ISOLATION AND CHARACTERIZATION OF ETEC CAUSING DIARRHOEA IN CALVES AND MAN

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DEDICATION

I dedicate this work to the soul of my mother, to my father, brothers, sisters and my colleagues with love and gratitude.

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ABSTRACT

Field and laboratory investigations were carried out in Khartoum State to estimate the prevalence and to characterize enterotoxigenic *E.coli* (ETEC) associated with diarrhoea in calves and human infants. Faecal samples were collected from 146 diarrhoeagenic calves from different conventional dairy farms at four different areas. Stool samples from 72 children with diarrhoea at different pediatric wards were also collected.

*E.coli* were isolated from 90 faecal calf samples. Only 19 isolates (13%) were found MRHA. Fifteen *E.coli* strains were also isolated from human stools and only 3 strains (4.2%) were found mannose resistant (MRHA). The identification of MR and MS (MRHA and MSHA) haemagglutination was found a reliable method to differentiate between the adhesive fimbriae and common pili of *E.coli* isolates. The pattern of HA produced by MRHA *E.coli* strains isolated from diarrhoeagenic calves or children were not produced by *E.coli* of their normal flora. Therefore, MRHA was very specific and a reliable indicator for the presence of colonization factors which is an important attribute to virulence associated with ETEC. Furthermore, the MRHA isolates were found adhesive to brush border cells (enteroctye). Both HA ability and adhesion to brush border of calf isolates were inhibited by an antiserum raised against the strain MkhE14a that have HA titre of 64. However, the
HA titre of the various strains were restored at high dilution of the antiserum (1/512) indicating the titre of the prepared antiserum equal to 256. The antiserum prepared against the bovine strain failed to inhibit HA and adhesion of human strains to brush border cells this has indicated the specificity of receptor and adhesion factors interaction. HA has also divided the *E.coli* of calf origin into 19 MRHA strain (21.1%) and 51 strains of MSHA (56.7%) and 20 HA negative *E.coli* isolates (22.2%).

Likewise, MRHA strains of human origin were 3 isolates (20%) and MSHA were 9 (60%) and HA negative strains were 3 (20%).

Optimal temperature for the expression of adhesion factors was found to be 37°C for the various MRHA isolates. Growth at 25°C resulted in reduction of HA titre and HA ability was lost completely by growing the isolates at 20°C.

The toxigenicity of MRHA was first studied on Vero cell cultures. Only culture supernatants of 5 isolates (22.7%) from the total MRHA and (4.8%) from the total *E.coli* isolates were found positive, producing frank rounding of the cells. These isolates were considered LT producing strains. These include four bovine isolates (18.2%) (JETE8, MkhE14a, HiTE16 and HkuE91) and only one human BLKE 19 (4.5%). These toxigenic strains were further tested in the adult rabbit ligated test for toxin production as measured by fluid accumulation ratio (FAR). MRHA *E.coli* strains that failed to induce rounding of Vero cells or induced fluid
accumulation in the ligated-gut model were tested for toxigenicity in suckling mouse model, giving gut weight/carcass weight ratios ranging from 0-1 to 0-3. Heat inactivated culture supernatant of the same strains were also positive in the suckling mouse model suggesting the toxin is heat stable. With the view to simplify the diagnosis of ETEC, LT producing strains were individually tested in Elek’s test. Antiserum against BLKE19 toxoid gave a weak precipitation lines with toxoid of LT producing strains. Continous precepitation line was obtained with two colonies of BLKE19 strain. This clearly confirmed the specificity of the prepared antiserum and the inclusion of linocomycin had facilitated the release of LT. Following characterization of isolates the prevalence of ETEC in calves was estimated as 13% and that in children was 4.2%. Other causes of diarrhoea were discussed. High incidence of ETEC was encountered in the area of JeblToria (4.79%) in comparison to AlMerkiat (2.74%), and Al Hitana (1.37%).
The study was conducted to evaluate the prevalence of E. coli in Jordan, particularly among children with diarrhea and normal flora samples. A total of 146 samples were collected from patients with diarrhea, and 72 samples were collected from healthy children. The study found that 90% of diarrheal samples contained E. coli, while 19% of healthy samples contained the bacteria. The prevalence of adhesive factors in the diarrheal samples was 4.2%, compared to 3% in healthy samples. The study concluded that E. coli is a significant cause of diarrhea in Jordan and underscores the importance of proper hygiene and sanitation practices.
MRHA (MkhE14a) 1.25 % %21.1 %56.7 %22.2 %20 %60 %22.7

MRHA (Verocell) (LT$_s$)
(ARLT) توأمة يش عد، (Verocell) (DZRF) (MRHA) (suckling mouse test) يوزف ضم محل، (ETEC) (Elek’s test) (BkE₁₉) (Lincomycin)
Introduction

Sudan is one of the richest African countries in animal resources. Its population was estimated as 30 million in 2002 and its animal population was estimated as more than one hundred and thirty million heads. These large numbers of animals account for more than 20% of the Sudan foreign trade (Ministry of Animal Resource Report, 2002). As a result of drought (1983) people migrated from rural areas and settled around the big cities. This has increased the demand for milk and other milk products. Large numbers of small dairy farms were established around these towns and small cities to satisfy this demand. Some of these farms practice traditional farming (Dukha), and few are following ingestive system. However, in both cases hygienic measures and good management practices are almost absent. The low hygienic standard and the absence of good management have resulted in some important health problems. One of the most important health problems in both animal and human, in underdeveloped countries, is diarrhoeal diseases.
Diarrhoea is a common manifestation of a complex of diseases, which affect calves and children. Diarrhoea is also the major causes of morbidity and mortality among preschool children in developing countries. In the Sudan the mortality rate among children was estimated as 9% and more than half of this percentage was due to diarrhoeal diseases (AboAlgasim, 2003). Mortality rate among calves was estimated to be 10% and in some months it may reach 100% due to diarrhoea, and is considered the major cause of economic losses in intensive, modern and conventional farming systems (Ali, 2000).

The entric diarrhoeal diseases have many common clinical and epidemiological characteristics but different aetiology. Treatment and control of diarrhoea is usually difficult and unrewarding because the cause of the diarrhoea is difficult to determine quickly and accurately. This is considered the major stumbling blocks in the diagnosis of the aetiology of diarrhoeas. Hence accurate and specific aetiological diagnosis of infectious diarrhoeal diseases in claves and preschool children is difficult
to obtain. Some reasons for this difficulty are multiplicity of infectious agent involved; the apparent nonspecific signs and lesions caused by some agents. For instance, colibacillosis, calf – scours or *E.coli* infection is one aetiological diagnosis commonly applied to calves with diarrhoeal disease. However, the diagnosis is frequently made with little supportive evidence. This is concerned primarily with the study of ETEC, the primary pathogen that cause acute diarrhoeal disease in calves as well as in children in this country. Nonetheless, few studies were conducted in the Sudan to investigate the aetiology of diarrhoea in both calves and man.

Analysis of the *E.coli* K-12 genom sequence also shows that about 2% of its DNA consists of mobile genetic elements, including phages, plasmid and transpons (Blattner *et al.*, 1997). These elements are responsible for the continuous evolution of the bacterial genetic repertoire, providing significant diversity in *E.coli* strains. In this regard, pathogenic *E.coli* appears to have evolved from non pathogenic stains by acquiring new virulence factors by horizontal transfer of
accessory DNA, which is often organized in clusters (Pathogenicity island) in the chromosome or on plasmids (Hacker et al., 1997). In this context, it seems that most pathogenic *E. coli* do not have a single evolutionary origin, but instead emerged as a result of different events of DNA transfer and that even strains capable of causing the same disease do not constitute a monophyletic group (Pupo et al., 1997). *E. coli* pathogenic variants are represented by strains of specific serogroups possessing a particular set of virulence factors which are responsible for different clinical manifestations in animals and man, including several types of diarrhoea and extra-intestinal infections (Puente and Finlay, 2001).

Acute bacterial diarrhoeal diseases are a worldwide problem of enormous magnitude. Moon et al. (1976) demonstrated that entero-toxigenic strains of *E. coli* must possess two attributes to cause diarrhoea in claves: the ability to produce entrotoxins and the ability to colonize the small intestine. Elaboration of the toxin alone is not sufficient to cause diarrhoea. They also demonstrated that adherence of *E. coli* to
the surface of the small intestinal mucosa was required for the proliferation of the organism and the colonization of the small intestine. These two attributes also have been shown to be essential to the production of diarrhoea in man (Satterwhite et al., 1978).

There has been a tremendous development in our knowledge of ETEC during the last 30 years. However, the exact nature of the problem of ETEC diarrhoea in claves and man in the Sudan could not be determined due to the lack of adequate facilities for laboratory diagnosis of ETEC. This largely due to the fact that tests employed for determinations of ETEC are by no means simple. There are no biochemical markers by which ETEC can be easily distinguished from non-ETEC strains, and therefore no selective media can be used to differentiate these strains in primary plating. The method of blindedly picking colonies that resemble *E.coli* is relatively insensitive when compared to other bacterial entric pathogen; the recognition of which can be facilitated by enrichment or growth on highly selective media. ETEC diagnosis is probably
at least 10-100 times less sensitive than for other entric pathogen
and probably a million times less sensitive than *V.cholerae*
recognition (Sack ,1981). Nonetheless, in this study a number of
bioassay and immunoassay tests were employed for the
characterization of ETEC isolated from diarrhoeagenic claves
and children with view to study the magnitude of diarrhoea
causd by ETEC in animals and man.

The isolates identified as *E.coli* will characterize by:

1. Mannose-resistant haemagglutination (MRHA).

2. Adhesion to brush border cells.


5. Infant mouse test.

6. Bicken test (Elek’s Test).
CHAPTER ONE

Literature Review

1.1 Historical background:-

For centuries acute diarrhoea has been an important disease of infancy and childhood. “Cholera infantum” was recognized in many countries but was clearly differentiated clinically and epidemiologically from Asiatic cholera. Nevertheless cholera infantum was observed to have a high mortality and to occur in
epidemic form, particularly in warm seasons of the year (Lambert, 1979).

In 1885 the German pediatrician, Theobald Escherich, isolated bacterium coli (\textit{E.coli}), from cases of infantile entritis and in the remaining years of the nineteenth century this organism was reported in numerous epidmics of infantile diarrhoea in hospitals.

It was soon realized that 	extit{E.coli} occurred in the faeces of healthy infants as well as in those with acute diarrhoea and attempts were made to differentiate virulent or pathogenic strains. Animal experimentation proved useless but Lesage (1897) used a serological approach and demonstrated that antiserum from an acute cases agglutinated \textit{E.coli} but not from other patient. His work was confirmed by other investigators.

In animal the ability of \textit{E.coli} to cause diarrhoeal diseases was first suggested in late 1800 and early 1900 by several veterinary workers studying the calves scours (Nocard and Lectainche. 1898; Joest 1903; Titze and Orcutt 1908). From that
time veterinarian and scientist have intended to run parrelled investigations of diarrhoea in young animals and human infants.

Adam (1923) attempted to identify \textit{E.coli} types which were responsible for infantile diarrhoea but failed with a serological approach and therefore used biochemical tests to differentiate the various types of \textit{E.coli}. This approach has only a limited success and Goldschmidt (1933) returned to a serological approach to \textit{E.coli} “identification”. She introduced the use of slide agglutination to trace the \textit{E.coli}; an approach which has become established in clinical laboratory practice. Using this serotyping technique Gold Schmidt was able to study the epidemiology of infantile enteritis in institution and to identify the importance of symptomless carriers of the epidemic strains.

During the next 15 years little progress was made until Bray (1945) investigated an outbreak of enteritis in London hospital and showed that a particular serological type of \textit{E.coli} was the epidemic agent. In the same year certain strains of \textit{E.coli} were isolated from children 5-8 years of age with diarrhoea at school in London. Workers found that these strains invade the epithelial
cells and cause diarrhoea. Giles and Sangester (1948) investigated diarrhoea epidemics in babies in Aberdeen and identified two serological types which were named Aberdeen alpha and beta.

In 1956 the emphasis was slowly shifted by several medical and veterinary workers to enterotoxins produced by previously recognized and new strains of _E. coli_. De et al., (1953) working in Calcutta, showed that whole broth cultures of several _E. coli_ isolates (including three enteropathogenic _E. coli_ serotypes from patients with diarrhoea) resulted in a fluid secretory response in the rabbit upper small bowel. This response didn’t occur with a majority of strains isolated from healthy controls, when large bowel loops were used. Taylor and Bettelhaim (1966) demonstrated that viable organisms were not required to produce this secretory response and showed that enterotoxigenicity correlated poorly with the classically recognized serotypes. Meanwhile several veterinary workers demonstrated additional enterotoxigenic _E. coli_ strains associated
with animal diarrhoea (Moon et al., 1970: Smith and Halls, 1968).

Other workers demonstrated an association of enterotoxigenic *E. coli* with acute undifferentiated diarrhoea in adult patients from Bengal. These organisms were usually not of classically recognized entro pathogenic serotypes and were present only during acute illness. The enterotoxigenic material from culture filtrates of these organisms caused upper small bowel secretion and appeared to be heat-labile, non dialyzable and precipitable in 40% ammonium sulphate (Gorbach et al., 1971).

Further clarification of the entrotoxigenic material produced by *E. coli* suggested that at least two types of entrotoxins were produced: one a heat-labile entrotoxin (LT) that has subsequently been demonstrated to be much like cholera toxin and the other a heat stable entrotoxin (ST) (Moon and Whipp, 1971). Both entrotoxins have been found to be plasmid-encoded traits which appeared to be separable from the equally important plasmid encoded adherence traits for pathogensis (Smith and
Halls, 1968). Dean (1972) recognized that ST require a different assay model, the suckling mice assay (Giannella, 1976) and it bears no antigenic cross reactivity with LT or cholera toxin (Gyles, 1972).

In the past three types of *E. coli* were recognized

Enterotoxigenic *E. coli*, Enteropathogenic *E. coli* and Enteroinvasive *E. coli*. EPEC was recognized in Aberdeen (1947-1948) workers identified strains of *E. coli* causing dilemma in infants at summer month and produced toxin that differ from toxin produced by ETEC, which was isolated from children between 5-8 years of ages in London in 1944-1945 (Lambert, 1979).

At present eight types of *E. coli* are recognized

Enterotoxigenic *E. coli*, Enteropathogenic *E. coli*, Enteroinvasive *E. coli*, Enteroaggregative *E. coli*, Diffusely adhering *E. coli*, uropathogenic *E. coli*, Enterohaemorrhagic *E. coli* and *E. coli* that causes sepsis and meningitis.
1.2 Classification of Escherichia:-

Escherichia is a genus of the family entrobacteriaceae and tribes Eschericheae. The genus includes the following species:

1.1.1 *E. coli*:

Like many other entrobacteria contains numerous serotypes some of which are associated with certain infections in man and animals, some are particularly associated with diarrhoeal diseases while others causes a variety of extra intestinal infections (Ørskov and Ørskov 1976).

1.1.2 *E. adecarboxylata*:

It was described by Leclerc (1962); strains were isolated from clinical speciemens.

1.1.3 *E. fergusonii*:

It was isolated from animal and human clinical materials.

It was proposed by Farmer *et al.* (1985).

1.1.4 *E. hermanii*:

It was isolated particularly from wounds; it was described by Brenner *et al.* (1982).

1.1.5 *E. blattae*:
It was isolated from the intestinal tract of cockroaches and has not been reported in clinical material (Barrow and Feltham, 1993).

