children in developing countries is either minimal or not yet well defined. They include:

- **Viruses**: Norwalk agent, enteric adenoviruses.

- **Bacteria**: *Aeromonas hydrophila, enteroadherent Escherichia coli, enteroinvasive Escherichia coli, enterohaemorrhagic Escherichia coli, Plesiomonas shigelloides, Vibrio cholerae* non-O group 1, *Vibrio parahaemolyticus, Yersinia enterocolitica.*

  Protozoa: *Giardia lamblia, Entamoeba histolytica, Isospora belli.*

  Enteric pathogens can also be found in about 30% of healthy children under 3 years of age, making it difficult to know whether a pathogen isolated from a child is actually the cause of that child's illness. This is especially true for *Giardia lamblia*, cysts of which are found nearly as often in healthy children as in those with diarrhoea; it is also true for enteropathogenic *E. coli* or *C. jejuni* isolated from children older than 1 year. On the other hand, *Shigella* and rotavirus are rarely identified in healthy children; their presence in a child with diarrhoea is strong evidence that they are the cause of the illness (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

2.1.5 Pathogenetic mechanisms

Microbial agents cause diarrhea by a number of mechanisms, several of which are considered below:

Viruses

Viruses, such as rotavirus, replicate within the villous epithelium of the small bowel, causing patchy epithelial cell destruction and villous shortening. The loss of normally absorptive villous cells and their temporary replacement by immature, secretory, crypt-like cells causes the intestine to secrete water and electrolytes. Villous damage may also be associated with the loss of disaccharidase enzymes, leading to reduced absorption of dietary disaccharides, especially lactose. Recovery occurs when the villi

regenerate and the villous epithelium matures (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016

Bacteria

Mucosal adhesion: Bacteria that multiply within the small intestine must first adhere to the mucosa to avoid being swept away. Adhesion is caused by superficial hair-like antigens, termed pili or fimbriae that bind to receptors on the intestinal surface; this occurs, for example, with enterotoxigenic *E. coli* and *V. cholerae* 01. In some instances, mucosal adherence causes changes in the gut epithelium that may reduce its absorptive capacity or cause fluid secretion (e.g., in infection with enteropathogenic or enteroadherent *E. coli)* (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

- **Toxins that cause secretion.** Enterotoxigenic *E. coli, V. cholerae* 01 and possibly other bacteria, e.g., *Salmonella,* cause intestinal secretion by producing toxins that alter epithelial cell function; these toxins reduce the absorption of sodium by the villi and may increase the secretion of chloride in the crypts, resulting in net secretion of water and electrolytes. Recovery occurs when the intoxicated cells are replaced by healthy ones after 2-4 days.

- **Mucosal invasion**. *Shigella, C. jejuni* and enteroinvasive *E. coli* cause bloody diarrhoea by invading and destroying mucosal epithelial cells. This occurs mostly in the colon and the distal part of the ileum. Invasion is followed by the formation of microabscesses and superficial ulcers, and hence the presence of red and white blood cells, or frank blood, in the stool. Toxins produced by these organisms cause tissue damage and possibly also mucosal secretion of water and electrolytes

(http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

Protozoa

Mucosal adhesion. *G. lamblia* and *Cryptosporidium* adhere to the small bowel epithelium and cause shortening of the villi, which may be how they cause diarrhea causing microabscesses and ulcers, in much the same way as *Shigella*. This only happens, however, when the infecting strain of *E. histolytica* is virulent. In about 90% of human infections the strains are non-virulent; in such cases there is no mucosal invasion and no symptoms occur, although amoebic cysts are present in the faeces (http://www.who.int/child-adolescent-health/New_Publications Accessed: November 7th, 2016).

2.2 *Escherichia coli*

Despite the fact that *Escherichia coli* as a commensal bacteria can be found in intestinal microflora of a variety of animals including man, not all the strains are harmless, and some can cause debilitating and sometimes fatal diseases in humans as well as mammals and birds (Belanger *et al.*, 2011). Pathogenic strains are divided into intestinal pathogens causing diarrhea and extraintestinal *E. coli* (ExPEC) causing a variety of infections including urinary tract infections (UTI), meningitis and septicaemia in both humans and animals (Kaper *et al.*, 2004). Cystitis and pylonephritis that can lead to urosepsis is caused by Uropathogenic *E. coli* (UPEC) which accounted for approximately 80% of the estimated 130-175 million human UTIs (Russo and Johnson, 2003). Furthermore, ExPEC are the primary Gram-negative bacterial pathogens associated with neonatal meningitis and are the second

overall cause of the disease after group B Streptococci (Furyk *et al.*, 2011). Severe neurological lesions resulting from infection with meningitis-associated *E. coli* (MNEC) leads to death in 20-40% of infected infants (Furyk *et al.*, 2011). Nosocomial bloodstream infections in hospitals and nursing homes may be caused by ExPEC strains which may also be the cause of respiratory, UTI or bacteraemia in long-term hospitalized patients (Ron, 2010). Resistance to antimicrobials has made combating these infections a major problem worldwide (French, 2010).

2.3 Bacteriology and Classification of *Escherichia coli*

2.3.1 Bacteriology

Escherichia coli is a gram negative rod-shaped bacteria which possesses adhesive fimbriae and a cell wall that consists of an outer membrane containing lipopolysaccharides, a periplasmic space with a peptidoglycan layer, and an inner cytoplasmic membrane. Some strains are piliated and capable of accepting and transferring plasmid to and from other bacteria. Such property enables *E. coli* under bad conditions to survive (Furyk *et al.*, 2011).

Even though it has extremely simple cell structure, with only one chromosomal DNA and plasmid, it can perform complicated metabolism to maintain its cell growth and division. *E. coli* can produce soluble amyloid oglimers and precipitate them as curli because *E. coli* have specific nucleation-precipitation machinery. The network of fibers can bind *E. coli* to their host cells (Ron, 2010).

Escherichia coli can cause severe infections in many animals such as humans, sheep, horses, dogs etc. The one that is only found in humans is called enteroaggregative *E. coli*. Urinary tract infection for example, can be caused by ascending infections of urethra in both adult male and female. *E. coli* O157:H7 is one of the most infective

strains. It belongs to enterohemorrhagic strain of the *E. coli* and can lead to bloody diarrhea and kidney failure. It produces a toxin which catalytically inactivates 605 ribosomal sub-units of most eukaryotic cells, blocking mRNA translation and thus causing cell death (Furyk *et al.*, 2011).

Escherichia coli is the head of the large bacterial family, enterobacteriaceae, the enteric bacteria which are facultative anaerobes, physiologically, *E. coli* is versatile and well adapted to its characteristic habitats. It can grow in media with glucose as the sole organic constituent. Wild type *E. coli* has no growth factor requirements and metabolically it can transform glucose into all of the macromolecular components that make the cell. The bacterium can grow in the presence or absence of oxygen. Anaerobically, it will grow by means of fermentation producing characteristic mixed acids and gas (Kim *et al.*, 2014).

Escherichia coli can respond to environmental signals such as chemicals, pH, temperature, osmolality etc. For example, it can sense the presence or absence of chemicals and gases in its environment and swim towards or away from them. Or it can stop swimming and grow fimbriae that will specifically attach it to a cell surface or receptor (Ron, 2010). It is a consistent inhabitant of the human intestinal tract and hence is often present in feaces which has led to tracking the bacterium in nature as an indicator of fecal and water pollution. Over 700 antigenic types (serotypes) of *E. coli* are recognized based on O, H and K antigens. Hence, the serotype O157:H7 (O refers to somatic antigen, H refers to flagellar antigen) is uniquely responsible for causing Hemolytic Uremic Syndrome (HUS) (Kim *et al.*, 2014).

2.3.2 Classification of *Escherichia coli*

Domain: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacteriales

Family: Enterobacteriaceae

Genus: *Escherichia*

Species: *E. coli*

Escherichia coli are classified based on their unique virulence factors and can only be identified as these traits. Hence, analysis for pathogenic *E. coli* usually requires that the isolates first be identified as *E. coli* before testing for virulence markers (Hodges and Ravinder, 2010).

2.4 Pathogenesis of *Escherichia coli*

An altered movement of ions and water following an osmotic gradient is at the heart of diarrheal diseases (Belanger *et al.*, 2011). Under normal conditions, the capacity of gastrointestinal tract to absorb fluid and electrolytes is tremendous and from 8-9 liters of fluid presented to intestine daily, only 100-200 ml are excreted in the stool (French, 2010). Enteric pathogens, however, can alter this balance towards net secretion leading to diarrheal disease (Hodges and Ravinder, 2010). Diarrheagenic *E. coli* strains are among the most common etiologic agents of diarrhea and based on their specific virulence factors and phenotypic traits are divided into enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), Vero toxin-producing Shiga toxin-producing *E. coli* (VTEC/STEC) which include its well-known subgroup enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), and diffusely adherent *E. coli* (DAEC).

2.4.1 Enteropathogenic *Escherichia coli* (EPEC)

History

Until the 1970s serotyping was the only means of distinguishing EPEC strains from those of normal flora, since no biochemical, microbiological or animal tests were available for their differentiation (Levine, 1987). The 12 serogroups originally recognized by the World Health Organization as EPEC or the classical EPEC were; O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158 (Hermandes *et al.*, 2009). Current classification of EPEC however, is based on the presence of specific virulence genes, which the use of molecular techniques has shown to be present in serogroup/serotypes other than classical ones as well (Ochoa *et al.*, 2008).

Pathogenesis

The distinctive histopathology induced by this group of *E. coli* is termed attaching and effacing (A/E) lesions and is caused by the intimate attachment of bacteria to the intestinal epithelial cells and effacement of enterocyte microvilli (Chen and Frankel, 2005).

Formation of the micro ulcers and exfoliation of the cells at the site of EPEC attachment was first described in experimentally infected pigs (Stanley *et al.*, 1969) and subsequently in biopsies from infected infants (Chen and Frankel, 2005). A protein called intimin mediates the bacterial attachment to outer cell membranes and is encoded by *eae* gene which along with all other genetic elements required for this phenomenon are located on the locus of enterocyte effacement (LEE), a large genomic pathogenicity Island which was discovered in 1995 (McDaniel *et al.*, 1995). The *eae* is one of the genes currently used for the molecular diagnosis of EPEC.

Pathogenesis of these bacteria however is many faceted which has not been fully unraveled as yet and may involve factors other than those directly responsible for A/E lesions as well as more specialized intestinal cells (Cravioto *et al.*, 1979; Nataro and Kaper, 1998).

Detection

Originally, HEp-2 cell-adherence assay performed with serologically defined EPEC strains showed that 80% of these strains adhere to HEp-2 cells in vitro (Cravioto *et al.*, 21979). The HEp-2 assay has been modified often since its first description. including such variations as extending the incubation time to 6 h or changing the growth medium during the incubation.

However, collaborative studies have shown that the assay performed essentially as first described provides the best ability to differentiate among EPEC, EAEC, and DAEC isolates (Nataro and Kaper, 1998).

After the introduction of the term "attaching and effacing" actin accumulation under the attached bacteria was demonstrated using *ex vivo* culturing of human intestinal biopsies (Knutton *et al.*, 1987). Staining this electron dense material produced the actin fluorescent assay (FAS) which enabled researchers to detect the ability of a strain to produce A/E lesions in vitro (Knutton *et al.*, 1989). It should however be noted that a negative FAS result may depend on the cell type used and the bacteria should be confirmed as nonpathogenic by alternative methods (Hernandes *et al.*, 2009).

The localized adherence pattern of EPEC strains was shown to be associated with the presence of a 60 MDa plasmid called pMAR2 from which a DNA fragment of 1 kb was isolated which has been used extensively in epidemiological studies (Nataro *et al.*, 1985; Levine *et al.*, 1988; Bouzari *et al.*, 2000).

The presence of the *E. coli* adherence factor (EAF) plasmid carrying *bfp* operon, encoding the type IV bundle-forming pilus (BFP), and *per* operons, a transcriptional activator called plasmid encoded regulator (Per) is the basis of typical and atypical classification of EPEC strains (Trabulsi *et al.,* 2002).

Recently in a study done in Iran, multiplex PCR was used to differentiate between tEPEC, and aEPEC and PCRRFLP for H typing of conventionally serogrouped isolates (Bouzari *et al.,* 2011) showing the ease and applicability of this method for rapid screening of large number of isolates.

Epidemiology

Although EPEC are among the most important pathogens infecting children less than 2 years of age in the developing world, but the prevalence may vary depending on differences in study population, age group, diagnostic criteria and diagnostic tools used (Ochoa and Barletta, 2008). Over the last several decades, the significance of EPEC infection has declined in published literature. The decline might be due to interventions, particularly breast-feeding promotion, or to the overestimation of these organisms in earlier studies that used O- or O:H typing compared to the recent ones, in which molecular methods and/or adherence assays was used for EPEC diagnosis (Okeke, 2009; Ochoa and Contreas, 2011). A study conducted in the south of Iran (Katouli, 1988) using serological test alone for identification of EPEC reported an isolation rate of 30.7% for this pathotypes in children of less than 3 years old with diarrhea. The isolates belonged to 12 different serogroups, of which O128 and O126 were the most prevalent. A similar study published 2 years later reported EPEC as the most common pathogen among patients admitted to 4 children's hospital in Tehran and those attending an outpatient clinic in Sanandaj with an isolation rate of 26.7 and 20.1% respectively (Katouli *et al.,* 1990). Recent studies dealing with diarrhea in

children under 5 years of age have reported varying rates of isolation for EPEC ranging from 12.6-44.9% showing a significant association between EPEC isolation and diarrhea (Alikhani *et al.*, 2007, and 2008). A noticeable reduction in isolation rate of EPEC was reported in 2005 in which 200 children suffering from acute diarrhea with age-matched controls were studied in Tehran and EPEC was isolated from 6% of patients and 5% of controls (Salmanzadeh *et al.*, 2005).

Various studies carried out in Iran and abroad have shown a significant association between EPEC and infant diarrhea compared to controls (Alikhani *et al.*, 2007, 2007, and 2008), but similar isolation rates from diarrheal cases and controls have also been documented. However, in none of these studies the typical and atypical EPEC have been separately addressed, except for the work by Alikhani *et al.*, 2008). The data obtained showed a significant association between both typical and atypical EPEC and diarrhea. In both developed and developing countries an increasing isolation rate for a EPEC has recently been reported, but the epidemiological association of this group with childhood diarrhea is controversial and requires further studies (Ochoa and Contreas, 2011).

In developing countries healthy carriage of enteric pathogen is shown to be common and has been attributed to the interplay of many factors including host susceptibility (related to the child's age, breastfeeding, nutritional and immunological status), bacterial virulence factors (different virulence genes), and environmental factors (poor hygiene, and high fecal contamination). However, data from a recently developed quantitative real-time PCR (qRT-PCR) has shown a significantly higher bacterial load in EPEC associated diarrheal samples compared to controls (Ochoa and Contreas, 2011). Prolonged and persistent episodes of diarrheal disease (diarrhea > 14 days) constitute a significant portion of the global burden of diseases in children (Ochoa and

Contreas, 2011). A recent systematic review of the illness in developing countries has shown that EPEC, ETEC and EAEC are the main pathogens associated with this complication, and are responsible for 30-40% of all persistent diarrheal episodes in children (Abba *et al.,* 2009).

In developed countries, aEPEC is the most common pathogen isolated from children with persistent diarrhea accounting for more than half of the episodes (Nugen *et al.*, 2006). The role of aEPEC in persistent diarrhea in most countries has not been dealt with systematically.

2.4.2 Enterotoxigenic *E. coli* (ETEC)

History

ETEC is the most important but under-recognized bacterial cause of diarrhea or cholera-like disease in all age groups in areas with poor sanitation and inadequate clean water. Furthermore, of the estimated one billion yearly international travelers. 20-60% of those traveling to low-income countries will suffer from travelers' diarrhea (Hill and Beeching, 2010). In approximately 30-70% of traveler's diarrhea bacteria are the causative agent, of which ETEC are the most commonly detected (Wiedermann and Kollaritsch, 2006). Development of rabbit ileal loop assay which led to the discovery of cholera toxin was also used for pure cultures of *E. coli* isolated from stools and small bowels of children and adults showing similar symptoms to cholera. Live cultures and culture filtrates of these strains when injected into isolated rabbit ileal loops produced strong choleralike secretary response leading to the discovery of the heat-labile enterotoxin of *E. coli* and recognition of ETEC pathotype in 1968 (De *et al.*,1956; Sack, 2011).

Pathogenesis

ETEC strains adhere to intestinal epithelial cells via a heterogenous group of proteinacious surface structures termed colonization factors (CFs) which can be fimbrial, non-fimbrial or fibrillar (Croxen and Finlay, 2010). The more recent nomenclature refer to these structures as coli surface (CS) antigen, but some of the old names still persist such as colonization factor antigen I (CFA/I). Despite the fact that more than 25 CFs have so far been identified, on many strains no CF is detected which might be attributed to the technique (s) used for detection, true lack of CFs or as yet unidentified ones (Isidean *et al.*, 2011). Following the initial adhesion and colonization, ETEC strains cause diarrhea by producing heat-labile (LT) and/or heat-stable (ST) enterotoxins, which are plasmid-encoded (Johnson and Nolan, 2009). ETEC bacteria produces the small STs as a 72-amino acids preprotoxin which is processed into an 18-19 amino acid active toxin called STa and a 42 amino acid toxin referred to as STb. STa is produced by both human and animal strains, whereas STb is mainly detected in strains of veterinary origins (Nagy and Fekete, 2005). LT like the closely related cholera toxin is a member of AB5 family of toxins which are heterohexameric molecules consisting of five B subunits and a single A subunit (Beddoe *et al.*, 2010). The A1 domain constitutes the active toxin and is linked to the A2 domain via a disulfide bond (Fleckenstein *et al.*, 2010). The A2 fragment is the helical portion of the molecule and anchors the A subunit to the B pentamer which binds irreversibly to GM1 ganglioside as receptors on cell surface (Nagy and Fekete, 2005). The toxin is then internalized and the A subunit ADP-ribosylates the stimulatory guaninenucleotide- binding protein, increasing the levels of intracellular cyclic AMP resulting in diarrhea.

Detection

Diagnosis of ETEC is based on the production of LT and/or ST and the rabbit ileal loop and infant mouse physiological assays were initially used as gold standards for the identification of these enterotoxins respectively. These tests are difficult to perform and time consuming and for a while efforts were made to use serotyping for this purpose, but soon it became clear that a large number of serotypes could be enterotoxigenic and therefore not applicable (Qadri *et al.*, 2005).

In 1974 it was found that LT produces morphological changes on Y1 adrenal and Chinese hamster ovarian cell lines that were neutralizable by antitoxin (Donta and Smith, 1974; Guerrant *et al.,* 1974). Although these tissue culture tests were used in preference to the animal models, but these assays were only useful for LT detection and not available in all laboratories making ETEC detection problematic.

Enzyme-linked immunosorbant assay, passive latex agglutination, immune-precipitation in agar and Biken test were developed subsequently and were found to be specific (Yolken, *et al.,* 1977; Honda *et al.*, 1981). PCR has revolutionized clinical diagnosis of pathogenic microorganisms and was used in 1994 for detection of ETEC strains (Wever *et al.,* 1994) but prior to the advent of PCR methods radioactively and non-radioactively labeled probes were used for detection of enterotoxin genes and the method was shown to be both sensitive and specific (Hill *et al.,* 1983).

Different methods have been used for CF detection, including mannose-resistant agglutination of certain species of erythrocytes, serological tests initially using polyclonal sera (Ahren *et al.,* 1986) which were subsequently replaced by monoclonal antibodies and eventually molecular methods (Sommerfelt *et al.*, 1996). These methods were used in a study conducted in Iran to characterize ETEC strains with ten years difference in isolation date and a high degree of agreement was observed

between the results obtained using different detection methods (Shahrokhi *et al.*, 2011). In 1992 however, both mannose-resistant hemagglutination and polyclonal antisera for CFA/I and CFA/II were used for detection of these antigens among Iranian ETEC isolates and it was concluded that hemagglutination was not specific enough for characterization of these fimbriae (Katouli *et al.*,1992). Besides determination of the toxins and CFs, serotyping, i.e. determination of O serogroups associated with the cell wall lipopolysaccharides and H serogroups of the flagella, has been applied for identification and characterization of ETEC (Orskov *et al.*, 1978). However, as shown in studies conducted in different countries, clinical ETEC isolates may belong to a large number of serotypes making this method unsuitable for identification of these bacteria. Furthermore, ETEC serotype profiles may change over time (Qadri *et al.*, 2005).