1.1.6 *E. vulneris:*

It was also described by Brenner *et al.*, (1982) as a new species to include group of strains, many of which were isolated from human wounds.

1.3 Normal Habitat of *E. coli:*

*E. coli* is a world wide in distribution. Many *E. coli* are part of normal flora of the intstinal tract of human and animal. Some species are free living occuring in soil, water and vegetations (Carter, 1985). *E. coli* becomes established in the intestine shortly after birth when the sterile intestine of the fetus is seeded with bacteria derived from the mother and the environment.

*E. coli* passes easily and reaches the intestine because in newborn animal and human the stomach pH is nearly neutral. *E. coli* continues throughout adult life as the major facultiatively
anaerobic species of bacteria in the intestine and is usually the dominant isolate on aerobic culture of faeces or intestinal contents. Most strains of *E. coli* are harmless commensals but others are virulent pathogens that affect intestine or extra-intestinal sites (Gyles, 1993).

### 1.4 Resistance of *E. coli:*

*E. coli* survives in the external environment for months. It is more resistant to the physical and chemical factors than other bacteria. *E. coli* is killed comparatively rapid by all methods and preparations used as disinfectant, at 55°C the organism perishes in one hour and at 60°C it perishes in 15 minutes (Ali, 2000).

### 1.5 Mode of Transmission:

Transmission of *E. coli* through ingestion of contaminated food water, milk and vegetables. Infection by *E. coli* require large dose $10^9$ cfu of bacteria to be ingested or inoculated (Lambert, 1979).

### 1.6 Diseases caused by *E. coli:*

*E. coli* causes various diseases in human and animals including several types of diarrhoea (watery diarrhoea, cholera
like diarrhoea, watery to dysentry like diarrhoea and watery to mucoid diarrhoea) urinary tract infections, sepsis, haemorrhagic colitis, haemorrhagic uraemic syndrome and meningitis in man (Nataro and Kaper 1998).

In animal *E.coli* causes profused watery diarrhoea in most animal specieses, oedema, haemorrhagic colitis, septicema and mastitis as showed in table (1) and (2).

1.7 Morphological Characterisitic of *E.coli*:–

*E.coli* is Gram negative rod-shaped bacterium ranging in size from 0.2 - 0.5 micrometers.

1.8 Isolation and cultural characterization of *E.coli*:

1.8.1 Media for isolation of *E.coli*:

Three types of media can be used for primary isolation of *E.coli*:

1.8.1.1 Differential or selective media:

1.8.1.1.1 MacConkey’s agar medium:

It is used to detect coliform and enteric pathogens from faecal samples based on their ability to ferment lactose. Lactose fermenting bacterial species like *E.coli* gives pink to red
colonies while other non-lactose fermenting organisms give
colourless to transparent colonies after an overnight incubation
at 37°C on this medium.

1.8.1.1.2 Eosin and methylene blue medium (EMB):

It is used for the isolation of lactose fermenting Gram-
negative organism like *E.coli*. Eosin and, methylene blue
medium consist of peptone base with lactose, sucrose, eosin and
methyline blue. Eosin and methylene blue serve as indicators for
fermentation as well as inhibiting Gram- positive organisms.

On EMB media *E.coli* like lactose fermenting organisms
produces a black precipitate. Colonies will be either black or
posses drak centers with transparent colourless peripheries after
an overnight incubation at 37°C.

1.8.1.1.3 Cystiene lysine electrolyte deficient (CLED)

medium:

It is also a selective medium for lactose fermenting
organisms. It is consist of peptone base agar with lactose and L-
cystiene, restriction of electrolytes inhibits swarming by proteus,
lactose with bromthymol blue indicator is differential for
coliform. Lactose fermenting species give yellow colonies and the media is changed to blue color.

1.8.1.2 Enriched media:

Blood agar is used for first isolation of *E.coli* from systemic infection. Blood agar is constituted of tryptose, soldium chloride, heart infusion, agar and 5% sheep blood.

*E.coli* is an aerobe and facultative anaerobe. On blood agar *E.coli* produces 1-4mm in diameter colonies after an overnight incubation at 37°C. The colonies may appear mucoid and some strains are haemolytic due to production of haemolysin.

1.8.1.3 Basic media:

Nutrient agar is used for subculturing of *E.coli* from differential, selective or storage media (slant) prior to perform biochemical and serological identification (Monica 2000).

1.9 Maintenance and Preservation of *E.coli*:

*E.coli* can survive well in holding media as modified Carey-Blair medium for several weeks to month without lossing its plasmids (Sack 1981). Storage of strains in liquid broth media supplemented with 15% glycerol as cryopreservative at -
70°C gives good stability of the enterotoxin properites as well as of the surface adhesion. Storage of strains on Dorset egg medim at 4°C is a good alternative for liquid broth media.

Lypholization of *E.coli* strains also give a good stability of plasmid for years (Sack 1981).

### 1.10 Biochemical characterization of *E.coli*:

*E.coli* is oxidase negative, catalase positive, most *E.coli* are indole test positive, mannitole positive, sorbitol positive, citrate utilization test negative, glucose fermentation positive, oxidation fermentation positive, urease and H₂S production negative. Some *E.coli* are motile others are not motile.

The most important biochemical test used is production of acid and gas from lactose when inoculated into MacConkey broth and incubated in water bath at 44°C for 48hrs (Barrow and Feltham 1993).

### 1.11 Antigenic Structure of *E.coli*:

#### 1.11.1 The Cell Wall:

The cell wall in all Gram -negative bacteria are more complex than that of Gram positive bacteria. Like all Gram-
negative bacteria *E.coli* cell wall consists of preplasmic space, monomeric peptidoglycan, lipoprotein, outer membrane, outer membrane protein (OMP) and lipopolysaccharide (Prescott *et al* 1999).

**1.11.1.1 Periplasmic space:**

It is the layer between the plasma membrane and outer membrane. It is filled with a loose network of peptidoglycan, which is a gel than fluid filled space. Periplasmic space constitutes about 20-40% of the cell walls (30-80 nm). Periplasmic space contains many proteins that participate in nutrient acquisition, example binding proteins involved in transport of material into the cell. Periplasmic space also contains enzymes involved in peptidoglycan synthesis and modification of toxic compounds that could harm the cell (Inouye, 1979)

**1.11.1.2 Peptidoglycan (mucopptide or murein):**

*E.coli* cell has a 2-7 nm peptidoglycan (PG) layer surrounded by a 7-8 nm thick outer membrane. Peptidoglycan comprises a disaccharide polymer joined by Peptide Bridge
attached to one sugar. Peptidoglycan is responsible for rigidity of bacteria cell wall. The structure of PG can be divided into:

A. **Backbone:** composed of alternating N-acetyl glucose amine and N-acetylmuramic acid.

B. **Tetra-peptide side chains:** it is attached to N-acetyl muramic acid and has certain features: $R_1$ is L-alanine, $R_2$ is D-glutamate, $R_3$ is diaminopimelic acid in this position lipoprotein of cell wall is linked, $R_4$ is D-alanine.

C. **Cross-linked chains:** Five amino acids link the tetrapeptide chain. Direct peptide linkage between the diaminopimelic amino group of one side chain and the carboxyl group of the terminal D-alanine of second side chain (linkage between $R_3$ and $R_4$). According to cross-linkage PG can be classified into chemotype B or I, II, III. Gram negative bacteria have peptidoglycan of type AI$d$ (Osborn,1980).

**1.11.1.3 Lipoprotein:**

It is the most abundant protein of cell wall. Lipoprotein molecules cross the outer membrane and peptidoglycan layers. The lipid component consisting of diglyceride thioester linked to
a terminal cystine, which inserted in the outer membrane. Its function is to stabilize the outer membrane and anchors it to the peptidoglycan layer (Prescott et al. 1999).

1.1.1.4 Outer membrane:

The outer membrane is a phospholipid bilayer in which the phospholipids of the outer leaflet are replaced partially by lipopoly-saccharide. The outer membrane is a fluid mosaic containing a set of specific proteins (OMP) embedded in phospholipid matrix. These proteins are pores forming proteins and non-pore forming proteins (Osborn, 1980).

A. Matrix proteins (Porin Protein):

Act as hydrophilic pores, they include outer membrane protein F and are called transmembrane protein, they penetrate both faces of the outer membrane and permit free diffusion of hydrophilic solutes.

B. Lam B and Tsx:
These proteins are responsible for specific permeation of oligo-saccharides of the maltose series and nucleosides but they appear to act as pores rather than carrier proteins.

Lam B responsible for most of transmembrane diffusion of maltose, maltodextrins and some specific solutes passage, whereas Tsx is responsible for transmembrane diffusion of nucleosides and some amino acids (Osborn, 1980).

C. Non-porins proteins:

They include outer membrane A (OMPA), which participate in the anchoring of the outer membrane to peptidoglycan layer and also is a sex pilus receptor in F-mediated bacterial conjugation (Rogers, 1983).

D. Minor proteins:

It contains a set of less abundant proteins, it is evolved in transport of specific small molecules like vitamin B₁₂ and iron-siderophore complexes. They show highly affinity for their substrate. Minor protein include limited number of enzymes e.g. protease, phospholipase as well as some penicillin-binding protein. It transports iron by making it soluble. Proteins of the
outer membrane are synthesized on ribosomes and transfer to the outer membrane (Osborn, 1980).

1.11.1.5 Lipopolysaccharides (LPS):

LPS is attached to outer membrane by hydrophobic bonds. It is formed of a complex lipid called lipid A to which is attached a core polysaccharide which in turn attached to outer cell membrane by hydrophobic bonds. It is synthesized on the cytoplasmic membrane and transports its final exterior position. Lipopolysaccharide consist of three components (Prescott et al., 1999):

a. **Lipid A:**

Consist of a chain glucosamine disaccharide units connected by pyrophosphate bridge to which are attacked a number of long chain fatty acids (β-hydroxy). Lipid A is responsible for the toxicity of LPS endotoxin (Prescott et al., 1999).

b. **The O side chain (O antigen):**
It is a short polysaccharide chain extending outward from
the core. It has several peculiar sugars and varies in composition
between bacterial strains. Although O side chains recognized by
host antibodies, bacteria can resist host defense by rapidly
changing the nature of their O side chains to invade the immune
system of the host (Prescott et al., 1999).

c. The core polysaccharide:

It is specific chain, which link to the extra polysaccharide
KDO (keto deoxy octonic acid) or oligosaccharide. Lipid A and
O side chain are linked through core polysaccharide chain
(Prescott et al., 1999, and Osborn, 1980). The LPS act as an
adjuvant enhances the immune response for antigens, activate
complement system and it is a virulent factor in Gram negative
bacteria. It causes septic shock and it has other biological
functions.

1.11.2 Fimbriae and/or Pili:

Fimbriae or Pili are protein poles (sticks) arrange
perpendicular to the surface of bacteria and projecting out into
extracellular space. They are tiny hollow projections. They are
used to attach bacteria to surfaces and are not involved in movement. A pilus is composed of sub-units of protein known as pilin. According to Duguid (1980) bacteria have two kinds of pili:

1. **F-pili or sex pili:**
   They are long pili found in low number and used to attach one bacterium to another typically of the same species. F-pili are involved in the transfer of plasmids from one bacterium to the other though a process called conjugation (Duguid 1980).

2. **Fimbriae (adhesion factor):**
   They are short pili found in large numbers up to hundreded and used to attach bacteria to surfaces. Fimbriae can be important contributers to ability of the bacteria to cause disease since they secure the attachment of the bacteria to the host. Fimbriae are rod-like structures 5-7 nm in diameter but some may be less than 4 nm thin. The adhesive potential of the fimbriae is determined by the adhesin being located either at the tip of each fimbria or along the length of the structure (Hultgren *et al.*, 1993).
1.11.2.1 Types of Fimbriae:

1.11.2.1.1 Type One Fimbriae or Common Type fimbriae:

They are produced by many different strains of *E.coli*, both commensal as well as pathogenic. Type one fimbriae mediate adherence to many surfaces including human type A (erythrocyte), guinea pig, chicken and bovine (erythrocyte). The attachment mediated by type-1 fimbriae is inhibited by low concentration of D-mannose, (100 µg/ml in PBS) i.e. it is mannose sensitive (Salit and Gotschlich, 1977).

1.11.2.1.2 K88 (F4):

Are fine fimbriae produced by certain serotypes of *E.coli* that caused diarrhoea in pigs. The attachment of (F4) to RBC and or epithelial cells is not impaired by D-mannose, i.e Mannose-resistant, (Gaastra and De Graaf, 1982). Strains of porcine ETEC that possese the K88 fimbriae are able to adhere efficiently to the intestinal mucosa of neonatal pigs, thereby attaining high population densities in the gut.

1.11.2.1.3 K99 (F5):
K99 are similar in structure and function to K88 but are found on bovine and ovine ETEC. The production of K99 (F5) is plasmid mediated and temperature dependant, and adherence occurs with intestinal epithelial cells of younger but not older animals.

Synthesis of (F5) antigen is repressed by certain components (alanine and glucose) in complex media, and a minimal medium supplemented with casamino acids is usually used for production of (F5). The main component of K99 is adhesin and binds to its receptor, the ganglioside glycolipid Neu5Gc-a(2-3)-Gal-β(1-4)Glc-β(1-1)cermide (Gyles, 1993).

1.11.2.1.4 987 P fimbriae (F6):

They are plasmid-mediated fimbriae found on porcine and bovine ETEC. F6 is associated with diarrhoea in neonatal but not in older animals. A cluster of genes adjacent to the genes for heat-stable entrotoxin (Sta) has been found to encode the proteins necessary for synthesis and expression of (F6) (Schifferli et al., 1991). It is difficult to demonstrate (F6) on strains grown in vitro.
1.11.2.1.5 F41 Fimbriae:

They are responsible for colonization of the intestine of pigs, lambs and calves. They occur on bovine and porcine ETEC that lacks F4, F5 and F6 (Korth et al., 1992).

1.11.2.1.6 Colonization Factor Antigen I and II:

CFA/I and CFA/II are the best characterized of a number of specific pilus adhesions that promote colonization by human ETEC strains. These are plasmid-mediated pilus structures, which are mannose-resistant hemagglutinins, and are antigenically distinct from each other and from the fimbriae that promote attachment to the intestine of various animal species (Puente and Finlay, 2001).

1.11.2.1.7 Vir Pili:

They are identified on small percentage of strains of E.coli that cause septicemia in calves and lambs (Gyles, 1993).

1.11.2.1.8 Curli:

It is found in E.coli that cause mastitis. These pili play a role in the attachment and penetration of the bacteria to epithelium in the mammary gland (Todhunter et al., 1990).
1.12 Classification of pathogenic *E.coli*:

Pathogenic *E.coli* differ from the non-pathogenic *E.coli* by the presence of virulence factors organized in clusters in the chromosome or plasmids. According to the large variation in DNA content and to the difference in the distribution of genomic location (insertion site) of different virulence determinants, pathogenic *E.coli* were divided into eight major categories (Puente and Finlay, 2001).

1.12.1 Entero-toxigenic *E.coli* (ETEC):

ETEC causes watery diarrhoea, ranging in severity from mild and self-limiting to severe cholera-like profuse diarrhoea. Diarrhoea is usually without mucus, blood, pus, fever or vomiting. Death is due to dehydration; hypovolumic shock and acidosis (Sack, 1975).