Epidemiology

The first report of ETEC in a case series of infantile diarrhea in Iran was made in 1982 when Mohadjer *et al.* (Mohadjer *et al.*, 1982) detected LT and ST in the isolates using rabbit ileal loop, Y1 adrenal cell culture and infant mouse assay. The isolation rate for this pathogen was 7% and subsequently using rabbit ileal loop and infant mouse assay in a case series study ETEC was detected in 21.9% of diarrheal cases attending 12 outpatient clinics in Bandar Abbas (Katouli *et al.*, 1988). Detection of various toxin genes among 200 *E. coli* isolates from diarrheal cases in Tehran using Dig-labeled probes showed that LT and ST carrying isolates were the least frequent pathotypes (Bouzari *et al.*, 2007). Recently, however, PCR has been the detection technique of choice and the rate of ETEC in diarrheal cases in studies using this method varied from nearly 33% and 15.5% to less than 10% (Jafari *et al.*, 2008). Unfortunately, despite the availability of various techniques, there are still no simple,

readily available methods for identification of these organisms in minimally equipped laboratories or the field. Therefore, ETEC is not included in routine diagnosis of diarrhea in many laboratories.

2.4.3 Shiga toxin-producing/ Enterohaemorrhagic *E. coli* (STEC/EHEC)

History

The main virulence factor and the defining feature of this group is a phage-encoded potent cytotoxin the effect of which was shown to be neutralizable by anti-Shiga toxin of *Shigella dysenteriae* 1. The cell toxicity effect was also demonstrated on Vero cells resulting in a parallel nomenclature system of Shiga/Vero toxin-producing *E.coli* (STEC) and (VTEC) respectively (Karmali *et al.,* 2010). In 1983, an *E. coli* strain serotype O157:H7, was identified in association with outbreaks of a bloody diarrhea called hemorrhagic colitis (HC) leading to the recognition of EHEC as a new and increasingly important class of enteric pathogens causing intestinal and renal disease (Nataro and Kaper, 1985). The term enterohaemorrhagic *E. coli* (EHEC) is applied to those STEC serotypes that have the same clinical, epidemiological and pathogenetic features associated with the prototype strain *E. coli* O157:H7.

The high virulence of STEC strains such as O157:H7 is not only dependent on the virulence factors but partially also on the pathogen's ability to survive environmental stress conditions, such as resistance to low pH levels found in the gastrointestinal tract contributing to its very low infectious dose of 50-100 bacteria or lower (Viazis and Diez-Gonzalez, 2011). Among STEC serotypes, O157:H7 is associated with both outbreaks and sporadic cases of severe disease, but it has been shown that other serotypes may also cause human infections albeit variably (Coombs *et al.,* 2011).

This quantitative and qualitative difference in disease association among STEC has given rise to various classification schemes the simplest of which divides STEC into

E. coli O157 and non-O157. However, in view of the fact that the virulence potential of non- O157 might be genetically determined a seropathotype (SPT) classification has been proposed in which prior association with human epidemics, HUS, and diarrhea is considered (Coombs *et al.,* 2011). In this scheme, SPT-A includes O157:H7 and O157:NM, the most commonly isolated serotypes from outbreaks and HUS. SPT-B strains differ from group A in the frequency of isolation from outbreaks and HUS cases, SPT-C strains are only associated with sporadic cases of HUS, SPT-D are isolated from diarrheal cases and have not been encountered in outbreaks or HUS and SPT-E that have never been associated with human disease (Karmali *et al.*, 2003).

Furthermore, data collected using different methods of comparative genomic have suggested that several discreet genotypes differing in virulence exist within *E. coli* O157:H7 population and based on these data this serotype has been subdivided into nine clades (Zhang *et al.*, 2007; Manning *et al.,* 2008).

Recently a new Shiga toxin producing *E. coli* strain was identified in Germany causing one of the largest outbreaks of HUS worldwide. The perpetrator belonged to serotype 104:H4 which contained the virulence factors of typical EAEC and a Stx-2 producing prophage, but lacked the LEE pathogenicity island. This discovery has led to the emergence of a new pathotype for which the name Entero-Aggregative-Haemorrhagic *Escherichia coli* (EAHEC) has been suggested (Wu *et al.*, 2011; Brzuszkiewicz *et al.*, 2011). This event exemplifying the genome plasticity of *E. coli* has highlighted the need for public health surveillance of STEC infections and its important role in devising and implementing control measures.

Pathogenesis

Shiga toxin family with related structure and similar biological activity is composed of Stx1 which is essentially identical to the toxin of *Shigella dysenteriae* differing in a single amino acid and Stx2 with less than 60% amino acid homology to Stx1 (Caprioli *et al.*, 2005). Little sequence variation has been reported for Stx1 (Zhang *et al.*, 2002), but Stx2 has several subtypes which differ in biological activity and immunological reactivity (Fraser *et al.*, 20004). Shiga toxins similar to the heat-labile enterotoxin of ETEC belong to the AB5 family of the toxins and consist of a pentameric ring-shaped B subunit that is non-covalently attached to the A subunit. The B subunit interacts with globotriaosylceramides (Gb3s) on the surface of human intestinal mucosa and kidney epithelial cells resulting in the internalization of the toxin where the A subunit is activated causing cell death. Among the Stx2 variants, Stx2c has been isolated more frequently from HUS patients but Stx2e and Stx2f have been mainly isolated from pigs and birds and rarely from humans (Caprioli *et al.*, 2005). Moreover, a different AB5 toxin has been discovered in this group which differs significantly from other toxins in this group. This subtilase-like toxin (SubAB) was isolated from an HUS outbreak strain in Australia and shows greater cytotoxicity than Stx2 for a range of cell types including Vero cells (Beddoe *et al.*, 2010).

The EHEC genome contains the same locus of enterocyte effacement (LEE) as the EPECs and the intimate attachment of EHEC to host cells occur through interaction between an adhesin called intimin (*eaeA*), and Tir (translocated intimin receptor). This intimate attachment induces the characteristic attaching and effacing lesions (A/E), but the initial adherence of EHEC to colonocytes is not well defined (Croxen *et al.*, 2010). Sixteen potential fimbria-like operons, which have not been extensively studied have been recognized in STEC strains (Low *et al.*, 2006), and recently a pilus

involved in adherence and biofilm formation called hemorrhagic coli pilus, a type IV pilus, has also been identified in STECs (Xicohtencatl *et al.*, 2009). However, the intimate adherence as in EPECs is through the interactions between Tir and intimin. At least 29 distinct intimin types with heterogeneity in the C-terminal part of the molecule that is involved in binding to Tir in both STEC and EPEC have so far been identified (Mora *et al.*, 2009). The ability of STEC to produce A/E lesions is sufficient to cause non-bloody diarrhea but Shiga toxin is essential for the development of bloody diarrhea, HC, and HUC. Another toxin found in many STEC/EHEC isolates is the enteroaggregative heat-stable enterotoxin1 (EAST1) and usually two copies of the *astA* gene is present in the chromosome (Viazis *et al.*, 2011). The significance of this carriage in the pathogenesis of EHEC is unclear, but it has been suggested that some of the non-bloody diarrhea in person infected with these strains might be due to the production of this toxin (Gyles *et al.*, 2007). The primary virulence determinants of EHEC strains are chromosomally encoded, but plasmids might play an important role in the pathogenesis of EHEC strains. Plasmid pO157 is found in 99-100% of O157:H7 serotype isolated from human clinical isolates, most not all Stx-producing isolates. Presence of this plasmid has been correlated with hemolytic activity and adherence to intestinal epithelial cells, but the overall understanding of the role of plasmids in pathogenesis of STEC/EHEC strains is hindered due to the absence of a reliable model of human infection (Lim *et al.*, 2010).

Detection

Laboratory confirmation of STEC infection can be achieved by isolation and confirmatory tests using culture media, immunoassays, cell toxicity assays and PCR (Gould *et al.*, 2009). Screening of O157 relies on the strain's inability to utilize sorbitol rapidly, leading to the use of sorbitol-MacConkey agar (SMAC) as a

differential medium with added cefixime and tellurite (CT-SMAC) although in our setting addition of cefixime has not led to the prevention of other fecal associated microorganisms. More specific media have also been developed such as Rainbow agar, CHROMagar, and O157:H ID agar that are able to recover O157 along with sorbitol-fermenting O157 and non-O157 strains (Gould *et al.*, 2009). Tests conducted in our laboratory, however, has shown that to prevent the growth of some bacterial strains such as Salmonella which could produce similar colonies on CHROMagar plates the use of tellurite is necessary.

The identity of potential STEC isolates should be assessed by serotyping and Shiga toxins detection methods. Cell toxicity assay using Vero and HeLa cell lines for Shiga toxin in stool samples or broth enrichment is a very sensitive method since these cell lines have high concentrations of globotriaosylceramides Gb3 and Gb4 which are the receptors for Shiga toxin. Neutralization tests using antibodies against Stx-1 and Stx-2 confirms the results obtained, but this test despite high sensitivity is not routinely used due to its high cost, labor intensity and the expertise required. PCR however offers a fast and reliable method for detection of STEC which similar to immunoassay tests can be used directly with stool samples as well as isolated colonies and depending on the primers used can distinguish between *stx*1 and *stx*2 and detect *eae* and enterohemolysin (*hly*) genes.

Use of PCR on DNA extracted from whole stool however, is not recommended because of the low sensitivity (Gould *et al.*, 2009).

STECs are the only zoonotic *E. coli* pathotype and more than 380 different OH serotypes have now been isolated from humans with gastrointestinal disease and many of these as well as others have been recovered from animals. However, majority of human disease appear to be caused by a limited number of serotypes with

frequency varying depending on the location and the year (Gould *et al.*, 2009). Serogroups O26, O45, O91, O103, O111, O113, O121, O145 are listed as the most commonly encountered non-O157 STEC-associated O antigens (Karmali *et al.*, 2010; Gould *et al.*, 2009).

Epidemiology

Enterohaemorrhagic *E. coli* have been associated with several large outbreaks in US, Canada, Europe and Japan (Viazis *et al.*, 2011; Caprioli *et al.*, 2005), but apart from some sporadic reports on isolating this organism that have not been confirmed by any reference laboratory, no outbreaks or epidemics has been reported from Iran. Detection of this pathotype has been reported in various studies using different screening methods. Neutralization of Stx-1 and Stx-2 was used for identification of STEC strains in various provinces of Iran and showed a varying rate of detection for this pathotype in different locations but O157:H7 serotype was not isolated in any of these provinces (31, 89-90). PCR-detection of STEC isolates have been reported in various studies dealing with diarrhea in Iran with varying rates in different years and study groups (Sharifi-Yazdi *et al.*, 2011; Bonyadian *et al.*, 2010). Isolation of serologically confirmed O157:H7 was published in 2008, in a study conducted in Zahedan using sorbitol fermentation as primary isolation criteria and serotyping for confirmation. Of the 4 strains that gave positive reaction with antisera against O157, two were identified as O157:H7 (Fard *et al.*, 2008). However, detection of this bacterium requires an array of different tests and cultures, a combination of molecular and classic methods. Therefore any identification relying on a single method should be considered with caution. Moreover, serotyping for O and H determination especially interpretation of H serology results requires expertise and trained personnel

and in view of the fact that so far no epidemics or large outbreaks for this organism have occurred, isolation reports should be assessed more critically.

2.4.4 Enteroinvasive *E. coli* (EIEC)

History

Bacillary dysentery as opposed to dysentery caused by amoeba was described in 1887 and *Bacillus dysenteriae* as the causal agent was described in 1898 by Shiga during an epidemic of 89,400 cases (Peng *et al.*, 2009).

The medical importance of *Shigella* strains led to their separation from *E. coli* and the newly formed genus with its 4 species could be differentiated from *E. coli* on the basis of physiological and biochemical characteristics. However, the discovery of strains which could cause dysentery and were intermediate between *Shigella* and *E. coli* in biochemical characteristic in 1944 caused the separation of the two genera to be questioned (Van *et al.*, 2012). The ability of these strains which by now were called enteroinvasive *E. coli* (EIEC) to cause diarrhea was demonstrated in volunteer studies in 1971 (Nataro and Kaper, 1998). It has been shown that EIEC strains and *Shigella* species are biochemically, genetically, and pathogenetically very closely related so much so that it has been proposed that they should be classified as one species in genus *Escherichia* (Benner *et al.*, 1972; Lan *et al.*, 2004).

Pathogenesis

Acquisition of the invasive plasmid (pINV) encoding the ability to invade host tissues (Silva *et al.*, 1980) is probably the single most important event that has probably given rise to the evolution of both *Shigella* and EIEC from non-pathogenic *E. coli*. Nearly one third of this large single copy plasmid encodes IS elements and contains a 30 kb region enabling the bacteria to invade intestinal epithelial cells (Sansonetti, 1992). Many components of type three secretion system (T3SS) such as translocators,

transcriptional activators, some effectors and chaperones are coded by this region with the expression of the Invencoded genes being regulated globally by VirB and MxiE (Johnson and Nolan, 2009). In addition to the genes of pINV many chromosomal genes which are not specific to *Shigella* spp. and are carried on the chromosome are required for pathogenesis (Sansonetti, 1992). Colonic mucosa is the infection site of *Shigella* and EIEC where invasion of M cells, macrophages and epithelial cells occur resulting in a watery diarrhea, which in severe cases may be followed by the onset of scanty dysenteric stools containing blood and mucus (Nataro *et al.*, 1995). EIEC strains may also produce a 63 kDa toxin designated Sen which contributes to the enterotoxic activity detected in the strains carrying the gene (Venkatesan *et al.*, 1989).

Detection

There are very few biochemical characteristics that differentiate *Shigella* and EIEC from each other and the two most convenient are mucate and acetate tests. EIEC may be positive for either or both, whereas with rare exceptions *Shigella* strains are negative for both (Lan *et al.*, 2004). Salicin fermentation and esculin hydrolysis have also been used to differentiate the two groups (Van *et al.*, 2012). The serotypes associated with EIEC include O28ac, O29, O112ac, O121, O124, O135, O136, O143, O144, O152, O159, O164, O167, and O173 of which O112ac, O124, and O152 are identical to O antigens present in *Shigella* species making identification on the basis of serotyping alone inadequate (Lan *et al.*, 2004). The ability to cause keratoconjunctivitis in guinea pig eyes and to form plaques in HeLa cell monolayers were the standard methods of identification for EIEC isolates. However, molecular methods have replaced these phenotypic assays (Knutton *et al.*, 1989) including amplification of a multicopy gene (4-10 copies) called *ipaH* with copies located on both plasmid and chromosome (Hartman *et al.*, 1990; Horakoya *et al.*, 2008). This

assay distinguishes EIEC and *Shigella* from other diarrheal pathogens but efforts have been made to develop molecular methods to discriminate between the two microorganisms resulting in development of conventional, multiplex as well as real-time PCR methods for this purpose (Yamazaki and Fukasawa, 2011; Kauer *et al.*, 2010).

Epidemiology

No epidemics and no recent reports of outbreaks caused by EIEC is found in the literature, although some references to older works dealing with outbreaks can be found and due to close similarity between the two organism misidentification is very probable especially in sporadic cases (Nataro and Kaper, 1998). A mPCR assay targeting *ipa*H was used recently to differentiate EIEC strains from other *E.coli* categories in cases of childhood diarrhea in Tehran (Sharifi *et al.*, 2011) showing a 13% isolation rate for *E.coli* of which 19.4% gave positive results with *ipa*H primers.

2.4.5 Enteroaggregative *E. coli* (EAEC)

History

This pathotypes is the most recently identified diarrheagenic *E coli* and is the second most common cause of travelers' diarrhea after ETEC in both developed and developing countries. EAEC are commonly being recognized as a cause of endemic and epidemic diarrhea worldwide and recently, has been shown to cause acute diarrheal illness in newborns and children in industrialized countries.

This organism has also been associated with persistent diarrhea. Diarrhea caused by EAEC is often watery, but it can be accompanied by mucus or blood (Weintraub, 2007).

The discovery of EAEC as well as diffusely adherent *E. coli* (DAEC) stemmed from the studies showing that EPEC adhere to HEp-2 cells in a distinctive pattern (Cravioto

et al., 1979). Examination of a collection of diarrheal *E. coli* strains that were not of EPEC serogroups showed that many of these strains also adhered to HEp-2 cells and the phenotype was different from that of EPEC (Nataro *et al.*, 1987). This pattern of adherence, which had been called "diffuse" was subsequently subdivided into aggregative and true diffuse adherence (Bouzari *et al.*, 1994). *E. coli* showing aggregative adherence (AA) are autoagglutinating, but their hallmark is aggregative adhesion, which involves the formation of a stacked-brick pattern on HEp-2 cells. In a study in Iran Bouzari *et al.* (Boisen *et al.*, 1992) reported that 32% of diarrheagenic *E. coli* isolated from infants and children which did not belong to any known *E. coli* pathotypes formed AA pattern on HeLa cells and showed a significant prevalence in children with diarrhea compared to controls.

Pathogenesis

Lack of suitable animal models and the heterogeneity of virulence factors caused the paucity of details regarding the EAEC transmission, pathogenicity and epidemiology. However, colonization of intestinal mucosa, mucoid biofilm formation and elaboration of various enterotoxins, cytotoxins and mucosal inflammation are considered the major features of EAEC pathogenesis (Huang *et al.*, 2006). Colonization of intestinal mucosa by the EAEC occurs via aggregative adherence fimbriae (AAF) encoded by a 55-65 MDa plasmid named pAA. The first one of which, aggregative adherence fimbriae I (AAF/I), was cloned and characterized from EAEC prototype strain 17-2 (Scaletsky *et al.*, 1984; Czeczulin *et al.*, 1997). A probe derived from this adhesin did not recognize O42, the second EAEC prototype and subsequently a new fimbria was characterized in this strain called AAF/II (Bernier *et al.*, 2002).

Although two other adherence factors (AAF/III and AAF/IV) as well as a non-fimbrial adhesin have been described but some strains are encountered that do not contain any of these known fimbriae despite showing AA phenotype which is indicative of the as yet uncharacterized adhesins. (Scaletsky *et al.,* 1984), Similar to ETEC strains adhesion of EAEC to intestinal tissue is mediated by antigenically heterogenous adhesins and multiple carriage of AAFs by an EAEC strain has been rare (Bouzari *et al.,* 2005). A transcriptional activator known as "AggR," encoded by pAAs, regulates the biogenesis of AAFs (Sheikh *et al.,* 2001) and is the major EAEC virulence regulator controlling diverse virulence genes encoded by pAAs as well as by chromosomes (Scaletsky *et al.,* 1984). Adherence of EAEC to the mucosa is characterized by the formation of a thick, aggregating mucus layer inside which they survive and this biofilm production has been attributed to the activity of *fis* and *yafK* genes (Nataro *et al.,* 1992). However, a secreted 10 kDa protein encoded by pAA and called antiaggregation protein (Aap) or dispersin, facilitates the movement of bacteria across the surface of the cells for subsequent aggregation and adherence (Johnson and Nolan, 2009). Dispersin is highly immunogenic and is translocated via an ATP binding cassette (ABC) transporter complex (the Aat apparatus), both these genes have been used for identification and classification of EAEC isolates, but it has been noted that dispersin gene (*aap*) can be detected in DAEC as well as non-pathogenic *E.coli* (Wakimoto *et al.,* 2004).

Detection

The ability of EAEC to form biofilm has been utilized in an assay which has been suggested as a screening test in both clinical and epidemiological studies (Weintraub, 2007). Formation of biofilm however, was shown to be method dependent and strongly influenced by culture media, leading to the conclusion that considering the

experimental variables the results need to be interpreted cautiously (Henderson *et al.*, 1999).