<table>
<thead>
<tr>
<th>Type of <em>E.coli</em></th>
<th><em>Disease</em></th>
<th>Virulence factors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ETEC</td>
<td>Diarrhoea (most specieses)</td>
<td>Entrotoxins/pili</td>
</tr>
<tr>
<td>--------</td>
<td>---------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>EPEC</td>
<td>Diarrhoea (pig)</td>
<td>Attachment/effacement/pili</td>
</tr>
<tr>
<td>VTEC</td>
<td>Oedema disease (pigs)</td>
<td>Verotoxin/pili</td>
</tr>
<tr>
<td>EIEC</td>
<td>Hamorrhagic colities (calves)</td>
<td>VT₁, VT₂ attachment/effacement</td>
</tr>
<tr>
<td>Septicemic E.coli</td>
<td>Septicemia most specieses</td>
<td>Serum resistance, iron scavenging, lipopolysaccharide Alpha haemolysin</td>
</tr>
<tr>
<td>Mastitic E.coli</td>
<td>Mastitis</td>
<td>Opportunistic</td>
</tr>
<tr>
<td>unknown</td>
<td>Pyometra (cats)</td>
<td></td>
</tr>
</tbody>
</table>

ETEC: Enterotoxigenic *E.coli*.

EPEC: Enteropathogenic *E.coli*.

VTEC: Verotoxigenic *E.coli*.

EIEC: Entero invasive *E.coli*.

VT₁: Verotoxin-1.

VT₂: Verotoxin-2.
# Table (2) *E. coli* that are pathogenic for human

(Puente and Finlay 2001)

<table>
<thead>
<tr>
<th>Type of <em>E. coli</em></th>
<th>Disease</th>
<th>Virulence factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterotoxigenic <em>E. coli</em> (ETEC)</td>
<td>Watery to cholera-like diarrhoea</td>
<td>Heat-labile (LT), heat-stable (ST), colonization factors (CF₃)</td>
</tr>
<tr>
<td>Enteroinvasive <em>E. coli</em> (EIEC)</td>
<td>Watery diarrhoea to dystentery</td>
<td>Ipas, type III secretion (Mix and Spa). Vir G/IcsA</td>
</tr>
<tr>
<td>Enteropathogenic <em>E. coli</em> (EPEC)</td>
<td>Watery diarrhoea</td>
<td>Esps, Type III secretion (Sep and Esc), Intimin, Tir, and BFP</td>
</tr>
<tr>
<td>EnteroHaemorrhagic <em>E. coli</em> (EHEC) or Verotoxigenic <em>E. coli</em> (VTEC)</td>
<td>Haemorrhagic colitis. Hemolytic uremic syndrome (HUS)</td>
<td>Above EPEC factors and Shiga Toxin, haemolysin</td>
</tr>
<tr>
<td>Enteroaggregative <em>E. coli</em> (EAEC)</td>
<td>Watery to mucoid diarrhoea</td>
<td>AAFadhesins.EAST-1, Pet, Pic, hemolysin</td>
</tr>
<tr>
<td>Diffuse adhering <em>E. coli</em> (DAEC)</td>
<td>Watery diarrhoea</td>
<td>F1845 and ADIA-1 Fimbriae.</td>
</tr>
<tr>
<td>Uropathogenic (UPEC)</td>
<td>Urinary tract infection</td>
<td>Type I pili, P pili, A fimbrial adhesins (Afa). a Haemolysin. CNF-1</td>
</tr>
</tbody>
</table>
1.12.1.1 Prevelance of the disease caused by ETEC:

In human all ages are susceptible to the disease, the disease is less common in breast fed infants. During the weaning the disease is common (Guerrant et al., 1975). In adult the disease usually affect the travellers usually from developed countries, where there is a high hygienic standard, to the developing countries where there is a low hygienic standard. The risk of the disease among travellers varies according to the exposure of traveller to the causal agents and susceptibility to them (Black, 1990).

In calves the disease is restricted to those under 4 months of age. The incidence of the disease is high in summer and autumn months in tropical countries. In non-tropical countries the incidence is high during warmer season of the year (Holland, 1990).

1.12.2 Enteroinvasive E.coli (EIEC):
It causes a watery diarrhoea, or dysentry like diarrhoea, with mucus, blood, pus and fever.

1.12.2.1 Virulence factors of EIEC:

EIEC secretes invasion plasmid antigens (Ipas) A-D for invasion of the host cell (colonic epithelium). To export the invasion plasmid antigen out of the bacteria it utilizes specialized secretion system designated the type III system. For cell-to-cell spreading EIEC trigger an actin-mediated motion, the vir G protein also called IcsA is sufficient for this process (Kocks et al., 1995).

1.12.3 Enteropathogenic E.coli (EPEC):

It is the predominant cause of infant diarrhoea world wide and affects children under 6 years. It causes watery diarrhoea of varying severity with vomiting and low fever often accompanying fluid loss (Levine and Edelman, 1984).

1.12.3.1 Virulence factors:

EPEC in order to develop lesion it requires three stages. First stage is characterized by the initial non intimate attachment to epithelial cell surface in a pattern termed localized adherence
LA is associated with the production of type IV fimbriae known as bundle-forming pili (BFP) (Giron et al., 1991). During the second stage a set of EPEC-secreted proteins (ESPs) triggers the activation of signal transduction pathways leading to a complex response by the epithelial cell. Finally and during the third stage an outer membrane protein called intimin allows EPEC to attach intimately to the host cell membrane on interaction with its translocated intimin receptor called “tir” (Lia et al., 1997).

1.12.4 Enterohaemorrhagic or Vertoxigenic *E.coli*:

It causes bloody watery diarrhoea, abdominal pain, fever and vomiting. In severe cases it leads to haemolytic anaemia (Brunder et al., 1997).

1.12.4.1 Vilulence factors:

For EHEC to cause disease three major virulence attribute, the capacity to causes formation of attaching and effacing lesions (A/E), mediated by the genes encoded within the LEE (molecules found on the surface of bacteria or interact with the host cell), the expression of Shiga toxin; and the presence of a
60-MDa plasmid that encodes a haemolysin which stimulate bacterial growth in the gut by releasing haemoglobin from red blood cells (Brunder, 1999).

1.12.5 Enteroaggregative *E.coli*:

It causes persistent pediatric watery diarrhoea often mucoid in nature. There is often low-grade fever, but no vomiting. Grossly bloody stool can occur.

1.12.5.1 Virulence factors of EAEC:

EAEC strains are heterogenous collection of pathogenic *E.coli* that share certain chromosomal and plasmid - borne genes. They adhere to cultured cells in small clumps or aggregates through aggregative adherence fimbriae (AAF1 and AAF11). EAEC strains produces two types of enterotoxin, plasmid - encoded heat - stable toxin designated as entero - aggregative *E.coli* heat-stable toxin - 1 or EAST-1 and a plasmid - mediated entrotoxin “Pet”. Some EAEC strains produce chromosomally encoded protein known as “Pic”, a protein involved in intestinal colonization (Eslava *et al*., 1998).

1.12.6 Diffusely adhering *E.coli* (DAEC):
It causes persistent watery diarrhoea in children between 2-5 years.

1.12.6.1 Virulence factor of DAEC:

DAEC has been associated with four different adhesins while toxins have not been described. DAEC strain C 1845 express the F 1845 fimbriae adhesins while DAEC strain 2787 expresses an outer membrane fimbrial adhesin denominated AIDA-1 (Peiffer et al 1998).

1.12.7 Uropathogenic E.coli:

It causes urinary tract infections.

1.12.7.1 Virulence factors of UPEC:

In order to successfully colonize and establish urinary tract infection, UPEC encode several adhesins, both are fimbrial or non-fimbrial adhesin (afimbrial adhesion Afa-l and Afa-III). Fimbrial adhesin like type one pili and pylonephritis-associated pili (P.Pili). Many UPEC produce haemolysin, which may be involved in kidney disease in addition to cytotoxin necrotizing factor (CNF-1) (Johson 1991).

1.12.8 E.coli that causes neonatal sepsis and meningitis:
In addition to gastrointestinal and urinary tract infection, some strains of *E.coli* can cause septic (invasive) diseases in newborn (Quagliarello and Scheld 1992)

### 1.12.8.1 Virulence factors:

*E.coli* that causes septic diseases produce a polysaccharide capsule that blocks complement and antibody deposition on the bacterial surface and thus avoid uptake and clearance by antibody-mediated immune events (Robins *et al*., 1974).

*E.coli* that cause meningitis and sepsis often produces type-1 pili, the presence of type-1 doesn’t increase virulence factor but help in intial colonization of the oropharynx of the neonates. Some *E.coli* associated with new born meningitis express S-fimbriae that mediate binding to glycoprotiens. The S-fimbria are expressed *in vivo* in blood and cerebrospinal fluid, and mediate binding to cells lining the choroid plexuses and brain ventricles and in order *E.coli* to invade central nervous system and cross blood brain barrier (Korhonen *et al*., 1985).
1-13 Pathogenesis of ETEC: -

Two factors are essential for ETEC to cause diarrhoea, colonization factor and toxin production.

1.13.1 Colonization factors:

ETEC attachment to an intestinal surface is mediated by colonization factor antigens (CFAs), coli surface antigens (CSs), and putative colonization factors (PCFs), which are generally referred to as colonization factors (CFA) (Cassels and Wolf, 1995). These structures are
Candy (1980) The role of mucosal adhesion in the pathogenesis of *E.coli* induced diarrhoea.

essential for ETEC to colonize the small intestine, which is a central step in ETEC’s virulence. At least 20 different and antigenically distinct CFs have been described in human ETEC, and these are found in varying combinations along with LT, ST, or both. The main CFs associated with human ETEC strains include CFA/I, constituted by a single fimbrial structure, and CFA/II and CFA/IV, which can be a combination of a particular
set of CSs (Cassels, 1995). CFA/II can express CS3 alone or in combination with CS1 or CS2, while CFa/IV strains can express CS6 alone or mixed with CS4 or CS5. CFs dictate host and tissue specificity, since animal ETEC CFs are not found in human ETEC isolates. The main CFs associate with calves ETEC is K99 (F5). Morphologically, CFs can be subdivided into four major groups: rigid rode (e.g. CFA/I), bundle forming (e.g. longus), fimbriar and non fimbrial adhesins (Cassels and Wolf, 1995).

ETEC CFs are encoded within standard fimbrial operons (Kroglet 1991). These operons usually encode 4-8 proteins whose functions include regulation, the major subunit that forms the adhesin, and accessory factors that include a perplasmic chaperone and an outer membrane molecule usher. The CS₁ to express its function it require Coo operon which is composed of Coo B, Coo A, Coo C, and Coo D genes.

Coo B act as a chaperone-like protein and is required for pilus assembly but is not found in the final pili structure. Coo A is the major structural subunit. Coo C is an outer membrane
protein and is involved in transmembrane transport of CS$_1$. CooD that is located at the tip of the pilus and is involved in the adherence determines the initiation of pilus assembly and modulates the number of CS$_1$ pili on bacterial cells. Unlike Pap and type 1 pili, which produce minor tip adhesion, the major structural subunit (the stalk protein) also function as the major adhesin. Operons are usually flanked by transposons, and are mainly contained on plasmid that also contain LT and/or ST.

The intestinal receptors that CFs bind include sialoglycolipids such as GM2, sialic acid containing glycoconjugates, a sialogangliosides, and several other glycoconjugates found on the cell surface (glycolipids and glycoproteins) as reviewed by (Cassels and Wolf (1995).

The oligosaccharides expressed on mammalian cell surface vary widely, providing an extensive range of options that might contribute to the host and tissue specificity for the ETEC CFs.

1.13.2. Toxin Production:

ETEC produce two types of entro-toxins.
1.13.2.1 Labile toxins (LTs):

Labile toxins are high molecular weight proteins, they are of two form LT-1 and LT-11. Labile toxin1 (LT-1) is the predominant form and is quite similar to cholera toxin (80% identity) (Domenighini et al., 1995). LT-1 is oligomeric in structure with one enzymatic A subunit and five B subunits are arranged symmetrically in a ring-like structure that binds the ganglioside GM1 and weakly to ganglioside GD1b.

**Mode of Action:**

The A subunit is proteolytically cleaved into two domains- A\(^1\) and A\(^2\) - that remain linked by a disulfide bond and span the centre of the ring. The toxin is endocytosed, and the A subunit reaches the bottom surface of the epithelial cell after escaping the endocytic vesicle. The A\(^1\) peptide transfer an adenosine diphosphate-ribosyl (ADP-ribosyl) group from nicotine amide adenine dinucleotide (NAD) to the \(\alpha\)-subunit of the guanosine 5- triphosphate (GTP) binding protein (Gs). This modification of Gs \(\alpha\)- subunit inhibits its intrinsic GTP-ase activity which result in permanent activation of adenylate cyclase, leading to
accumulation of intra-cellular levels of cyclic adenosine monophosphate (cAMP).

As cAMP accumulates inside the intestinal cells, the cAMP dependant kinase (A-Kinase) is activated which then results in phosphorylation of apically located chloride channel proteins (cystic fibrosis trans membrane conductance regulator protein (CFTR)), this causes channel opening, and chloride ion efflux out of cells along with a block in ion and fluid absorption into cells, resulting in a net osmotic imbalance which result in watery diarrhoea (Goldstein et al., 1994).

LT-11 shows less identity to LT-1 and no identity to B sub unit. LT-11 binds best to gangloisides GDIIb or GDIIa and it is found mainly in animals but not in human (Gyles 1993).

1.13.2.2 Stable Toxins (STs):

STs are of two types STa and STb, they are cysteine-rich molecules that can form three intramolecular disulfide bonds. STs are made from larger precursors (72 amino acids) that can be cleaved as the molecule transits out of the bacterium (Takeda et al., 1979).
**Mode of action of STs:**

The membrane receptor for Sta is guanylate cyclase C (GCC), which is located in the apical membrane of intestinal cells (Shulz *et al.* 1990). Because of the apical location of guanylate cyclase, ST-mediated cell activation is quite rapid. GCC activation leads to accumulation of intracellular cGMP levels, the cGMP-dependent activation of protein kinase A (PKA) and the PKA-dependent phosphorylation and activation of the cystic fibrosis transmembrane conductance regulator (CFTR), which finally leads to increase chloride secretion and blockage of sodium chloride uptake, resulting in diarrhoea (Goldstein *et al.*, 1994). STb is primarily found in animal pathogens and it has also been isolated from human ETEC isolates. STb bears no sequence similarity to Sta, although it has four cystine rich molecules. STb affects neither cAMP or cGMP levels nor stimulates chloride secretion instead it elicit an intestinal response characterized by secretion of bicarbonate to which human cell lines seem to be insensitive (Weikel *et al* 1986).
1.14 Diagnosis of ETEC Infection: -

Diagnosis depends on a combination of both clinical and laboratory findings.

1.14.1.Clinical findings:

1.14.1.1 In Man:

ETEC affects new born, infants and adult (Traveler’s diarrhea). In new born and infants ETEC cases sever watery diarrhea, abdominal pain, nausea and in sever cases dehydration occur leading to acidosis and death. In adults ETEC causes what is known as travelers diarrhoea. Traveler’s diarrhoea usually affects person traveling from developed countries with high hygiene standards to countries with lower hygiene standards, usually tropical countries. The symptoms usually begin within 14 days of arrival. The one set of diarrhoea is abrupt and the stools are loose or watery but rarely contain blood or mucus. Other symptoms include malaise, fever, nausea and vomiting. The illness usually a self-limiting and subsidies within two or three days but a majority of suffers have to endure a much more sever illness (Lambert 1979).
1.14.1.2 In Calves:

ETEC commonly causes diarrhoea in calves less than 1 week of age but it can be a problem in calves as old as 2-3 weeks (Acres 1985) Older calves may also be affected by ETEC in the presence of certain agent like rotavirus (Gyles 1993). The disease is characterized by profuse watery diarrhea, dullness, inappitance in severs cases dehydration occur and acidosis.

1.14.2. Laboratory diagnosis: -

After isolation and identification of *E. coli* the following tests are usually performed.

1.14.2.1 Detection of colonization factors:

In human two distinct colonization factors have been found among ETEC CFA/1 and CFA/11, whereas in calves K99 (F5) is specific for intestinal adhesion. Colonization factors can be detected using haemagglutination and haemagglutination inhibition by D-mannose (100 µg/ml PBS) or specific antisera (Jones *et al* 1979 and Burrows and Sellwood 1976).

1.14.2.2 Enterotoxin assay of ETEC isolates:

1.14.2.2.1 *In vivo* Bioassays:
A. Rabbit ileal loop (The adult rabbit ligated gut test):

It is a successful animal model developed by De and chalterjee (1953). These authors demonstrated that a loop prepared by injection of segments of the small intestine of the adult rabbit with *V. cholerae* showed after 18-24 hours accumulation of fluid into the ligated intestine. The first attempt to standardize this test was made by Burrows and Musteikis (1966) to compare the reactivity in the loop of different animals, these authors introduced the fluid accumulation ratio (FAR) that relates the volume of fluid accumulates in the loop to the length of the loop. Improved anesthesia and surgical procedures and injection of rehyderation fluid to compensate for the body fluid loss were introduced by Ibrahim (1984). The LAGT was used to study the toxigenicity of ETEC (Ibrahim, 1984).

B. Adult Rabbit Open gut Model:

It had recently been developed for study of ETEC infections. This model involves transient ligating of the ileum of the animal, producing a temporary ileal mechanical obstruction at the time of inoculation of the organism in the upper small
bowel. Severe diarrhea with a high mortality rate then results following ETEC challenge (Spira et al., 1981).

**C. Infant mouse Test:**

It is developed by Dean *et al* (1972) and modified by Giannella (1976). It is still the only practical way to detect the ST entrotoxin which is not immunogenic. ST preparation is inoculated intragastrically into suckling mice and the suckling mice are examined after 3-4 hrs. The ratio of intestinal weight to remaining body weight is then determined separately or in-groups of three to six mice each, depending on the laboratory and the mouse strain used (Greenberg and Guerrant, 1981).

**D. Infant Rabbit:**

Seven to nine-day-old infant rabbits are used for the detection of both ST and LT. One ml of broth culture is inoculated into the small intestine, either directly or through a surgically implanted gastric tube; the animals are sacrificed after 6 hrs. The whole bowel is dissected to determine the fluid to-
whole weight ratio. If the ratio is > 0.2 the test is considered positive (Dulta and Habbu 1955).

**E. Rabbit Skin Test:**

It is used to detect the effect of LT and ST on Rabbit skin permeability assay. Twenty-four hours after an intracutaneous injection of toxin, a blue dye (Evans’ blue) is given intravenously to demonstrate the area of increased skin permeability (bluing area) (Moon and Whipp, 1971). ST provides a qualitatively different type of skin permeability than LT.

**F. Perfused intestinal loops:**

This method used for detection of entrotoxins (LT and ST) and other agents that affect fluid secretion, (Nalin et al 1974). For several years, intestinal perfusion have been done in canine and more recently, in rat isolated small bowel segments *in vivo*. This model provides an opportunity to detect decreased absorption even in the absence of net secretion (Nalin *et al* 1974; Klipstein *et al* 1978).
1.14.2.2.2 In Vitro Bioassays:

1.14.2.2.1 Tissue Culture Assay:

This assay used for detection of LT. It is based on the fact that LT stimulates adenylate cyclase or cAMP pathways resulting in a morphological changes. LT was found to cause the rounding of mouse adrenal tumor Y₁ cells, Chinese hamster ovary (CHO) cells and African green monkey kidney cells (Vero) respond with characteristic morphological changes to LT (Speirs et al., (1976)).

Speirs et al. (1976) used the continuos cell line, Vero cells (African green monkey kidney cells) for the bioassay of ETEC LT. The Vero cells showed characteristic morphological changes in response to culture filtrate of toxigenic strains of *E.coli*. The cells were transformed from spindle shaped cells to round cells refractile and with several filamentous tendrils. The sensitivity of Vero cells was described as adequate. First evidence of response in Vero cells to LT was noted at 4 hour and elicited response in 50% to 100% in vero cell appear after 18 hrs and the cellular changes persisted for at least 3 days. The
morphological effects were recorded as 1, 2, 3 or 4 corresponding roughly to $\leq 25$, 50, 75 or $\geq 90\%$ cells affected respectively. They also reported that stock culture of Vero cells required no maintenance for periods up to 3 weeks and growth medium can be replaced with PBS at the time of the test. Therefore, Vero cells were found the simplest and most economical cell line for testing toxigenicity of \textit{E.coli} strains.

1.14.2.2.3 Immunologic assays:

1.14.2.2.3.1 Enzyme-Linked Immuno Sorbent Assay (ELISA):

Enzyme immunoassay referred to as enzyme-linked immuno sorbent assay (ELISA), originally described by Engvals and Perlman (1971), have revolutionized diagnostic microbiology. The assay can be designed to detect microbial Ags for instant, bacterial toxin or Abs. The exquisite sensitivity of the method enables less than one ng microbial Ag/ml to be detected. A wide variety of different assay procedures are used including direct, indirect, sandwich (Ag-capture) and competitive ELISA. It is application for assaying enterotoxin
was subsequently elaborated by Holmegren and Svennerholm (1973) by coating polystyrene tubes with LT. An interesting modification of the indirect ELISA for the detection and quantitation of cholera toxin (CT) and *E. coli* LT has been described by Svennerholm and Holmegren (1976) and Sack *et al* (1980) who used microtitre plates coated with gangoliside (GM1) the natural receptor for CT and *E. coli* LT. This method compared favourably with similar assays using anti CT coated plates (Ag- capture ELISA) as reported by Sack *et al* (1980). In both system an enzyme labelled Ab is added and its presence being reflected by hydrolysis of the subsequent added substrate. The colour produced can be quantitated by ELISA reader. The major advantage of ELIAS is the stability of the enzyme-Ab conjugate, which has self-life of years, and safe to handle and the test is simple to perform (Ibrahim 1984).

1.14.2.2.3.2 Solid phase Radio Immuno Assay (RIA):

The RIA techniques have become an extremely important tool in biomedical research and clinical practice. The technique was developed by Yallow and Berson (1960) for measurement
of human insulin. Nevertheless, RIA techniques have been in general use for many years and their usefulness for detecting extremely small amounts of immuno reactant Ags or Abs is well acknowledged. The exquisite sensitivity of the method enables less than one ng of microbial Ags per ml to be detected. It is therefore, not surprising that RIA has been utilized for assaying enterotoxins particularly in situations where very high sensitive test is desired. Competitive liquid-phase RIA was described by Hejtmancik et al (1977) primarily for the analysis of the antigenic mosaic of cholera toxin (CT) rather than testing strains for toxigenicity. However, Ceska et al (1978) developed a solid-phase competitive RIA for the detection of E.coli enterotoxin in microtitre plates coated with anti-CT. Test samples (culture filterates) and $^{125}$I-labelled purified E.coli enterotoxin were added. After incubation the plates were washed and residued radioactivity was measured in gamma counter. Greenberg et al (1977) used direct non-competitive RIA for quantitation of E.coli LT using microtitre plates coated with antiserum to CT. Test samples were then added and after incubation and washing
an indicator Abs were added (anti-ct $^{125}$I labelled IgG).

Following incubation and washing radioactivity was measured in gamma-counter. Major objections to RIA include expensive gamma counter, expensive isotope with short-half life, the presence of radiation hazards and the disposal of radioactive waste.

1.14.2.2.3.3 Elek test:

Various assay methods for LT have been developed. However, many of these assay methods are unsuitable for routine clinical purposes because are laborous, cumbersome and require tissue culture set-up or surgical intervention in animal models (Honda et al., 1981). Elek test was developed as a simple and reproducible assay method that can be widely used in clinical laboritories. The test is a modification of test originally developed by Elek (1948). ETEC were tested for the production of LT in agar using antiserum placed in wells. The presence of precipitation lines indicates the production of toxin. Antibiotics like polymxin or lincomycin impregnated in disks
were placed over the colonies to facilitate the secretion of the toxin into the medium.

1.14.2.2.3.4 Passive immune haemolysis:

*E. coli* cells were cultured at 37°C for 24 h with shaking in Evan’s broth, then the culture was centrifuged and the precipitate was mixed with polymyxin B solution, and incubated at 37°C for 30 min. with gentle shaking. Then the mixture was centrifuged and the supernatant fluid was used as LT preparation (Evans and Evans 1977). A sample of the toxin was mixed with 2% sheep blood cell suspension in certain buffer containing 0.9% NaCl and incubated at 37°C for 30 min.

Hyperimmune serum and complement were added, and incubated. The mixture was centrifuged. The extend of haemolysis was measured by reading the absorbance of the supernatant fluid. Sample which gave an absorbance at 420 nm or greater than 0.3 were considered to be positive (Honda *et al* 1981).

1.14.2.2.4 Genetic Assay (DNA Probes):
ETEC strains were among the first pathogenic microorganisms for which molecular diagnostic techniques were developed. As early as 1982, DNA probes were found to be useful in the detection of LT-and ST-encoding genes in stool and environmental samples (Natro & Kaper 1998). Since that time, several advances in ETEC detection have been made, but genetic techniques continue to attract attention and use. The LT polynucleotide probe provides good sensitivity and specificity when labelled with radioisotopes or with enzyme, non-isotopic detection systems. Several different protocols have been published in which non-isotopic labelling methods have proven useful for LT detection (Natro and Kaper 1998). Now a highly reliable alkaline phosphates based detection system is in use as polynucleotide probe colony blot hybridization.

ST polynucleotide probes have had problems of poor sensitivity and specificity, presumably because of the small size of the gene. For this reason, oligonucleotide probes which are generally more sensitive and specific for ST detection have been developed. Recently, a trivalent oligonucleotide probe has been
proposed which may be of use in detecting the genes encoding LT, ST, and the EHEC Shiga toxin genes (Natro and Kaper, 1998).

Several PCR assays for ETEC are quite sensitive and specific when used directly on clinical samples or on isolated bacterial colonies.

1.15 Serotyping:

It becomes clear that ETEC belong in general to a group of serotypes quite distinct from that of EPEC serotypes (Sack, 1981). Nevertheless, because enterotoxin is plasmid mediated it has been postulated that any *E.coli* serotype could theoretically be enterotoxigenic. Although the number of reported ETEC serotypes is steadily expanding, there remain, a relatively small group of serotypes (Table 3) which are usually enterotoxigenic and have a wide geographical distribution (Merson *et al.*, 1980a). It has been postulated that pools of antisera might therefore be useful in recognizing these serotype.
Table (3) Serotype characteristic of the diarrheogenic *E.coli* categories (Ørskov et al 1982)

<table>
<thead>
<tr>
<th>Category</th>
<th>Serogroup</th>
<th>Associated H antigen (s)</th>
<th>Category</th>
<th>Serogroup</th>
<th>Associated H antigen (s)</th>
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however, a considerable number of ETEC were found which could not be identical serotypes, with these pools (Sack 1981). Since there are marked geographical difference antiserum pool should be adjusted for different geographical areas.

### 1.16 Treatment and control of ETEC infection:

#### 1.16.1 Treatment:

Clear liquids are recommended for persons with diarrhea to replace dehydration and loss of electrolytes. According to world health organization 3.5g of sodium chloride, 2.5g of sodium bicarbonate, 1.5g potassium chloride and 20g of glucose or surcose per liter of clean water are recommended for oral rehydration. Isotonic intravenous rehydration may become necessary if the patient is unable to tolerate the solution because of vomiting or lethagy (Greenberg and Guerrant 1981). Traditional remedies with salty liquid as chicken soup are also effective. Bismuth subsalicylate compound reduces the number of bowel movement, which result in reduction of electrolyte loss. All antimotility agents e.g imodium and Lomotil can effectively relieve pain associated diarrhoeal and cramps.
Antimotitly should be avoided in persons with high fevers or bloody diarrhoea and should be discontinued if diarrhoeal symptoms persist more than 48 hs (Rowland 1981).

Antibiotics can shorten the duration of diarrhoeal illness especially if given early, but they are usually not required. Because of resistant of ETEC to common antibiotics, the decision to use an antibiotic according to sensitivity test should depend upon the severity of illness and the risk of adverse reaction such as rash and antibiotic associated colitis. Fluoroquinotones have been shown to be effective therapy. On the other hand treatment of calves with diarrhoea follow the same principles. Calves, which respond and recover, usually show marked improvement from intravenous and or oral fluid therapy within 24-42 hrs (Radostits 1975). They start to urinate and dry matter content in their faeces will increase, while calves which don’t respond will not hydrate normally, they may not begin to urinate because of irreversible renal failure, their faeces remain watery, they remain depressed and not well enough to
suck or drink and continued intravenous and or fluid therapy beyond 3 days is usually advised.

Oral or parental antibiotic therapy is routine useful in calves with diarrhoea. Broad-spectrum antibiotic oxytetracycline, neomycin, chloromphenicol and chemotherapeutic such as nitrofurans are used widely for treatment of calf with diarrhoea. Ideally drug of choice will depend on the sensitivity of the causative bacteria to the drug.

1.16.2 Prevention of ETEC in Human:

In young prevention can be by encouraging breast-feeding, avoiding drinking contaminated water, avoiding uses of contaminated instrument and through vaccination. For adults (travelers) infection can be prevented by avoiding foods and beverages that can be contaminated with bacteria.

In developing countries food can be made safe to eat by thorough cooking and by keeping it hot. Fruits and vegetables should be cleaned by the user, water used for drinking or washing vegetables in these countries should be bottled, boiled or chemically treated with iodine, chlorine or another
disinfectants. Good sewage management can prevent spreading of infection from one person to another. Bismuth subsalicylate preparations can reduce the risk of becoming ill with ETEC infection.

1.16.3 Control of calve diarrhoea:

Control of the disease at economical minimum amount should be the aim of the herdsman. Reasons for high mortality are commonly difficult to identify but include: inadequate colostrum intake, high stocking rate in the calf pens, concentrated calving frequency resulting in high percentage of susceptibility, newly introduce pathogen to which the cows and hence the calves have no immunity and inadequate calf management (Radositis 1975).

Effective control of diarrhoea will be accomplished by applying the following principles (Oxnder et al 1973):
1. Removal of the calf from the contaminated environment:

   Dairy calves should be removed immediately at birth to an individual calf’s pen, which was previously cleaned, disinfected and left vacant for a few days.

2. Removal of infectious agents from the environment:

   Occurrence of acute diarrhoea in young calves is usually proportional to contamination with entropathogenic bacteria as well as to the concentration of calves in the pen and occupation time of the calf born.