The aggregating nature of this pathovar has made serotyping in many cases impractical and the fraction that can be serotyped belong to a wide range of O:H types, making serotyping of little use in EAEC diagnosis (Harrington *et al.*, 2006). Bacterial adhesion is followed by the secretion of various toxins of which the plasmid-encoded toxin (Pet), a serine protease causing cytoskeletal rearrangements and EAST1, an activator of guanylate cyclase, are regulated by AggR. Within EAEC group, different pathogenicity islands have been identified including *she* pathogenicity island of *Shigella*, containing enterotoxin and mucinase genes, and *Yersinia* high-pathogenicity island, containing the yersinibactin siderophore gene (Baudry *et al.*, 1990). None of these genes however is present in all the EAEC strains and many of them are not specific for this *E. coli* category which makes developing an alternative method to HEp-2 cell adherence assay difficult. In 1990 a diagnostic probe obtained from the aggregative plasmid of strain 17-2 was reported by Baudry *et al.* (Bouzari *et al.*, 2001). The cryptic 1-kb probe known as "CVD432" or aggreagative probe (AA) which was later shown to correspond to the site of Aat transporter complex performed variably in different locations. This probe was used by Bouzari *et al.* (Schmidt *et al.*, 1995) on a collection of 98 HeLa cell assay-confirmed EAEC isolates of which only 46 (46.9%) reacted with the probe. A PCR method using primers based on the probe sequence was developed by Schmidt *et al.* (Alizadeh *et al.*, 2007) and its sensitivity and specificity was reported as similar to the AA probe. This method has been used by several authors in Iran for detection of EAEC in patients with diarrhea but only one report used PCR in combination with HeLa cell adherence (Boisen *et al.*, 2012). This group used CVD432 PCR for the preliminary

screening of the isolates which were further analyzed for adherence to HeLa cells and found that the PCR method showed 100% sensitivity and 98.4% specificity.

Heterogeneity among EAEC strains in their carriage of putative virulence factors have been well established (Scaletsky *et al.,* 1984), but in view of the pivotal role played by *aggR* in regulating a large number of virulence factors and its location on pAA, strains positive for this gene are called "typical EAEC" and strains lacking pAA, but showing the characteristic stacked-bricks phenotype in HEp-2 cell adherence assay are considered "atypical EAEC" (Scaletsky *et al.,* 1984). Typical EAEC has been associated with diarrhea, but in an extensive genomic analysis of EAEC strains isolated from a case-control study conducted in Mali with children under the age of 5 suffering from moderate to severe diarrhea presence of *aggR* regulon genes was not correlated with diarrhea (Le and Servin, 2006).

The importance of EAEC in diarrheal diseases in various epidemiological and clinical settings and the unusual degree of heterogeneity among EAEC isolates in carrying various putative virulence factors has been well documented. However, data pertaining to the role of individual factors and their contribution in conferring distinct clinical outcomes are required for a true assessment of EAEC as a human pathogen (Boisen *et al.,* 2012).

2.4.6 Diffusely adherent *E. coli* (DAEC)

DAEC is a heterogenous group that generates a diffuse adherence pattern on HeLa and HEp-2 cells and has been associated with the watery diarrhea that can become persistent in young children in both developing and developed countries as well as recurring urinary tract infections (Servin, 2005). It has been shown that the relative risk of diarrhea associated with DAEC increases with age of children from 18 months to 5 years. The intestinal carriage of these strains has also been reported to be

widespread in older children and adults. The consequences of this persistence are unknown, but several observations have suggested a potential role in the development of chronic inflammatory intestinal disease (Servin, 2005).

Two types of adhesins mediating the DA pattern have so far been described dividing the DAEC strains into AIDA-I-dependent group and those that their adhesins is encoded by a family of related operons, which include both fimbrial and afimbrial adhesins. These groups of proteins are collectively designated Afa-Dr adhesins (Nowicki *et al.*, 1987).

The first afimbrial adhesin (*afa*) operon belonging to this group was characterized and sequenced in 1984 (Nowicki *et al.*, 1987), and subsequently another operon in this family as well as the adhesins receptor were described (Nowicki *et al.*, 1990). AIDA-I is a 100 kDa outer membrane protein which is associated with DA phenotype and was described by (Benz *et al.*, 1992) who also showed that this adhesin was not commonly encountered among DEAC isolates. The *afa/dr/daa* operons are genes that arise and are expressed in a variety of genetic backgrounds (Servin, 2005) and the pathogenesis of DAEC seems to be predominantly mediated through Afa/Dr adhesin interactions with host cells. In addition a secreted autotransporter toxin (Sat) has also been implicated in pathogenesis, but nevertheless, the implication of Afa/Dr DAEC strains in diarrhea remains controversial. Phenotypic detection of DEAC is based on the mannose-resistant diffuse adhesion of these strains to cultured epithelial HEp-2 or HeLa cells (Bouzari *et al.*, 1994). The adhesion assay however, is not specific for Afa/Dr DAEC detection, since other pathogenic *E. coli* including EPEC strains may show this pattern of adhesion (Bouzari *et al.*, 2000).

Other phenotypic assays have also been developed, but none has proved convenient and universal to be used for identification of all Afa/Dr DAEC isolates (Labigne *et*

al., 1984). Colony hybridization using various probes have also been developed and used in epidemiological studies (Le *et al.*, 2001), but this technique is laborious and time consuming and not suitable for use on individual strains. Design of PCR methods that allow identification of all known Afa/Dr adhesins has been achieved (Le *et al.*, 2001), but even with this simpler and faster method no report of Afa/Dr DAEC isolation in Iran has been published (Le *et al.*, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Equipment

The equipment that was used include: Incubator (BIO-RAD Laboratories, USA); Electronic Heating Block (WEALTEC Corp); Micro-centrifuge (DENILLE 260D); Elite 300 plus (WEALTEC), Ultraviolet Trans-illuminator (EUROPE GmbH); Dano Drop; Microwave (Cleaver Scientific Ltd. CSL-AG500); Refrigerator (African Advice.Com); Autoclave (Fedegari Autoclavi SpA, Italy); Weighing Balance (Scale Tech. South Africa); Hot-air Oven (Sree Valsa Enginneering Company, India); Centrifuge (Beijing Era Beili Centrifuge Co; Ltd. BEILI); Pipette Adjustable (Qindao Hiprove Medical Technologies Co; Ltd. China); and Microscope (HOVER LABS™ India).

3.1.2 Media

Media such as Eosine Methylene Blue agar (BIOTEC Laboratories Ltd, Ipswish, United Kingdom); Sorbitol MacConkey agar and MacConkey agar (without salt) (BIOTEC Laboratories Ltd, Ipswich, United Kingdom); Luria-Bertani Broth (Inqaba Biotec, South Africa); Simmons Citrate Agar (BIOTEC Laboratories Ltd, Ipswish, United Kingdom); Peptone Water (BIOTEC Laboratories Ltd, Ipswish, United Kingdom); Agarose gel (Gibco Life Technologies, Paisley, United Kingdom); and Nutrient agar (Merck KGaA, Darmstadt, Germany) and Methyl red- Voges Proskauer (MR-VP) media (BIOTEC Laboratories Ltd, Ipswish, United Kingdom) were used.

3.1.3 Glass ware

Glass ware such as Petri dishes; Bijou bottles; conical flask; Measuring cylinder; and Beaker were used.

3.1.4 Reagents

Sodium Chloride (NaCl) (Himedia Laboratories Pvt. Ltd, India), Ethanol (Fluka Sigma-Alduch Chemie Gmbti Buscits Switzerland), Kovac's reagent (Himedia Laboratories Pvt. Ltd, India), Methyl red, Ethanol Potassium hydroxide (KOH) (BDH laboratory England), Crystal violet and Safranin (Himedia Laboratories Pvt. Ltd. India), Potassium iodide and Iodine crystal (Himedia Laboratories Pvt. Ltd, India). Ethidium bromide (Inqaba Biotec, South Africa), Trisboris EDTA, Molecular marker (1-kb DNA ladder; Gibco/BRL) and Beta-Napthol (Burgoyne Burbidges and CO. India Mumbai)

3.1.5 Primers

The primers, the gene sequence and their amplicon sizes for different pathotype of E. coli as described by Nguyen *et al.* (2005) were used. The primers were product of Inqaba biotec, South Africa as shown in Table 3.1

3.1.6 Reference strains of *Escherichia coli*

The reference strains of diarrheagenic and non-diarrheagenic *E. coli* and their target genes were provided by the Collaboration Project between Sweden and Nicaragua on Diarrhea and is as shown in Table 3.2

Table 3.1: Primers and Aplicon size for different pathotypes of *Escherichia coli*

Primer	Target gene	Primer sequence	Amplimer size (bp)
LT	*eltB*	5'-TCTCTATGTGCATACGGAGC-3'	322
		5'-CCATACTGATTGCCGCAAT-3'	
ST	*estA*	5'-GCTAAACCAGTAGAGGTCTTCAAAA-3'	147
		5'-CCCGGTACAGAGCAGGATTACAACA-3'	
VT1	*vt1*	5'-GAAGAGTCCGTGGGATTACG-3'	130
		5'-AGCGATGCAGCTATTAATAA-3'	
VT2	*vt2*	5'-ACCGTTTTTCAGATTTTGACACATA-3'	298
		5'-TACACAGGAGCAGTTTCAGACAGT-3'	
Eae	*eaeA*	5'-CACACGAATAAACTGACTAAAATG-3'	376
		5'-AAAAACGCTGACCCGCACCTAAAT-3'	
SHIG	*Ial*	5'-CTGGTAGGTATGGTGAGG-3'	320
		5'-CCAGGCCAACAATTATTTCC-3'	
BfpA	*bfpA*	5'-TTCTTGGTGCTTGCGTGTCTTTT-3'	267
		5'-TTTTGTTTGTTGTATCTTTGTAA-3'	
EA	*pCVD*	5'-CTGGCGAAAGACTGTATCAT-3'	630
		5'-CAATGTATAGAAATCCGCTGTT-3'	

LT= Enterotoxigenic *E. coli* (ETEC); ST= Enterotoxigenic *E. coli* (ETEC); VT= Enterohemorrhagic *E. coli* (EHEC); Eae= Enterohemorrhagic *E. coli* (EHEC); SHIG= Enteroinvasive *E. coli* (EIEC); BfpA= Enteropathogenic *E. coli* (EPEC); EA= Enteroaggregative *E. coli* (EAEC).