3. Increasing non-specific resistance:

   This depends primarily on day to day care management of the herd and particularly young claves. Factors such as nutrition of the dam during pregnancy, environmental temperature, humidity of calf pens feeding schedule, other disease in the herds, season of the year and the amount of colostrum ingested and how soon after birth. Early feeding of colostrum during the first 36-40 hour of life is very important because after this period the absorption of immunoglobultin will not take place.
4. Increasing specific resistance:

New born calves deficient in immunoglobulin they received their maternal immunity through colostrum to protect calves and therefore the dams should be vaccinated.

1.16.4 Vaccination against ETEC:

Development of a vaccine against *E.coli* -induced diarrhoea is complicated by the existence of several different diarrheal diseases caused by *E.coli*. These diseases differ in their pathogenesis as well as in the virulence attributes and antigenic make up of the *E.coli*, which cause them. It appears unlikely that these different diseases can ever be controlled by a single vaccine. On the other hand, it is realistic to expect that the principles which apply to development of a vaccine for one of these diseases will apply in part to the others (Moon and Runnel, 1981).

1.16.4.1 Vaccination in Animals:
Several groups have demonstrated that vaccination can protect offspring that were suckling vaccinated dams. There is little or no transport of antibodies across the placenta in some species of animal (Cattle and swine). However, colostrum and milk from vaccinated dams contain antibodies, which can provide passive protection. Under some regimes, protection of sucklings can be enhanced if dams are vaccinated orally than parentally (Collins, 1974). Protection following oral vaccination has also been demonstrated. Pregnant cows should be inoculated parenterally with purified F5 or 987-type fimbriae. Newborn calves suckled on immunized mothers were significantly protected against death from diarrhoea. Non-living vaccine containing components to stimulate both antibacterial and antitoxic immunity were added to animal feed. Multiple oral doses from non-living vaccine are required to prime the secretory immune system and assure a good local antibody response (Porter and Linggood, 1983).

Vaccination is coming into routine use as part of programmes to control ETEC infections in farm animals in
Most, but not all of the field trials that have been reported, have presented evidence that vaccination can reduce morbidity or mortality due to naturally occurring ETEC infection (Porter and Linggood, 1983). ETEC are antigenically diverse and there are diseases (viral, bacterial, protozoon and unknown causes) which are clinically similar to ETEC infection in animals. Thus, the elements which have probably determined that apparent success or failure of ETEC vaccines in the field are antigenic matching and diagnostic accuracy. Both efficacy and use of vaccination are likely to increase if more is learnt about mucosal immunity in general and about protective antigens of ETEC. Selective pressure exerted by widely used vaccine may accelerate the emergence of new ETEC serotype. Thus continued efficacy of a vaccine may require occasional adjustments in antigen content as the disease evolves. The ultimate impact of vaccines on animal production will depend on their cost-effectiveness as compared to the alternate means of control (Porter and Linggood, 1983).
1.16.4.2 Vaccination in Human:

Mammary secretion of antibodies against endemic pathogens appears to be a universal mechanism for maintaining passive immunity in the gut of the mammalian neonate.

Colostrum and breast milk from some women provides antibodies protection against ETEC, which ceases at weaning. Furthermore, parenteral or oral vaccination can boost antibody production by the human mammary gland (Moon and Runnel, 1981). It seems likely that breast-fed human infants could be immunized against ETEC infections by vaccinating their mothers. Vaccination by purified CFA/I and CFA/II fimbrial vaccines can produce protection against ETEC in human (Levine et al., 1979). Levine et al. (1983) undertook to assess type I somatic pili as a potential immunizing agent in the hope that inclusion of this antigen might broaden the spectrum of protection of a future polyvalent fimbrial antigen vaccine. Another approach toward prevention of ETEC infection in humans involves the use of alternated five strains of *E.coli*
bearing several critical antigens. After ingestion of a single dose this strain colonize the small intestine and stimulate the immune response without causing significant reactions (Karper and Basel, 1983). In spite of the technical complications posed, it might be feasible to protect bottled-fed infants by adding antibody to formulae or even vaccinating animals to stimulate them to produce milk, which contains antibody against the appropriate protective antigens of ETEC.

CHAPTER TWO

Material and Methods

2.1 Collection of Samples: -

2.2.1 Animal faecal samples:

Soft to watery faecal samples were collected from diarrhoeagenic calves age between one week to six months, from conventional dairy farms around Khartoum States. Calves in these farms have a history of passing soft to watery faeces for a period of two to seven days. Faecal samples were collected during a period of 4-6 months (from April to October). Four
major areas were covered in this study in western and eastern of Khartoum State.

From the western part 31 samples were collected from conventional farms at Jebel Toria, 35 samples from Almerkheiat area and 23 from Alhitana area. From the Eastern part of the state 57 samples were collected from Hilat Kuku area.

All feacal samples were obtained directly from diarrhoeagenic calves or obtained following thumbing the calves under the tail using disposable gloves to stimulate defeation. Samples were collected in sterile MacCarteny bottles and labelled and kept on ice. All samples were cultured 2-3 hours after collection or kept frozen at-20°C.

2.1.2 Human Stool Samples:

Human stool samples were collected from pediatric wards at different hospitals. Twenty-seven stool samples were collected at Omdurman pediatric wards and 31 samples from AlBulk hospital, pediatric wards. Six samples from Khartoum hospital, pediatric wards, two samples from Khartoum North
hospital, pediatric words and six stool samples from Ahmed Ghasim pediatric hospital.

All stool samples were obtained from diarrhoeagenic children in sterile MacCarteny bottles and kept on ice. Samples were cultured 2-3 hours after collection or kept frozen until cultured.

2.2 Culture Media:

All media were dispensed under aseptic conditions in the media room. For preparation of sterile media room, phenolic disinfectant and 70% alcohol were used for disinfecting floor and benches and the room was irradiated by ultra violet light for complete sterilization.

The media were either prepared from the original ingredients or obtained in a dehydrated form.

2.2.1 Solid media:

2.2.1.1 MacConkey’s agar No. 3 medium:
According to the manufacture fifty two grams (gm) of the medium which consist of (20gm) peptone, (10gm) lactose, (5gm) Bile salt, 5gm sodium chloride,( 0.075gm) neutral red and (12gm) agar were added to one litre distilled water and dissolve by boiling. The pH was adjusted to 7.1 and the medium was sterilized then distributed in 20ml amounts in sterile Petri dishes.

2.2.1.2 Nutrient agar medium:

Dehydrated nutrient agar (oxoid) was prepared according to the manufacture’s instruction. This medium consist of beef extract (10gm), peptone (10gm), sodium chloride (5gm) and agar (2%). Twenty-eight grams of the medium were dissolved in one litre of distilled water. The rehydrated medium was boiled to dissolve ingredients and the pH was adjusted to 7.4, sterilized, and then distributed into sterile Petri dishes.

2.2.1.3 Dorset egg medium:
This medium consists of fresh egg mixtures (yolk and white) 750ml (about 16 eggs) and nutrient broth (oxoid) 250ml. This medium was prepared according to Barrow and Feltham (1993). The eggs were washed thoroughly with soap and water, soaked in 70% Alcohol for 30 minutes, then laid on a sterile surface, the shell was broken at the narrower end with a sterile knife and the contents were let to fall into sterile flask and the set was shaken thoroughly to break up the yolk and to produce homogenous mixture. The nutrient broth medium was suspended in 250ml of distilled water, boiled to dissolve completely and then added to the egg mixture and mixed well avoiding bubbles formation. The medium was then distributed in sterile MacCarteny bottles in amounts of 5-7ml each, then sterilized. This media should be used fresh.

2.2.1.4 Simmon’s Citrate medium (Difco):

The dehydrated medium of (Difco) consist of ammonium dihydrogen phosphate (1gm), magnesium sulphate (0.2gm), K2HPO4 (1gm), sodium citrate (2gm), sodium chloride (5gm), bacto-agar (15gm) and bacto-bromothymol blue (0.08g).
According to the manufacture (24gm) from the media were dissolved in one litre of distilled water, pH was adjusted to 6.8 then sterilized and distributed into sterile MacCarteny bottles and allowed to solidify in a slope position.

2.2.1.5 Evans’ medium:

It was prepared according to Evans et al (1973) and solidified by adding 2% agar. This medium consist of casamino acid (Difco) (20gm), yeast extract (Difco) (6gm), sodium chloride (2.5gm), KH$_2$ Po$_4$ (8.7gm) and trace salt solution (appendix 1) pH was adjusted to 8.5 with 0.1 NaOH, sterilized and 2.5ml (20%) glucose solution was added under aseptic condition then distributed into sterile petri dishes (20ml/plate) left to solidify then stored at 4°C untill it was used.

2.2.2 Liquid media:

2.2.2.1 Peptone water (oxid):

It was prepared according to the manufacture: by dissolving (10gm) of peptone and five gram of sodium chloride in one liter distilled water mixed well, distributed into sterile
tubes each tube contain 5-7ml and then sterilized by autoclaving.

### 2.2.2.2 Robertson’s cooked meat medium:

The medium was prepared according to Barrow and Feltham (1993). Thousand grams of minced, fat free beef were added to one liter of 0.05 N-NaoH, boiled for 20 minutes. The pH was adjusted to 7.5 then filtered through gauze or muslin.

The meat was left to dry. The dry meat fragment were distributed to a depth of 2.5cm in sterile screw capped bottles then five ml nutrient broth was added and then sterilized.

### 2.2.2.3 Glucose-phosphate broth:

This was used for methyl red (MR) test and for Voges-Proskauer (VP). It was prepared according to method described by (Barrow and Feltham 1993).

The ingredients of this medium were (5.0gm) peptone, (5.0gm)-dipotassium hydrogen phosphate, (100 ml) distilled water and (5.0gm) glucose. Peptone and phosphate were added to DW and steamed to dissolve, and the pH was adjusted to 7.5 then (5.0 gm) glucose was added and sterilized.
2.2.2.4 MacConkey’s broth (oxoid):

It consist of peptone (20gm), NaCl (5.0gm), bile salts (5.0gm) Bromcresol purple (0.2%) solution and lactose (20gm).

According to the manufacture (5.0gm) from the medium dissolved in (100 ml) distilled water the pH was adjusted to 7.4. The medium then sterilized and distributed into sterile test tubes containing Durham’s tubes. Each tube should receive 5-7 ml from the medium.

2.2.2.5 Carbohydrate fermentation media:

According to Barrow and Feltham (1993). Nine hundreds ml of peptone water were used in preparation of this medium.

The pH was adjusted to 7.1-7.3 then (10ml) of Anadrad’s indicator was added. The solution of the sugar used for a test was prepared by dissolving (1 gm) of sugar in (9ml) D.W. The specific sugar was added to the mixture of peptone water plus indicators, mixed thoroughly sterilized and, distributed into sterile test tubes containing Durham’s tubes.

2.2.2.6 Evans’ broth:
It was prepared according to Evans’ et al (1973) (20gm) Casamino acid (Difco), (6.0gm) yeast extract, (2.5gm) sodium chloride, (8.7gm) KH$_2$ PO$_2$ and (1ml) trace salt solution (appendix 1) were mixed in one litre distilled water the pH was adjusted to 8.5 using NaOH and then sterilized. After cooling (2.5ml) sterile (20%) glucose solution was added. Then the media was dispensed into sterile test tubes 5-7ml / tube.

2.2.3 Semi solid media:

2.2.3.1 Hugh’s and leifson’s (O.F) medium:

Prepared according to Barrow and Feltham (1993): two grams of peptone, (5.0gm) of sodium chloride, (0.3gm) dipotassium hydrogen phosphate (K$_2$HPO$_4$) and 3.0gm agar were dissolved in one litre distilled water by heating in a water bath at 55°C. The pH was adjusted to 7.1 and the medium was filtered then 15ml of 0.2% aqueous solution of bromothymol blue was added as indicator. The medium was sterilized. Sterile glucose solution was added aseptically to give a final concentration of 1% then the medium was mixed and distributed aseptically into sterile test tubes (5-7ml/tube).
2.2.3.2 Motility medium:

This medium was prepared as described by Barrow and Feltham (1993). It consists of (10gm) peptone, (3.0gm) meat extract, (5.0gm) sodium chloride, (4.0gm) agar and (80gm) gelatin. The gelatin was soaked in one litre of distilled water for 30 minutes, then the other ingredients were added, and heated to dissolve the ingredients. The medium was distributed into sterile test tubes in 10ml amounts then sterilized.

2.3 Reagents: -

2.3.1 Tetra methyl-p-Phenylene diamine dihydrochloride:

This was obtained from Hopkin and Williams, London, it was prepared fresh as 1% aqueous solution and used for oxidase test.

2.3.2 Hydrogen peroxide (H_2O_2):
It was obtained from British Drug House Chemical (BDH) company, England. It was prepared as 3% aqueous solution and used for catalase test.

### 2.3.3 Potassium hydroxide (KOH):

It was obtained from Hopkin and Williams, London it was prepared as 40% solution and used in Voges Proskauer test.

### 2.3.4 Kovac’s reagent:

It was composed of five grams of p-dimethylaminobenzaldehyde, (75ml) iso-amyl alcohol and (25ml) concentrated hydrochloric acid. It was prepared according to Barrow and Feltham (1993). The aldehyde was dissolved in alcohol by gentle warming to 56°C. The mixture was cooled and followed by the addition of the acid. The reagent was stored at 4°C in a dark bottle. It was used for detection of indole production.

### 2.3.5 Methly red reagent:

It was prepared according to Barrow and Feltham (1993) by dissolving 0.04 grams of methly red in 40ml ethanol
and diluted with distilled water to 100ml. It was used in methyl red test.

2.3.6 Alpha-naphthol solution:

This was manufactured by British Drug House Ltd England. It was prepared as 5% solution and used for Voges-Proskauer test.

2.4 Sterilization:

2.4.1 Sterilization of equipments:

Glassware such as MacCarteny, bijou, universal bottles and flasks, were sterilized in the autoclave (15lb/square inch) for 15 minutes at 121°C. Petri-dishes, graduated pipettes, tubes and surgical equipments were sterilized in hot air oven at 160°C for 90 minutes.

Micropipette and tips were sterilized by autoclaving at 115°C for 10 minutes and dried at 56°C.

2.4.2 Sterilization of culture media:

Nutrient agar, MacConkey’s agar, cooked meat media, Simmon’s citrate agar, Evans’ medium, Voges-Proskauer medium, MacConkey’s broth and peptone water were sterilized
by autoclaving at 121°C for 15 minutes (15lb/square inch). Hugh and leifson’s (O.F) medium and motility media were sterilized by autoclaving at 115°C for 20 minutes (10lb/square inch). Carbohydrate media were sterilized by autoclaving at 5lb/square inch for 10 minutes (110°C). Dorset egg medium was sterilized by tyndallization for three successive days, it was inspissated by slow heating at 75°C in slope position and maintained at this temperature for one hour. The procedure was repeated for two successive days.

2.5 Culture methods:

2.5.1 Primary isolation:

Faecal samples were streaked onto MacConkey’s agar plates.

2.5.2 Incubation conditions:

All plates were incubated at 37°C for 24 hours at aerobic condition.

2.5.3 Sub-culturing of the isolates:
Typical and well isolated colonies were picked by means of a wire loop and either streaked onto a surface of fresh solid medium plate or inoculated into a liquid medium.

2.5.4 Examination of cultures:

All cultures on solids and semi-solid media were examined with the naked eye for growth, colonial characteristic and any changes in the medium. The liquid media were examined for turbidity, change of colour, formation of pelicle or sediment and accumulation of gas in case of carbohydrate media.

2.5.5 Purification of cultures:

This was obtained by sub-culturing of typical and well isolated colony on MacConkey’s agar plate or nutrient agar plate. Growth was checked for purity by examining smears stained by Gram’s method under the microscope.

2.5.6 Preservation of isolates:

The isolates which were confirmed as pure were preserved on nutrient agar plate, Dorset egg media or cooked meat media. This was performed by inoculation of these media and then incubation at 37°C for 48 hours prior to storage at 4°C.
2.6 Staining techniques: -

2.6.1 Preparation of smears:

This was done by spreading a loop full of liquid culture or an emulsified colony on a clean dry glass slide. The smears were allowed to dry in air and then fixed by gentle flaming.

2.6.2 Staining method:

2.6.2.1 Gram stain:

This was done according to Barrow and Feltham (1993).

2.7 Identification of isolated bacteria: -

2.7.1 Cultural Characteristics:

The growth characteristic of a bacterium in MacConky’s agar medium is essential for identification. The colonial shape, size and colour are useful criteria for isolation of *E.coli*.

2.7.2 Primary tests:

The purified bacterium was identified according to criteria outlined by Barrow and Feltham (1993), Gram stain, morphology and other tests specified below.

2.7.2.1 Motility:
The tested strain was inoculated into the motility medium using straight wire and incubated at 37°C for 48 hours. Motile bacteria spread away from the inoculated line.

2.7.2.2 Aerobic growth:
Cultured media were incubated aerobically.

2.7.2.3 Oxidase test:
The organism was grown in a medium free from glucose and nitrate (nutrient agar medium). A filter paper was placed in a Petri dish and 2-3 drops of fresh 1% tetra methyl-p-phenylene diamine dihydrochloride were dropped on the paper. The test organism was taken by sterile glass rod and smeared across the surface of the paper. Positive reaction was indicated by formation of dark purple colour within 10 seconds.

2.7.2.4 Catalase test:
The organism was grown on nutrient agar and a drop of 3% aqueous solution of hydrogen peroxide was placed on a clean slide. A colony of test culture was placed on a drop of hydrogen peroxide. The test was considered positive if the organism produces gas.
2.7.2.5 Oxidation Fermentation test (O.F):

Two tubes containing Hugh and leifson’s medium were inoculated with the test organism by stabbing with straight wire. The medium in one of the inoculated tubes was covered with a layer of sterile liquid paraffin oil to a depth of one cm. The tubes were incubated and examined daily for 14 days. Change of colour to yellow in both tubes indicated a fermenting organism, yellow colour in the open tube only meant that the organism was oxidative.

2.7.3 Other biochemical tests (Secondary tests)

2.7.3.1 Indole test:

The organism was grown in peptone water and after 24-48 hours incubation, (0.5ml) of Kovac’s reagent was added. Positive result was indicated by formation of red ring.

2.7.3.2 Methyl-red test:

Glucose-phosphate medium was inoculated with the test organism and incubated for 48 hours. 5-6 drops of methyl-red (0.02%) indicator were added and the tube was shaken. The development of red colour indicated positive reaction.
2.7.3.3 Voges-Proskauer (VP) test:

This test is used for detection of production of acetyl-methyl-carbinol by the organism. Glucose phosphate medium was inoculated with the isolate under the test and incubated for 48 hours. Then 0.6ml of (5%) a-naphithol followed by (0.2ml) of (40%) potassium hydroxide (KOH) aqueous solution was added. It was well shaken and put in a slop position and examined after 15 minutes. A positive reaction was indicated by formation of bright pink colour.

2.7.3.4 Fermentation of sugars:

Tubes containing the various peptone water sugars were cultured with the test organism. Positive reactions were indicated by changes of colour to red or bright red due to acid production. Durham’s tubes were examined for gas production.

2.7.3.5 Citrate utilization test:

Simmon’s citrate medium was inoculated with the test organism and incubated at 37°C for up to 48 hours, positive test
was recognized by bluish colour. Negative test was considered after further incubation up to 14 days.

**2.7.3.6 EijKman test (modified 44°C fermentation test):**

MacConkeny’s broth was inoculated with the organism under the test, warmed at 37°C then incubated in a water bath at 44°C ± 1°C for 48 hours. Production of acid and gas was regarded positive result.

**2.8 Storage and labelling of bacterial isolates:**

*E.coli* positive strains were carefully labelled and preserved on dorset egg medium, strains that were not identified as *E.coli* were stored on cooked meat medium.

Isolates of *E.coli* were labelled according to the source of the isolates.

**2.9 Detection of colonization factors (Fimbriae):**

**2.9.1 Bioassay:**

**2.9.1.1 Haemagglutination test:**

All *E.coli* positive isolates were subjected to this test. HA test preparations were done according to (Sedlock *et al* 1981)

**2.9.1.1.1 Preparation of bacteria under the test:**
Heavy culture of the various *E.coli* isolate were made on Evans’ solid media. The plates were incubated at 37°C for 24 hours. Culture were harvested using sterile bent glass rod, in phosphate buffered solution (pH 7.2) (appendix 11), in a sterile test tubes. The number of bacteria was adjusted to $2.7 \times 10^9$ cfu/ml using Macfarlan’s standard tube number nine (appendix 11).

**2.9.1.1.2 Collection of blood:**

**Bovine blood:** bovine blood was collected from the jugular vein of healthy cows at university of Khartoum farm, sterile needles were used for collection of blood into a sterile MacCarteny bottle containing Alsever solution (appendix 11).

**Chicken blood:** was collected from the wing vein of a healthy chicken at the laboratory farm. Blood was stored at 4°C till use.

**2.9.1.1.3 Preparation of blood for the test (both bovine and chicken blood):**

Blood was centrifuged at 3000 rpm for 10 minutes then washed twice using sterile phosphate buffered solution. Two per cent suspension of RBC was made by adding (0.2ml) packed
erythrocyte to (9.8ml) PBS. The haemagglutination test was done as described by Sedlock et al., (1981): Two fold serial dilutions were made in microtitre plate from the bacterial suspension in PBS (50µl), and then equal volume (50µl) of 2% RBC were added to each well, mixed with gentle tapping of the plate and incubated at 4°C for 30 minutes. From piolet study better results were obtained by incubating the plates at 4°C rather than at room tempreature.

2.9.1.2 Haemagglutination in the presence of D-mannnose:

All Haemagglutination positive isolates were examined for their resistance to D.mannose. this test was done according to the procedure described by Sedlock et al., (1981): The D.mannose solution was made at a concentration of 100µg/ml PBS (appendix 11). Bacterial suspension were serially diluted (two fold serial dilutions) in V-shaped microtitre plates containing (50µl) of D.mannose in PBS in each well and incubated for 20min. at room tempreature. Then 50µl of 2% erythrocyte suspension were added to each well. The test was incubated at 4°C for 30 minutes then examined for haemagglutination activity.
Mannose resistant haemagglutinating isolates and the haemagglutination titres were recorded. Haemagglutination activities of the various *E. coli* MR strains were studied by growing each isolate at three different temperatures (37°C, 25°C and 20°C). MRHA isolates were used for further characterization.

2.9.1.3 Haemagglutination inhibition by antiserum:

2.9.1.3.1 Production of polyclonally-restricted antiserum against adhesion factors:

A. Preparation of the antigen:

MKhE14a was selected for the production of antiserum against the adhesion factors that agglutinated bovine RBC and it was mannose resistant and most likely a fimbriated strain. Ten plates of Evan’s medium containing 2% agar were inoculated with MKhE14a and incubated for 18h at 37°C. The growth was checked for purity by Gram stain. Plates were harvested in 0.5% formalin- saline and the harvest was allowed to stand for 24 h at 4°C to kill all the bacteria.

The harvest was washed three times in PBS and the number of bacteria was adjusted to $2 \times 10^9$ cfu/ml using Macfarlen’s tube number nine.
B. Animals used:

Two New-Zealand white Rabbits from (Pharmcutical company, Bulsam, Khartoum North) were used for antiserum production. The animals were kept for adaptation period of 1 week. Each rabbit received intravenous injection of (0.5ml) of the Ag at day zero and (1ml) at day 3 and (2ml) at day 6 and (2.5ml) at 9 day. Proof bleeding was made one week following last injection. The presence of strong antibody against MkhE14 adhesion factor was demonstrated by agglutintion test. MSHA strains were negative to the test. Rabbits were bled from the ear vein and serum was collected and kept at – 20°C.

2.9.1.3.2 Assay procedure:

The test was performed according to Freter (1980) two fold dilution of anti-adhesion serum in 50µl KRT were prepared in V-shaped microtitre plates viz. 1/2, 1/4, 1/8, 1/16, 1/32 etc. Then 50µl of the following bacterial strains JETE8, MKhE14a, HITE16, HKUE91 prepared as described for Haemagglutination were added to each serial dilution. The plates were incubated at 37°C for 30 minutes. Then 50µl of 2% bovine RBC were added and the plates were incubated at 4°C. Control wells without antiserum dilutions were included by adding the erythrocyte
suspension to the \textit{E.coli} test strains. Inhibition was indicated by absence of haemagglutination and RBC appears as a button on the microtitre wells.

\textbf{2.9.1.4 Adhesion to isolated rabbit brush border cells:}

This test was used for detection of adhesion ability of \textit{E.coli}. The test was performed according to Jones \textit{et al.}, (1979).

\textbf{A. Preparation of brush border cells:}

Brush border cells were prepared according to Jones \textit{et al.}, (1979). An adult rabbit (4 months) was killed by intravenous injection of pentobarbital. The small intestine was excised and the lumen was washed free of digesta with several changes of (0.85\%) Normal saline (appendix 111). The small intestine was partially filled with buffer solution A (appendix 111). The intestine was incubated at room temperature for 15 minutes in a bath of buffer solution B (appendix 111). The intestine was then drained and filled with the same buffer solution B and the epithelial cells were released by manipulation of the intestine. Epithelial cells were recovered at 4$^\circ$C by centrifugation at 5000 rpm for 10 minutes. The cells were suspended in 5mM EDTA pH 7.4 (appendix 111) and the brush borders cells were recovered by centrifugation at 5000 rpm for 10 minutes. Brush borders cells were purified by repeated alternative homogenization in 5mM EDTA pH 7.4 and centrifugation at 5000 rpm.
for 10 minutes, until the supernatent fluid was free of debris. Brush border cells were stored at 4°C in 5mM EDTA containing (10%) formalin. Such preparation could be used for at least 3 months after preparation. For the test procedure the number of brush border cells were adjusted to $2 \times 10^5$ cell/ml using haemocytometer.

**Test procedure:**

The MRHA bovine strains: JETE8, MKh14a, HITE16 and HKUE91 were prepared on solid Evans’ medium as described previously. The bacterial count for each strain was adjusted to $2 \times 10^7$ cfu/ml. Then 50µl containing $10^4$ cell from the adjusted brush border cells ($2 \times 10^5$ cell/ml) were added to 500µl of each test strain ($10^7$ cfu/ml) ie in the ratio of one brush border cell to $10^3$ bacteria. The mixture was incubated at 37°C for 15 minutes then centrifuged at 3000 rpm for 10 minutes. From each pellet three slides were prepared and the slide were left to dry, fixed with (5%) acetic acid alcohol and stained with Gram stain and examined under the light microscope (under oil emersion).

**2.9.1.5 Inhibition of adhison of calves MRHA *E.coli* to brush border cells:**

The strains JTE8, MKHE14a, HITE16 and HKU91 were used in this study. The test was performed as for adhesion of MRHA *E.coli* strain to brush border cells except that each strain was incubated with anti
adhesion serum for 30 minutes at 37°C before the brush border cells were added. Slides were prepared in triplicate as described before and stained with Gram stain.

2.10 Detection of labile toxin using Vero cells: -

2.10.1 Preparation of cell culture media:

The media used was Glasgow minimum essential medium with L-glutamine. This medium is a modified Eagle’s medium it was prepared according to the manufacture (Sigma) by dissolving the content of one bottle (100gm) into 2 liters of double distilled water. Stirred until dissolved, then (2.75gm) bicarbonate was added. While stirring the pH of the medium was adjusted to 0.1- 0.3 units below the desired pH since it may rise during filtration. The medium then sterilized by filtration using a membrane with a porosity of 0.22 microns. The sterile medium was distributed in suitable containers and stored at -20°C (as a stock). Sterility of medium was checked by inoculation of 2 drops from the medium into two vials containing thioglycolate medium (sterility test media). One vial should be kept at room temp and the other at 37°C for 7 days then examined.

The working tissue culture medium was prepared as follows: (200 ml) stock tissue culture, (25ml) of 1% yeast extract, (25ml) of 50% lactoalbumin hydrolysate, (8ml) tryptose phosphate broth, (1ml)
mycostatin, (1ml) penicillin and (1ml) streptomycin (appendix v) and complete to one litre with DDW. (Appendix IV). All additions were done aseptically from sterile solutions.

2.10.2 Preparation of Vero cell (passaging or subculturing):

Vero cells were obtained (from South Africa) in a tissue culture flask. For subculturing the following steps were performed.

1. The supernatant medium was discarded.

2. The cells were washed in PBS. Washing will remove the residual serum (which would inactivate the trypsin) and residual bivalent ions, which would inactivate the versene.

3. Trypsin was added (0.05-10%) to cover the culture and incubate at room temperature or at 37°C until the cells round up and start detaching from the surface. This can be detected by microscopic examination.

4. A suitable volume of growth medium was added. Then cells were removed from the surface of the container by inspiration and aspiration using Pasteur pipette and transferred to test tubes.

5. The cells were centrifuged at 3000 rpm for 15 minutes. The supernatent was discarded and the cells were resuspended in new medium.
6. The cells were counted and adjusted to $1 \times 10^6$ cell/ml in the medium containing 5% bovine serum.

2.10.3 Preparation of Toxin:

Mannose resistant Haemogglutinating strains of *E. coli* were inoculated into 10ml Evans’ broth and incubated at 37°C. For preparation of cell-free culture (according to Evans et al 1973), bacterial cells were removed by centrifugation at 6000 rpm for 30 minutes in a cold centrifuge.

2.10.4 Assay procedure:

Assay was done as described by Speirs *et al* (1977) with modification. Vero cells were grown in 96 well tissue culture plate by inoculation of each well with 100µl medium containing $1 \times 10^5$ Vero cells. The plate was incubated for 2-3 days at 37°C in a humidified atmosphere, resulting in confluent monolayers. Thirty minutes prior to the experiment, the culture medium was replaced by the same medium without calf serum. After 30 minutes 25µl of each of the toxin preparation to be tested was added per well. Tests were done in duplicates. Some wells were left uninoculated as a negative control. The plates were examined after 24 hrs
under microscope (power 100x) Heat labile toxin causes rounding of the Vero cells.

2.11 Detection of LT using adult Rabbit ligated gut test:

This test was done according to Evans’ et al (1973). E.coli isolates, which were MRHA and positive in Vero cells, were cultured on Evans’ broth and incubated at 37°C for 24 hours. In this test bacterial suspension was adjusted to $10^9$ cfu/ml for each test isolate.