Table 3.2 Reference *Escherichia coli* strains for Polymerase Chain Reaction

Category	Reference strain	Target gene(s)
ETEC	ATCC 35401	*eltB*, *estA*
EHEC	ATCC 43890	*vt1*, *eaeA*
EHEC	ATCC 43889	*vt2*, *eaeA*
EPEC	ATCC 43887	*eaeA*, *bfpA*
EIEC	ATCC 43893	*Ial*
EAEC	97R[a]	pCVD432-harboring strain
E. coli (negative control)	ATCC 11775	No virulence gene

ETEC= Enterotoxigenic *E. coli*; EHEC= Enterohemorrhagic *E. coli*; EPEC= Enteropathogenic *E. coli*; EIEC= Enteroinvasive *E. coli*; and EAEC= Enteroaggregative *E. coli*.

3.2 Methods

3.2.1 Sample Collection

A total of fifty (50) stool samples were collected from patients (0 to above 45 years) with cases of diarrhea attending, Keffi, General Hospital, Keffi and Federal Medical Centre, Keffi using sterile containers and transported to Federal Medical Centre Laboratory, Keffi, for analyses. Ethical clearance was obtained from Ethical Committee of the hospitals. The consent of the patients were obtained before sample collection.

3.2.2 Media and Sterilization

The media were prepared based on the manufacturer's instruction and sterilized using autoclave at 121°C and glass wares was sterilized using hot air oven at 160°C for 2 h.

3.2.3 Isolation and Identification

3.2.3.1 Isolation of *Escherichia coli*

The *E. coli* was isolated from the stool samples as follows; A loopful of stool samples were inoculated in MacConkey agar (MCA) and incubated at 37°C. The pinkish colonies on MCA agar were streaked on Eosine Methylene Blue (EMB) Agar and incubated at 37°C for 24 hours and after which greenish metallic sheen colonies on EMB agar were selected as presumptive *E. coli* (Cheesbrough, 2006).

3.2.3.2 Identification of *E. coli*

Gram Staining

The Gram Staining test for the suspected organism was carried out as described (Cheesbrough, 2006). Briefly, a small portion of cultural organism was transferred to

the slide, and emulsified in the drop of distilled water until a thin homogeneous film is obtained, then the wire loop was re-sterilized and thin homogeneous film was allowed to dry, fixed by passing through the flame, after the smear is made, air dried, heat fixed, it was then flooded with crystal violet for 1 minute, and then rinsed with distilled water. The stain was then flooded with Lugol's iodine for 1 minute, and rinsed with distilled water and then decolorized, rapidly with acetone until no more color appeared to flow from preparation and rinsed appropriately with distilled water. The stain was counter stained with neutral red for 1 minute, and rinsed with distilled water and allowed to air dried and viewed microscopically using x100 oil immersion objective. The organism retained the neutral red which indicates a Grarm-negative rod-shaped bacterium. Therefore *Escherichia coli* was confirmed as the colony been smeared.

Biochemical Test

Indole Test

The Indole Test for the suspected organism was carried out as earlier described (Cheesbrough, 2006). Briefly, a colony of organism from culture plate was inoculated unto 5ml peptone water and incubated at 37°C for 24 h. After which a three drops of Ḱovac's reagent was added to overnight peptone water culture. A positive reaction was indicated by the development of red ring colour in the reagent layer above the broth within 10 minutes observed.

Methyl Red/Voges-Proskauer Test

The Methyl Red Test for the suspected organism was carried out as described (Cheesbrough, 2006). Briefly, a pure culture of test organism was inoculated in to

MR-VP medium and incubates at 37°C for 24 h after which the culture was divided in to two portions. To the first portion three drops of methyl red was added and formation of red color indicated methyl red positive. To the second portion 10 drops of 40% (w/v) KOH was added follow by three drops of 5% (w/v) Beta-napthol and observe for 30 mins. Formation of yellow color indicated Voges-Proskauer negative.

Citrate Utilization

The Citrate Utilization Test for the suspected organism was carried out as described (Cheesbrough, 2006). Briefly, culture of the organism was inoculated as a single streak on the slant surface of citrate agar and was incubated at 37°C for 24 h; blue colour in the medium indicated the presence of alkaline products. No change in the colour of the test medium indicated a negative citrate utilization test.

Sugar Utilisation Test

The sugar utilisation test was carried out as follow; the suspected colonies of isolated organisms were inoculated into the medium containing various sugar such as glucose, lactose, manitol, dulcitol, sucrose, galactose, d-arabinose and maltose and incubated at 37°C for 48 h. The sugar fermentation was indicated by change of the colour of the medium from light pink to yellow.

3.2.4 Storage of Bacteria

The Bacteria were stored in the refrigerator at 4°C on nutrient agar slants and reactivated by sub-culturing on MacConkey agar and used in the molecular experiments.

3.3 Development of Multiplex Polymerase Chain Reaction (PCR) for Diarrheagenic *Escherichia coli*

3.3.1 DNA Extraction

The DNA of the *E. coli* isolates was extracted using a method earlier described by Nguyen *et al.* (2005). Briefly, 3 pure colonies of *E. coli* isolates from MAC agar plate was inoculated into 5 ml of sterile Luria Berthani broth in a Bijou bottle and incubated at 37°C for 7 h. and the 7h LB culture was transferred into a sterile Eppendorf tube and boiled at 100°C for 20 mins, and the culture was centrifuged at 10,000rpm for 10 mins to pellet the cell debris. The supernatant was used for PCR.

3.3.2 Multiplex PCR Assay.

The multiplex PCR assay for DNA extracted from *E. coli* isolates was carried out following a method described by Svenungsson *et al.* (2000). The DNA templates were subjected to multiplex PCR with specific primers for the detection of the following virulence markers: *eaeA* (structural gene for intimin of EHEC and EPEC), *bfp.4* (structural gene for the bundle-forming pilus of EPEC), *vt1* and/or *vt2* (Shiga toxins 1 and 2 of EHEC), *eltB* and/or *estA* (enterotoxins of ETEC), *ial* (invasion-associated locus of the invasion plasmid found in EIEC and *Shigella*), and pCVD (the nucleotide sequence of the EcoRI-PstI DNA fragment of pCVD432 of EAEC).

The multiplex PCRs were performed with a 25 µl reaction mixture containing 5 µl of template DNA, 0.2 µl of 18x PCR buffer II, 1.6 µl of a 1.25 mM mixture of deoxynucleoside triphosphates, 1.6 µl of 25 mM $MgCl_2$, 0.1 µl of 5 U of AmpliTaq Gold DNA polymerase (Inqaba biotec, South Africa) per µl, and a 0.2 µM concentration of each primer except primer VT1 (all primers were from Inqaba Biotec^{tm}, South Africa), which was used at a concentration of 0.4 µM. The

thermocycling conditions with a Gene Amp PCR system 9700 (AB Applied Biosystem) were as follows: 95°C for 5 mins, 94°C for 20 sec., 55°C for 30 sec., and 72°C for 30 sec. for 30 cycles, with a final 7mins extension at 72°C as described by Nguyen *et al.* (2005).

3.3.3 Agarose Gel Electrophoresis

The agarose gel electrophoretic assay for detection of amplified genes for different pathotypes of *E. coli* isolates was carried out following a method described by Nguyen *et al.* (2005). Briefly, 10 µl of PCR products stained with ethidium bromide was loaded into 1.0% (wt/vol) agarose gel (Gibco Life Technologies, Paisley, United Kingdom) wells with a molecular marker (1-kb DNA ladder; Gibco/BRL) run concurrently at 120 mV for 30 mins. The DNA bands were visualized and photographed under UV light 595nm.

3.4 Statistical Analysis

The data obtained from this study were analysed using chi-square test by the use of Smith Statistical Package (SSP) version 2.8 and the significance was determined at 5 % probability (that is P= 0.05).

CHAPTER FOUR

RESULTS

4.1 Study Population

The distribution of patients in relation to age and gender is as shown in Table 4.1

4.2 Isolation and Identification of *Escherichia coli* from Stool

Pinkish colonies on MacConkey agar and greenish metallic sheen colonies on Eosine Methylene Blue agar which were gram negative, rod-shaped, Indole positive, Methyl red positive, Voges-Proskauer negative, Citrate negative and Triple Sugar Ion positive were identified as *E. coli* isolates (Table 4.2)

4.3 Frequency of Detection of Different Pathotypes of *Escherichia coli* from Stool of Diarrheic Patients in Keffi, Nigeria

Out of fifty (50) stools sample collected, 50 (100%) of *E. coli* were isolated. Out of 50 isolates of *E. coli* from stool of diarrheic patients, 13(26.0%) were diarrheagenic distributed as follows: 11(22.0%) EAEC, 1 (2.0%) ETEC, 1 (2.0 %) EIEC, while no EHEC and EPEC were detected from the stool of diarrheic patients as shown in Table 4.3.1

The order of isolation frequency of EAEC in relation to age was 5-10 (50.0%) > 10-15 (40.0%) > 15-20 (20.0%) and 0-5 (17.2%) respectively. The frequency of isolation of EIEC was 20.0% in age 10-15 and 0.0% was observed in other age groups. For ETEC, the frequency of isolation was 2.9 % in age 0-5 and was not observed in other age groups as shown in Table 4.3.2. However, the frequency of isolation of EAEC in

relation to gender was high in female (36.4%) than male (10.7%) respectively as shown in Table 4.3.3.

The isolation frequency of ETEC was 4.5% in female and non was observed in male and for EIEC, the frequency of isolation was 3.8% in male and 0.0% in female respectively as shown in Table 4.3.2

Table 4.1: The distribution of patients in relation to age and gender

Age (Years),	Gender		Total
	Male	Female	
0-5	18	11	29
6-10	3	3	6
11-15	4	1	5
16-20	1	4	5
21-25	1	1	2
26-30	0	1	1
31-35	0	0	0
36-40	0	0	0
41-45	1	0	1
>45	0	1	1
Total	**28**	**22**	**50**

Table 4.2: Cultural, Morphological and Biochemical characteristics of *Escherichia coli* from patients with suspected cases of diarrhea in Keffi, Nigeria

Cultural Characteristics	Morphological Characteristics	Triple Sugar Ion	Biochemical Characteristic	Organism
Pinkish colonies on MAC agar, greenish metallic sheen colonies on EMB Agar	Gram Stain Morphology - Short rod shape	GLU LAC SUC + + +	IND MR VP CT + + - -	*E. coli*

MAC= MacConkey; EMB= Eosine Methylene Blue; IND= Indole; MR= Methyl Red; VP=Voges-Proskauer; CT= Citrate; - = Negative; + =Positive; GLU= Glucose; LAC= Lactose; SUC= Sucrose

Table 4.3.1 Isolation rate of different Pathotypes of *Escherichia coli*

No. of Samples	No. of *E. coli*	No. (%) DEC	No. (%) Pathotypes of *E. coli*				
			ETEC	EHEC	EPEC	EIEC	EAEC
50	50	13(26.0)	1(2.0)	0(0.0)	0(0.0)	1(2.0)	11(22.0)

P= 0.0535; χ^2= 5.8500

DEC= Diarrheagenic *E. coli;* ETEC= Enterotoxigenic *E. coli*; EHEC= Enterohemorrhagic *E. coli*; EPEC= Enteropathogenic *E. coli*; EIEC= Enteroinvasive *E. coli*; and EAEC= Enteroaggregative *E. coli.*

Table 4.3.2: Isolation rate of different Pathotypes of *Escherichia coli* in relation to Age

Age (Years)	No. of Samples	No. of *E. coli*	No. (%) DEC	No. (%) Pathotypes of *E. coli* ETEC	EHEC	EPEC	EIEC	EAEC
0-5	29	29	6(20.0)	1(3.4)	0(0.0)	0(0.0)	0(0.0)	5(17.2)
6-10	6	6	3(50.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	3(50.0)
11-15	5	5	3(60.0)	0(0.0)	0(0.0)	0(0.0)	1(20.0)	2(40.0)
16-20	5	5	1(20.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	1(20.0)
21-25	2	2	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
26-30	1	1	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
31-35	0	0	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
36-40	0	0	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
41-45	1	1	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
>45	1	1	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)	0(0.0)
Total	**50**	**50**	**13(26.0)**	**1(2.0)**	**0(0.0)**	**0(0.0)**	**1(2.0)**	**11(22.0)**

P= 0.9209; χ^2= 0.4908

DEC= Diarrheagenic *E. coli;* ETEC= Enterotoxigenic *E. coli*; EHEC= Enterohemorrhagic *E. coli*; EPEC= Enteropathogenic *E. coli*; EIEC= Enteroinvasive *E. coli*; and EAEC= Enteroaggregative *E. coli.*

Table 4.3.3: Isolation rate of different Pathotypes of *Escherichia coli* in relation to Gender

Gender	No. of Samples	No. of *E. coli*	No. (%) DEC	No. (%) Pathotypes of *E. coli*				
				ETEC	EHEC	EPEC	EIEC	EAEC
Male	28	28	4(14.0)	0(0.0)	0(0.0)	0(0.0)	1(3.8)	3(10.7)
Female	22	22	9(40.0)	1(4.5)	0(0.0)	0(0.0)	0(0.0)	8(36.4)
Total	**50**	**50**	**13(26.0)**	**1(2.0)**	**0(0.0)**	**0(0.0)**	**1(2.0)**	**11(22.0)**

P= 0.2495; χ^2= 1.3258

DEC= Diarrheagenic *E. coli;* ETEC= Enterotoxigenic *E. coli*; EHEC= Enterohemorrhagic *E. coli*; EPEC= Enteropathogenic *E. coli*; EIEC= Enteroinvasive *E. coli*; and EAEC= Enteroaggregative *E. coli.*

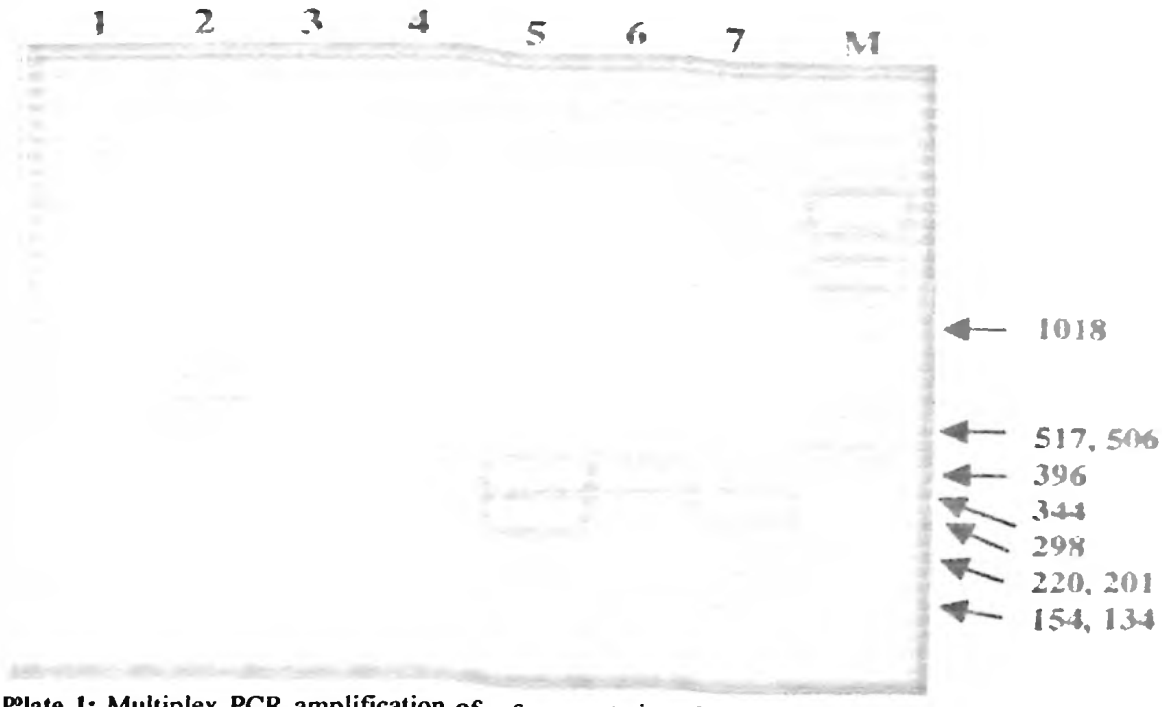


Plate 1: Multiplex PCR amplification of reference strains of diarrheagenic *E. coli* from pure cultures (Lane 1, *E. coli* ATCC 11775; lane 2, EAEC 97R; lane 3, EIEC ATCC 43893; lane 4, EPEC ATCC 43887; lane 5, EHEC ATCC 43889; lane 6, EHEC ATCC 43890; lane 7, ETEC ATCC 35401; lane M, marker (1-kb DNA ladder; Gibco/BRL). Numbers on the right are in base pairs).

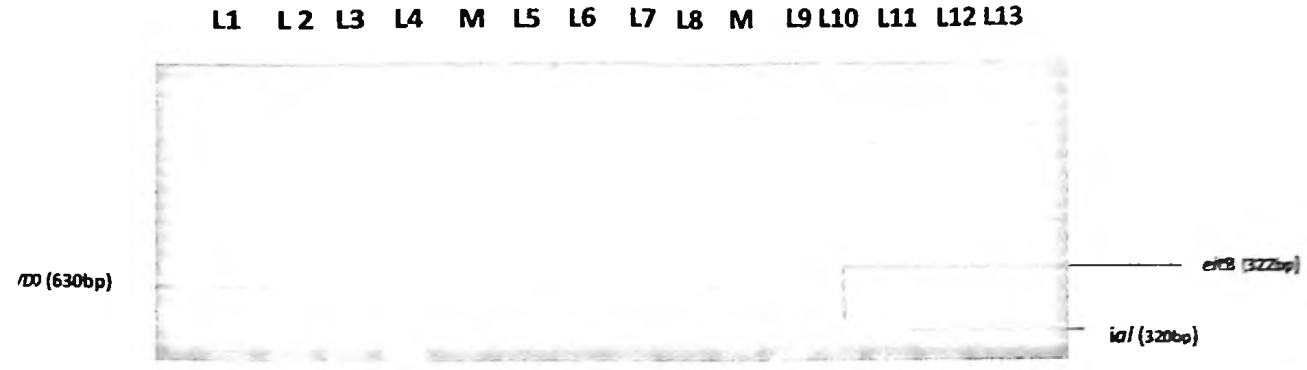


Plate 2: DNA Ladder for *Escherichia coli* isolates from stools of diarrheic patients in Keffi, Nigeria (L= Lane, L1= negative; amplification; L2-L4, L5-L9 and L12-L13= *pCVD* (EAEC); L11= *ial* (EIEC); L10= *eltB* ETEC; M= 100bp

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Diarrheagenic *Escherichia coli* (DEC) are important intestinal pathogens causing a wide variety of gastrointestinal diseases, particularly among children in developing countries (Ali *et al.*, 2014). Six pathogenic diarrhoeagenic *Escherichia coli* (DEC strains) are frequently detected worldwide which are Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Shiga toxin-producing *E. coli* (STEC) or Enterohaemorrhagic *E. coli* (EHEC), Enteroaggregative *E. coli* (EAEC) and Enteroinvasive *E. coli* (EIEC) (Nguyen *et al.*, 2005, Onanuga *et al.*, 2014, and Ali *et al.*, 2014.). These pathotypes have been described based on the genes mediating the virulence factors associated with the diarrheal diseases caused by them.

The isolation of *E. coli* in all (100%) stool samples is in agreement with studies reported by Onanuga *et al.* (2014); Sheikh *et al.* (2001); and Okeke *et al.* (2000); and confirms the fact that *E. coli* is a common bacteria isolated in stool of human (Aslani *et al.*, 2011). This high detection EAEC may be due to improper personal hygiene.