Two month-old rabbits (2kg) were acclimatized to feed and housing for one week. Then 24-48 hours before the test was performed, only water was supplied at libidum. One day prior to surgery, the skin over the abdomen was shaved. Laprotomy was done under general anaesthesia in a half open system as follows: The previously shaved skin was desinfected with a tincture of iodine (6.5g iodine and 3.5g NaI in 100ml 96% Alcohol). A mid-line incision about 5cm was made along the linea abla & the abdominal cavity was opened by cutting through the peritonium. The small intestine was brought out of the abdominal cavity on a sterile drape and was tied off 100cm cranial to the appendix. The bowel was tied off again at the iliocecal ligment. In the ileum 2-3 loops measuring 10±2cm were made & injected with one ml containing ($10^9$ cfu) from each test isolate. Like wise 2-3 loops were made in the jujunum and also injected. Interspacial segments of 2 cm in length were tied between
each tested loop. The interspacial loop served as a barrier to prevent possible leakage between the loops. Blood vessels were carefully avoided during ligation to ensure intact blood supply of the loops. The abdomen was closed using nylon sutures. The animal was allowed to recover from anaesthesia. Rabbit was killed 16 hrs later by I/V injection of an overdose of sodium pentobarbital. The abdomen was opened and small intestine was carefully removed and unravelled. The presence or absence of dilation was recorded as well as the length of dilated segment and fluid in each dilated segment was measured in milliliter. According to Sack (1975) the amount of fluid in ml/ length of the loop in cm > 0.4 recorded as positive.

2.12 Immuno assay of labile toxin:

2.12.1 Elek’s test: -

All E.coli isolates, which were positive in Vero cell assay and adult rabbit ligated gut test, were tested in Elek test.

2.12.1.1 Preparation of crude toxiod:

One hundred-ml amount of Evans’ broth was inoculated with two to three colonies of E.coli (BLKE19). The culture was incubated at 37°C for 48 hrs. Formalin was added to the culture to make the final concentration 0.5%. Then the bottle was allowed to stand for 50 hours at 4°C during this period the bacterial cells settled to the bottom leaving a clear supernatant. The supernatant was carefully decanted into sterile
centrifuged tubes and centrifuged at 6000 RPM for 15 minutes. The supernatant was put in a dialysis tube after it was boiled in water for 2 minutes. For concentration of toxoid, poly ethylene glycol 6000 Mr was added to cover the dialysis tube to facilitate removal of water and increase toxin concentration. The concentrated crude toxoid was kept on sterile MacCarteny bottle and stored at 4°C.

2.12.1.2 Determination of protein content of the crude toxoid:

Protein content was estimated according to method described by Lowery (1951). This method depends on the detection of tyrosine content of the protein, which is fairly constant in many proteins.

All reagents were prepared in distilled water.

1. 0.1 N-NaOH containing 2% anhydrous Na₂CO₃.

2. 2% sodium/potassium tartarate solution.

3. 1% CuSO₄ solution.

4. Folin ciocalteu phenol reagent (BDH) diluted 2:5 with distilled water.

Lowary solution was prepared by adding 50 ml of 0.1 N-NaOH containing 2% anhydrous Na₂CO₃ to 0.5ml of 2% sodium/potassium tartarate solution then 0.5 ml of 1% CuSO₄ was added.

Method:
Bovine serum albumin (BSA) was used as standard protein and was prepared by dissolving 50 mg of BSA in 50ml volumetric flask to serve as stock solution of one mg protein/mL and the following dilutions were made in test tubes 200µg/ml, 400µg/ml, 600µg/ml, 800µg/ml. Like wise, in separate test tubes 1/5, 1/10 and 1/20 dilution of the toxoid were made. From each dilution of the standard protein and the toxoid 0.1ml was pipetted into fresh test tubes and the volume was brought to 0.4 by addition of 0.3ml distilled water. All tests were made in duplicate. The final concentrations of the standard were 20, 40, 60, 80 and 100 micrograms protein. For blank 0.4ml of distilled water was added into two tubes. To each standard and dilution of toxoid 2ml of Lowry solution was added. Then the tubes were mixed well and incubated at room temperature for 10 minutes in order to allow protein to hydrolyse. Then to each tube 0.2ml of folin ciocalteu phenol reagent diluted 2:5 with distilled water was added. Test tubes were mixed and immediately kept in the dark for 30 minutes. Absorbance of each standard and sample (toxin) was read in spectrophotometer using one-cm path at 500nm.

A standard curve was blotted using protein value against Absorbance of the standard protein (Fig 2). The protein value of the toxoid was estimated from the standard curve. The Absorbance value of toxoid at dilution 1/5, 1/10 and 1/20 were found to be 0.95, 0.47, 0.23 respectively.
The Absorbance of 1/10 dilution was used to estimate its protein content from standard curve and was calculated as follows.

Corresponding protein value for Absorbance of toxoid (1/10 dilution) from standard curve.

\[
90 \times 10 \times \text{dilution factor (10)} = 9 \text{ mg/ml} \\
1000
\]

The protein content of the toxoid was found to be 9 mg/ml

2.12.1.3 Production of hyperimmune serum:

*E.coli* toxoid was used for production of antitoxin immune serum in two rabbits. The protein content of the toxoid was adjusted to 2.5 mg/ml and equal volumes of toxoid and Freund’s complete adjuvant were mixed. One ml of this Ag emulsion was injected subcutaneously at multiple sites at the thigh region after shaving. Two booster doses where given to the rabbits at the day 10 and 20. Proof bleeding test was done to determine the level of antibodies titre by Ouchterlony. Blood was collected from the ear vein one week later and sera were separated and kept at -20°C.

2.12.1.4 Elek’s Test procedure:
The test procedure was the same as described by Honda *et al* (1981). The strain BLUE19 was inoculated on to Evans’ agar medium to produce separate colonies. The plates were incubated for about 40 hours at 37°C. Paper discs of Lincomycin were placed on separate colonies. The plates were further incubated for several hours (4-6 h). After incubation 4mm well was made equidistant from two separate colonies (1 cm apart) and filled with the antiserum against *E.coli* (BLKE19) toxoid. The plates were incubated at 37°C over night then examined for precipitation lines. The strength of precipitation lines were scored as strong (+++), weak (+ +) and very weak (+) or negative (-).

(Fig 2) **The protein estimation curve using lowery method**

![Optical density graph](image-url)
**2.13 Invivo bioassay of stable toxin:**

Five isolates from MRHA *E.coli*, which were negative in both Vero cells and adult rabbit ligated gut test was examined using suckling mice test. Heat inactivated (90°C for 15 minutes) toxins from each test isolate were also included. The test was performed according to (Dean *et al.*, 1986). Infant mice at least 2 days old and not older than 4-5 day which
had a well-filled and hence clearly visible stomach were selected from several litters. During the test, the mice should be kept at room temperature. The mice were injected intragastrically with 0.1ml Evans’ blue-stained ST preparation using special canula, animals with no dye in the intestine or with dye within the peritoneal cavity at autopsy were discarded because it was not injected probably. Each strain was tested in three mice. The mice were kept at room temperature and killed after 3-4 hours by decapitation. The entire intestine (not including the stomach) was removed and inspected. The intestine of 3 mice per test were pooled and weighted and the ratio of gut weight to the combined weight of the remaining carcasses was calculated. A ratio of ≤ 0.08 indicated a negative result and a ratio of ≥ 0.09 indicated a positive result.

CHAPTER THREE

Results

3.1 Survey:-

One hundred and forty sex feecal samples were collected from different conventional farms in eastern and western
Khartoum state. *E. coli* positive strains were found to be 90 (61%).

Seventy two stool samples were collected from different pediatric hospitals in the Khartoum state. *E. coli* positive strains were found to be 15 strains (20%).

Distribution of isolates according to the source has been illustrated in tables (5) and (6)

3.2 **Microscopic and cultural characteristics of isolates:**

Microscopic examination of isolates revealed more or less short and thick gram-negative rods. Rods arranged singly and in pairs. Short chains were not found. Smears from some refrigerated cultures showed shorter rods.

3.2.1 **Growth on solid media:**

3.2.1.1 **MacConkey’s agar:**

On MacConkey’s medium colonial morpholoical study revealed large, profused, smooth pink colonies (2-3 mm in diameter) figure (3).
3.2.1.2 Nutrient agar:

On nutrient agar colonies were small round and more mucoid than on MacConkey’s agar figure (4).

3.2.1.3 Evans’ media:

On Evans’ media colonies vary in size, less mucoid, smooth and can easily be removed from the surface of the media.

3.3 Biochemical characterization of the isolates:

3.3.1 Oxidase test:

All the isolates were oxidase test negative.

3.3.2 Catalase test:

All isolates catalize hydrogen peroxide producing gas thatapper in the form of bubles and water.

3.3.3 Motility test:

Motility was detected by diffuse growth away from the line of inoculation and non-motile strains grew only along the line of inoculation.
In this study 72 out of 90 strains from animal source were found to be motile (80%). Whereas from the 15 human isolates, 14 strains (93%) were found to be motile.

3.3.4 sugar fermentation test:

On sorbitol all strains of *E.coli* ferment sorbitol with production of acid and gas. Acid was indicated by change in the colour of the sorbitol from white to pink. *E.coli* ferment glucose with production of acid and gas. Gas production was indicated by appearance of air bubles at the top of Durham’s tube.

3.3.5 Oxidation fermentation test:

All isolates of *E.coli* were oxidation and fermentation positive.

3.3.6 Indole test:

All strains of *E.coli* are able to produce indole when inoculated into medium containing tryptophan.

3.3.7 Voges-Proskauer (V.P) test:
All strains of *E. coli* were unable to produce acetyl methyl carbinol in phosphate glucose media and hence the colour of the media was not changed to red.

### 3.3.8 Methyl red test:

All strains of *E. coli* were methyl red positive.

### 3.3.9 Citrate utilization test:

All *E. coli* strains were unable to utilize citrate in Simmon citrate medium.

### Table (5) Animal *E. coli* isolates

<table>
<thead>
<tr>
<th>Animal source</th>
<th>No. of samples</th>
<th><em>E. coli</em></th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Western state</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jebel Toria (JET)</td>
<td>35</td>
<td>24</td>
<td>68</td>
</tr>
<tr>
<td>Al merkheit (Mkh)</td>
<td>23</td>
<td>13</td>
<td>56</td>
</tr>
<tr>
<td>Al hitana (HIT)</td>
<td>57</td>
<td>33</td>
<td>57</td>
</tr>
<tr>
<td><strong>Eastern state</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human source</td>
<td>No. of samples</td>
<td>No. of isolated <em>E. coli</em></td>
<td>Percentage</td>
</tr>
<tr>
<td>--------------------------------------------------</td>
<td>----------------</td>
<td>--------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>Omdurman hospital, pediatric ward (OMD)</td>
<td>27</td>
<td>5</td>
<td>18</td>
</tr>
<tr>
<td>Bulk hospital, pediatric ward (BLK)</td>
<td>31</td>
<td>4</td>
<td>13</td>
</tr>
<tr>
<td>Khartoum hospital, pediatric wards (KHT)</td>
<td>06</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td>Khartoum North (KTN)</td>
<td>02</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ahmed Ghasim pediatric wards (AhG)</td>
<td>06</td>
<td>3</td>
<td>50</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>72</strong></td>
<td><strong>15</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

Table (6) Human *E. coli* isolates
Table (7) Biochemical reactions for *E.coli* isolates

<table>
<thead>
<tr>
<th>Test</th>
<th>No. of the positive isolates</th>
<th>Percentage</th>
<th>No. of the negative isolates</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase</td>
<td>0</td>
<td>0</td>
<td>105</td>
<td>100</td>
</tr>
<tr>
<td>Catalase</td>
<td>105</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Motility</td>
<td>86</td>
<td>81</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Gas from glucose</td>
<td>105</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>105</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Indole</td>
<td>105</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MR</td>
<td>0</td>
<td>0</td>
<td>105</td>
<td>100</td>
</tr>
<tr>
<td>VP</td>
<td>105</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Citrate</td>
<td>105</td>
<td>105</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>O/F</td>
<td>105</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
3.3.10 Eijkman test:

All *E.coli* strains able to produc acid and gas as a result of fermentation of lactose at 44°C when inoculated into MacConkey’s broth. Acid was indicated by change in colour of medium from pink to yellow.

3.4 Haemagglutination and Haemagglutination with D-mannose (100µg/ml):

*E.coli* strains, which agglutinated bovine erythrocyte, were found to be 70 (77%). Haemagglutination mannose resistance strains were 19 (21.1%) from the total *E.coli* and 13% from the total number of samples collected (table 8).

*E.coli* strains that isolated from human and agglutinated chicken erythrocyte were found to be 12 (80%). *E.coli* that were mannose resistant haemagglutination were 3 (20%) from total
*E. coli* isolated and 4.2% from the total number of the samples collected (table 9).

### 3.5 Titres of Haemagglutination mannose resistant *E. coli*:

The titres obtained from MRHA strains of calf origin ranged from 64-16. Whereas titre from human origin ranged from 32-16 as shown in table (10) and Fig. (5).

### 3.6 Haemagglutination at different temperatures:

To study the effect of the temperature at which the culture media was incubated on hemagglutination ability of different strains of *E. coli*, MRHA strains were cultured on Evans’ media and incubated at 3 different temperatures (37°C, 25°C, and 20°C) (table 11).

### 3.7 Haemagglutination inhibition of *E. coli* strains by antiserum-

The Haemagglutination activities of *E. coli* strains JTE 8, MkhE 14a, HITE16 and HKU91 were inhibited by the antiserum against the adhesion factor. The erythrocytes appeared as a bottom in the all eight wells of the microtitre plate. This indicated clearly that the
titre of the antiserum was (1/246) and at high dilution (1/492) of
the antiserum the HA activity of the isolates were restored. The
antiserum is specific for calves and failed to inhibit human
strains.

3.8 Vero cell test: -

When all haemagglutination mannose resistant strains (22)
were tested in Vero cell five isolates (22.7%) were found
positive four were from calve origin and one (BLKE19) from
human origin (table 12 and fig. 6).

3.9 Adult Rabbit ligated gut test (ARLG):

The above five *E.coli* isolates were tested using adult
rabbit ileal loop method. Grossly distended ligated loops were
observed. The accumulated fluid in each loop was measured as
well as the length of the loop (table 12 and fig 7).
Table (10) Titres of various MRHA *E.coli* isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>HAT</th>
<th>Strain</th>
<th>HAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Animal isolates</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JETE 39</td>
<td>32</td>
<td>HKUE 61</td>
<td>32</td>
</tr>
<tr>
<td>JETE 7</td>
<td>32</td>
<td>HKUE 91</td>
<td>64</td>
</tr>
<tr>
<td>JETE 34a</td>
<td>32</td>
<td>HKUE 10</td>
<td>32</td>
</tr>
<tr>
<td>JETE 34b</td>
<td>32</td>
<td>HKUE 23</td>
<td>32</td>
</tr>
<tr>
<td>JETE 9</td>
<td>16</td>
<td>HKUE 1</td>
<td>16</td>
</tr>
<tr>
<td>JETE 35</td>
<td>32</td>
<td>HITE 11</td>
<td>32</td>
</tr>
<tr>
<td>JETE 8</td>
<td>16</td>
<td>HITE 16</td>
<td>32</td>
</tr>
<tr>
<td>MkhE 2</td>
<td>64</td>
<td>Human isolate</td>
<td>16</td>
</tr>
<tr>
<td>MkhE 14a</td>
<td>64</td>
<td>OMDE 15</td>
<td>32</td>
</tr>
<tr>
<td>MkhE 14b</td>
<td>16</td>
<td>BLKE 19</td>
<td>32</td>
</tr>
<tr>
<td>MkhE 22</td>
<td></td>
<td>AhGE 49</td>
<td></td>
</tr>
</tbody>
</table>

JETE: Jebel Torria *E.coli* strain.