The high frequency of detection of EAEC 11(22.0) observed in this study was not surprising and it is in agreement with another study reported elsewhere 7.2% (Nguyen *et al.*, 2005). EAEC was previously reported to be endemic in Southern Nigeria as well as in sub-Saharan Africa (Okeke *et al.*, 2003). So, our observation on the occurrence of EAEC 11(22.0) in Kefli concurs with what was reported in Southwestern Nigeria and elsewhere especially in the sub-Saharan Africa 18(7.2%) (Vila *et al.*, 2000).The frequency of detection of EAEC in this study is higher than that reported by Sani *et al.* (2015); but the detection of ETEC and EIEC was low as

study reported by Sani *et al.* (2015), (1.0%) and Nguyen *et al.* (2005) (1.0 and 1.9%) respectively The very low frequency of detection of diarrhoeagenic *E. coli* obtained in this study is in close agreement with the study conducted and reported by Chigor *et al.* (2010) who reported a prevalence of *E. coli* O157: H7 in children with diarrhea as 5.4% in Zaria, Nigeria. Also, Ogunsanya *et al.* (1994) and Olorunshola *et al.* (2000), reported a prevalence of 5% EHEC O157:H7 in humans, in Lagos, Nigeria. But it is in contrast with the study conducted by Nweze (2009), who 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 by Albert *et al.* (1999). Also, we observed that EHEC and EPEC were not detected in this study, reason may be so because isolation rate of different pathotypes of diarrheagenic *E. coli* have been reported to be vary in different geographical areas although other studies in other parts of the country reported low frequency of detection of EHEC and EPEC (Sani *et al.,* 2015). This is also in total disagreement with studies carried out in Southeast Nigeria, which reported that EPEC was the most isolated of all DEC pathotypes followed by EAEC, ETEC, EIEC and EHEC in that order (Nweze, 2009).

Outbreaks and sporadic cases of EHEC have been reported in developed countries of North America, Japan, Europe and even Australia (Effler *et al.*, 2001). However there have been few reports of sporadic EHEC in African countries. Three large EHEC outbreaks were previously reported in Swaziland, Central African Republic and the Cameroon (Effler *et al.*, 2001, Germanii *et al.*, 1997); but some authors criticized the methodology used in those studies as being nonspecific or insensitive (Okeke *et al.*, 2003). Despite this, our findings tend to align with the earlier observation that EPEC and EHEC may be rare after all (Nweze, 2009). The subjects employed in this study may be infected by other pathogens other than diarrheagenic *E. coli* since there are

different pathogens that can cause diarrhea in children and adults, including Rotavirus, *Salmonella* spp., *Shigella* spp., *Campylobacter jejuni*, *Entamoeba histolytica,* and *Giardia lamblia,* but were not investigated in this study. (Nweze, 2009).

The detection frequency of EAEC in respect to age was high in age 6-10 and 11-15 then 16-20. This observation is different from a study reported in Hanoi, Vietnam by Nguyen *et al.* (2005). Although the frequency of detection of EAEC gene of *E. coli* isolates in respect to age of patients was statistically insignificant. The EAEC genes were not detected in age > 21 years and this however disagree with other studies reported by Albert *et al.* (1999); Sani *et al.* (2015) and Nweze (2009). Though, we observed that age may not be a factor for prevalence of EAEC. From this study we also observed that EIEC and ETEC were detected in only age 11-15 and 0-5 respectively. And this seems to disagree with a study conducted by Nweze in 2009. Though, EIEC is also reported by researcher across all ages (Okeke *et al.*, 2003; Effler *et al.*, 2001, Germanii *et al.*, 1997). This study also revealed that, the order of frequency of detection of EAEC was high in female than male. The EIEC was detected only in male and ETEC was detected only in female. The frequency of detection of EAEC in relation to age and gender of diarrheic patients was statistically insignificant. This is also in agreement with studies carried out in Southeast Nigeria (Okeke *et al.*, 2003; and Nweze, 2009). Isolation rate of different pathotypes of diarrheagenic *E. coli* have been reported to be vary in different geographical areas (Okeke *et al.*, 2000).

5.2 Conclusion

The research was concluded with the following important findings:

i. DEC was found in Keffi; and only in children

ii. Frequency of isolation of DEC is low

iii. EAEC was the most prevalent whereas EPEC and EHEC were not isolated

iv. Isolation of EIEC and ETEC were very low

v. Frequency of EAEC was highest in children of age 0-5 years

5.3 Recommendations

i. Further studies on molecular epidemiology of different pathotypes of *E. coli* causing diarrhea should be carried out in other parts of Nasarawa State.

ii. Further studies on antibiotic susceptibility and molecular characterization of antibiotic resistant strains of different pathotypes of *E. coli* should be carried out.

iii. Further studies on DNA sequencing for different pathotypes of diarrheagenic *E. coli* in Keffi and other parts of Nasarawa State should be carried out.

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Appendix 1: Letter of Introduction and Ethical Clearance in General Hospital Keffi.

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MICROBIOLOGY UNIT

DEPARTMENT OF BIOLOGICAL SCIENCE

FACULTY OF NATURAL & APPLIED SCIENCES

NASARAWA STATE UNIVERSITY, KEFFI

Vice Chancellor: Prof. Mohammed A. Mainoma
Dean: Assoc. Dr. B. S. Jatau
H.O.D: Dr. J. C Onovo
Coordinator: Mr. Ishaleku David
Tel: +234-08068637760
E-mail, ishalekudavid@rocketmail.com

GENERAL HOSPITAL KEFFI
RECEIVED
DATE 16-6-2015
16-6-2015

2nd June, 2015

The Chief Medical Director
General Hospital,
Keffi.

18.6.15

Dear Sir,

INTRODUCTION OF MASTERS STUDENT FOR ACCESS TO GENERAL HOSPITAL KEFFI

I am pleased to write and introduce to you one of our masters student (Abimiku Rejoice Helma, NSU/NAS/MSC/MCB/002/14/15) who intends to use General Hospital Keffi as one of his sample collection / study center for the purpose of his mandatory final year research project on the topic "Isolation and molecular characterization of Diarrheagenic *Escherichia coli* among patients attending major hospitals in Keffi".

The student is to discuss his research plans and comply with your Institution's ethical requirements. No financial commitment is expected from your quarters. Looking forward to your kind approval.

Thanking you for your continuous support to our students.

Mr. Ishaleku David
(Coordinator, Microbiology)

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S 1/C
IL Refers

You may wish to assist the student N/c.

18/6/15
HEO ADMIN

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Seen and noted

18/06/15

Appendix 2: Ethical Clearance to Conduct Research in Federal Medical Centre, Keffi.

FEDERAL MEDICAL CENTRE

P.M.B. 004
KEFFI NASARAWA STATE,
HEALTH RESEARCH ETHICS COMMITTEE
047-620020, 620095, 08033970028
Website: http:www.fmckeffi.net
Email: fmc.keffi@gmail.com, info@fmckeffi.net

Reg. No. /21/12/2012
15th July, 2015

Our Ref: FMC/KF/HREC/064/15

Abimiku Rejoice Helma
(NSU/NAS/MSC/MCB/002/14/15)
Microbiology Unit,
Fac. Of National and Applied Sciences
Nasarawa State University, Keffi

RE : ETHICAL CLEARANCE TO CONDUCT RESEARCH AT OUR CENTRE

Your application dated 1st July, 2015 refers.

I write to convey full ethical approval for you to embark on the said research project at our centre.

This approval is for the period of four (4) months, July – October, 2015 only.

Titled: Isolation and molecular characterization of Pathotypes of Escherichia coli causing diarrhea in Keffi, Nigeria

This follows successful defense of your research proposal during the committee's ethical screening interview held on 14th July, 2015.

You are however expected to adhere strictly to the internationally acceptable ethical standards governing the conduct of research. Accordingly, all the rights and privileges of the volunteering participants must be respected.

No form of alteration shall be made on this approved protocol without the express permission of the committee.

Extracts of your findings should be forwarded to our committee at the end of the research work for filing and/or possible intervention please.

Thank you.

Signed: 15/7/15
Pharm. Envuladu, Elisha Y. (*FPC Pharm.*)
Secretary HREC

Appendix 3: Letter of Undertaking and Consent of the Subject in Federal Medical Centre, Keffi.

Microbiology Unit,
Department of Biological Science,
Faculty of Natural and Applied Sciences.
Nasarawa State University, Keffi.
16th July, 2015.

The Chairman,
Health Ethical Committee,
Federal Medical Centre,
Keffi.

Dear sir,

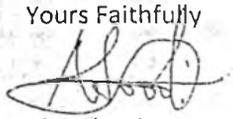
LETTER OF UNDERTAKING

I, Abimiku, Rejoice Helma a research student of Master Degree (MSC) with the matriculation number NSU/NAS/MSC/MCB/002/14/15 in Microbiology of the above named Institution write to carry out a research on the isolation and molecular characterization of pathotypes of *Escherichia coli* causing diarrhea in Keffi, Nigeria using your institution as a study centre.

I solemnly promise that all information gathered will be utilized for the purpose of the research only and strictly confidential.

Please accept the assurances of my highest regard.

Yours Faithfully



Abimiku, Rejoice Helma

08061671883

Appendix 4: Letter of Certification in Niger Delta University, Wilberforce Island, Bayelsa State.

NIGER DELTA UNIVERSITY

Wilberforce Island, Bayelsa State.

Our Ref.....

Your Ref.....

Dated 18th/01/2016

RE: LETTER OF CERTIFICATION

This is to certify that Mr Abimiku, Rejoice Helma Mat No: NSU/NAS/MSc/MCB/002/14/15 of the Department of Biological Sciences, Microbiology Unit, Nassarawa State University, Keffi carried out his MSc research work titled **"Isolation and Molecular characterization of different pathotypes of *E. coli* causing diarrhea in Keffi, Nigeria"** in the Molecular Biology Unit of the Department of Medical Laboratory Science, Niger Delta University under my supervision.

Tatfeng Mirabeau, PhD, FMLSCN
Prof. of Medical Microbiology and Immunology
Consultant molecular Biologist

P. M. B 071, Yenagoa, Bayelsa State e-mail: info@ndu.edu.ng
website: www.ndu.edu.ng