MkhE: Al merkheit *E.coli* strain.
HIT : Al Hitanna *E.coli* strain.
HKU : Hilet kuku *E.coli* strain.
OMDE : Omdurman *E.coli* strain.
BLK : Bulok *E.coli* strain.
AhG : Ahmed Gasuim *E.coli* strain.
HAT: Haemagglutination titre.

**Table (11) Haemagglutination titre of the various *E.coli* MRHA strains grown at different temperature**

<table>
<thead>
<tr>
<th>Strains</th>
<th>Incubated temperature</th>
<th></th>
<th>Strains</th>
<th>Incubated temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>37°C HAT</td>
<td>25°C HAT</td>
<td>20°C HAT</td>
<td></td>
</tr>
<tr>
<td><strong>Animal isolate</strong></td>
<td></td>
<td></td>
<td></td>
<td><strong>Human isolates</strong></td>
</tr>
<tr>
<td>JETE 39</td>
<td>32</td>
<td>4</td>
<td></td>
<td>HKUE 61</td>
</tr>
<tr>
<td>JETE 7</td>
<td>32</td>
<td>2</td>
<td></td>
<td>HKUE 91</td>
</tr>
<tr>
<td>JETE 34a</td>
<td>32</td>
<td>2</td>
<td></td>
<td>HKUE 10</td>
</tr>
<tr>
<td>JETE 34b</td>
<td>32</td>
<td>2</td>
<td></td>
<td>HKUE 23</td>
</tr>
<tr>
<td>JETE 9</td>
<td>32</td>
<td>4</td>
<td></td>
<td>HKUE 1</td>
</tr>
<tr>
<td>JETE 35</td>
<td>6</td>
<td>2</td>
<td></td>
<td>HIT 11</td>
</tr>
<tr>
<td>JETE 8</td>
<td>32</td>
<td>4</td>
<td></td>
<td>HiTE 16</td>
</tr>
<tr>
<td>MkhE 2</td>
<td>16</td>
<td>-</td>
<td></td>
<td>OMDE 15</td>
</tr>
<tr>
<td>MkhE 14a</td>
<td>64</td>
<td>8</td>
<td></td>
<td>BLKE 19</td>
</tr>
<tr>
<td>MkhE 14b</td>
<td>64</td>
<td>8</td>
<td></td>
<td>AhGE 49</td>
</tr>
<tr>
<td>MkhE 22</td>
<td>16</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All strains show no agglutination at this temperature.
JETE : Jebel Torria *E.coli* strain.

MkhE : Al merkheit *E.coli* strain.

HIT : Al Hitanna *E.coli* strain.

HKU : Hilet kuku *E.coli* strain.

OMDE : Omdurman *E.coli* strain.

BLK : Bulk *E.coli* strain.

AhG : Ahmed Gasuim *E.coli* strain.

HAT: Haemagglutination titre.

The intial number refer to the number given to each isolate

**3.10 Adhesion to isolated rabbit brush border cells: -**

Haemagglutinaion mannose resistant (MRHA) strains of *E.coli* were able to adhere to isolated Rabbit brush broder cells (fig.8). Adhesion of *E.coli* to brush border was difficult to quantitate. On the other hand mannose sensitive strains were not able to adhere to the brush border.

**3.11 Inhibition of adhesion to brush border cells: -**

The ability of the various MRHA strains to adhere to brush border cells was inhibited by antiadhesion serum prepared from MkhE14a in rabbits. The bacteria incubated first with antiserum failed to adhere to the brush border cells. The cells appear free of bacteria and most of the bacteria appear as clumps in between
the cells (fig.9) This antiserum is specific for bovine strain as it failed to inhibit adhesion of strains of human origin.

3.12 Elek test: -

Separate colonies of ETEC (BLE19) that produce LT toxin gave precipitation lines with antiserum placed in a well one cm away from the colony. The covering of the colonies with lincomycin containing discs has facilitated the development of the precipitation line. Continuous precipitation line was seen when the antiserum was placed one cm equidistant to two colonies indicating a reaction of identity. Results were scored as shown in table (12).

3.13 Suckling mice test: Results were illustrated in table (14).

Table (12) the Vero cell positive strains, their haemagglutination titre and Elek test reaction.

<table>
<thead>
<tr>
<th>Source</th>
<th>Titre</th>
<th>Vero cells</th>
<th>Elek Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>JETE 8</td>
<td>32</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MkhE</td>
<td>64</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Table (13) Rabbit ligated ileal loop test of Vero cells positive

_E.coli_ isolates, their source and their HA titre.

<table>
<thead>
<tr>
<th><em>E.coli</em> isolate</th>
<th>HA titre</th>
<th>Fluid accumulation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>JTE 8</td>
<td>32</td>
<td>0.5</td>
</tr>
<tr>
<td>MkhE 14a</td>
<td>64</td>
<td>0.8</td>
</tr>
<tr>
<td>HITE 16</td>
<td>32</td>
<td>0.7</td>
</tr>
<tr>
<td>HKU 91</td>
<td>64</td>
<td>0.8</td>
</tr>
<tr>
<td>BLKE 19</td>
<td>32</td>
<td>1.2</td>
</tr>
</tbody>
</table>

According to Sack (1975) fluid accumulation (FAR) ratio < 0.4 was considered negative.

* Precipitation lines scored as strong ++++, weak ++ or v- weak + or negative -
Table (14) Effect of the various MRHA *E. coli* strains on the infant mouse: -

<table>
<thead>
<tr>
<th><em>E. coli</em> isolates No.</th>
<th>Gut weight</th>
<th>Carcass weight</th>
<th>Gut weight/carcass wt.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKhE 14b</td>
<td>0.7225</td>
<td>6.7358</td>
<td>0.1</td>
</tr>
<tr>
<td>OMDE 15</td>
<td>0.6872</td>
<td>5.4325</td>
<td>0.12</td>
</tr>
<tr>
<td>HKUE 19</td>
<td>0.6961</td>
<td>6.0120</td>
<td>0.11</td>
</tr>
<tr>
<td>JETE 9</td>
<td>0.4987</td>
<td>4.9860</td>
<td>0.1</td>
</tr>
<tr>
<td>JETE 7</td>
<td>0.4963</td>
<td>1.6543</td>
<td>0.3</td>
</tr>
</tbody>
</table>

* All were negative in Vero-cells.

According to Dean *et al* (1976) when ratio of gut weight/carcass weight ≤ 0.08 it indicated a negative result,

when ratio ≥ 0.09 it indicated positive result.
Fig.3: Cultural characteristic of *E. coli* on MacConkey’s gar (No.3)  
Showing pink colonies.

Fig.4: Cultural characteristic of *E. coli* on nutrient agar.  
Showing large mucoid colonies.
Fig.5: Microtitre plate illustrating Haemagglutination of various *E.coli* strains

Fig.7: The effect of LT producing isolates on the intestine of adult rabbit. Note accumulation of fluid in each loop.
Fig. 6a: Vero cell before inoculation showing monolayer (100x)

Fig. 6b: Vero cell after inoculation with ALBE19. Note the rounding of the cells
Fig. 8: Adhesion of MKhE14a to isolated rabbit brush border cells. Gram stain (100x)

Fig. 9: Inhibition of adhesion of MkhE 14a to isolated rabbit brush border cells using MkhE 14a antiserum. Gram stain (100x)
Sudan owns 39 million heads of cattle raised under traditional pastoral conditions (Annual Bulletin of Animal Resources Statistics 2002). At present calves scour constitutes a major problem facing the veterinary Animal health authority leading to substantial financial losses due to annual deathes.

In man and according to world health organization diarrheal diseases are amongst the ten leading causes of death for children and resposible for 1-3 million deaths per year (Rohde and Northrup, 1976).

Calves scour and diarrhoal diseases in children caused by various agents including E.coli are becoming a health problem worldwide including the Sudan (Lambert, 1979 and Puent and Finlay, 2001). However, there was neither enough information regarding the significance of ETEC in naturally occuring cases of diarrhoeal diseases in both man and animals, nor regading the characteristic of the various isolates of ETEC in this country.

Routine culture and identification of E.coli from relevant
samples are all that are required for laboratory diagnosis of septicemia and infection of the urogenital tract due to *E. coli* (Johson, 1991). However, for enteric diseases it is necessary to use methods that distinguish the pathogenic from non-pathogenic *E. coli* strains. In our study the identification of mannose sensitive and mannose resistant haemagglutinating *E. coli* were used to differentiate the isolates carrying the adhesion factors (fimbriae) designated as K99 (F5) for calves *E. coli* isolates and CFA1 and CFAII for human *E. coli* isolates from the isolates carrying the common pili using bovine and chicken erythrocytes respectively (Bhattachajee *et al.*, 1978 Guinee *et al.*, 1976 Burrow *et al.*, 1976 and sedlock *et al.*, 1981). Therefore, the success of our present study relied heavily on a simple screening test to investigate the ability of our *E. coli* isolates to agglutinate erythrocytes in the presence of D-mannose. Haemagglutinating mannose resistant *E. coli* were considered possessing an important attributes of virulence, that is the adhesion factor as has been reported previously. Thus the result of this study confirm that the pattern of haemagglutination
caused by the presence of K99 (F5) and CFAI and CFAII are not produced by *E. coli* from normal flora and MRHA was specific indicator for the presence of colonization factors in these isolates. This is in agreement with finding of Blanco *et al.*, (1993). Therefore, our haemagglutination results provide a simple means for the identification of adhesive *E. coli*.

From 90 *E. coli* isolates from calves origin MR *E. coli* respresent 21.1%, MS *E. coli* were 56.7% and non-haemagglutinating strain were 22.2% our results are in agreement with the finding of Freter (1980) and Blanco (1993) who distinguished three groups of *E. coli* according to their pattern of Haemagglutination activity. Further more in this study 4°C was found the optimal termpareture for running Haemagglutination test. This is in agreement with previous finding of Duguid and Old (1980), and Sedlock *et al.* (1981). Moreover, the tempareture 37°C was found optimal for growth and the expression of haemagglutinions in the various isolates tested. The titre of haemagglutination was low in cultures grown at 25°C and completely lost in culture grown at 20°C (table11).
This might indicate that the genotype of adhesion is present but the phenotype is temperature dependant. These results are in agreement with previous finding of Duguid and Old (1980) and Ørskov et al. (1975). Furthermore, the attachment of MRHA E. coli to intestinal brush border provides a convenient system to study the adhesion of the various isolates in vitro. The test utilized the natural receptor on the enterocytes and is not sensitive to temperature, in contrast to haemagglutinin system.

The method of preparing brush borders was simple and gave good yields. Our preparation was contaminated with extraneous subcellular components but this caused no difficulty, because the appearance of brush border cells under the microscope was highly characteristic. The result described in this study, indicated that adhesion observed was mediated by adhesion factors. Antibodies raised against our E. coli isolates, MkhE14a, which was characterized as having HA titre of 64, successively inhibited adhesion of the various isolates to brush border cells. These findings are in agreement with the previous finding of a number of investigators, Jones and Rutter (1974), Jones and
Freter (1976) and Sellwood and Lees (1979). Hence the demonstration of these adhesion factors for calves *E. coli* isolates suspected of having enterotoxic colibacillosis tend to confirm our diagnosis. Other investigator had demonstrated the presence of adhesion factor using specific antibodies against bovine K99 (F5) and human CFAI and CFAII (Erwa, (1975), Guinee *et al.*, (1976) Merson *et al.*, (1980b) and Enour, (1993)). Nonetheless, during the last three decades our understanding of the pathogenesis of diarrhoeal diseases due to *E. coli* has advanced tremendously, yet the advances in human *E. coli* diarrhoea have been anticipated by studies in domesticated animals (Gyles 1993; Nataro and Kaper, 1998).

The adhesion to epithelial surface in the gut may be regarded as the first step with the process of bacterial association with mucosa (Candy 1980). Therefore, the instance where entrotoxigenic *E. coli* are suspected to cause or contribute to diarrhoeal diseases in calves or human infants, enterotoxigenicity (the second attribute of virulence) should be demonstrated. For the various MR *E. coli* isolates, laboratory
tests for toxigenicity was investigated on various bioassay systems: the Vero cell, rabbit-ligated gut test, infant mouse test, in addition to immunoassay the elek test.

In this study LT producing strains of *E.coli* were found to be five, 4 strains from calf origin and one from human origin (table 12) most of the other bovine isolates were ST producing strains. These findings were in agreement with the previous results reported by Isaacson *et al.*, (1981) and Pohl *et al.*, (1991). Most of our isolates which were negative in LGT were found reactive in the suckling mouse model. These finding were in agreement of the result of Ali (2000).

Following the different bioassay ETEC isolated from diarrhoeagenic calves represent about 13% of the total sample studied and 21.1% of the total number of *E.coli* isolated. This finding is in agreement with the incidence reported by Debnath *et al.* (1987) and Mellata *et al.* (1998). This low frequency of isolating ETEC from clinical cases of calf diarrhoea is probably due to involvement of other aetiological agents in the causation of calf scour (Lambert, 1979, Guinee *et al.*, 1979 and Debanth,
Nonethelss the prevalence of calf-scour due to ETEC in the four farms studied varies from 4.79% to 1.37% (table 8). Higher incidence was seen in Jebel Toria farm this variation might be due to difference in herds husbandry system and the hygenic measurment observation in each farm. On the other hand the low incidence of the isolation of ETEC of human cases (4.2% from the total number of samples and 20% from the total number of *E.coli*) was probably due to commencement of antibiotic treatment as well as to the involvement of other micro-organism including virus (rotavirus, calicivirus, adenovirms and coronavirus) other entero pathogen or even other *E.coli* type (Giannella, 1981). Entero pathogenic *E.coli* for instance is considered the primary cause of diarrhoea in human infant and preschool children (Puente and Finlay 2001). Erwa (1971) studied bacterial enteritis in Sudanese children and he isolated *E.coli* (27%), salmonella (2%), shigella (8.2%) and other enteropathogens. Despite of our limited number of samples both studies indicated other micro organisms were responsible for the causation of diarrhoea. Ahmad (1989)
studied the aetiology of diarrheal diseases in Juba in preschool children he found shigella was common in children above 6 months of age and *E. coli* was prevalent among children below the one year of age. Comprehensive laboratory studies of diarrheal cases in Bangladesh have revealed that Rota viruses and ETEC account for 50-60% of all diarrheal cases and 75-80% of diarrhoea cases in children below 5 years of age and both agents are very common in children below age of 2 years (Merson *et al.*, 1980b).

Elek test was revaluated in this study with a view to elaborate a simple and reproducible assay method which can be used in clinical diagnostic test for enterotoxigenic *E. coli*, highly labile toxin producing strains were detected in this assay by producing a relatively week precipitation lines. The inclusion of lincomycin has resulted in the release of the LT into the culture medium as has been reported by Honda *et al.* (1981). No precipitation lines were noticed in culture plates without lincomycin, nontheless a reaction of identity was noticed between anti-LT serum and colonies of two LT producing *E. coli* strains.
This reaction confirmed the specificity of our antiserum. The major advantage of this test is its ability to detect biologically and non-biologically active entrotoxin compared to the other bioassay system but it has low reproducibility.
Conclusion:

The identification of mannose-resistant and mannose-sensitive (MRHA and MSHA) haemagglutinating *E. coli* is a reliable method to differentiate between the adhesive fimbriae and common pili. The pattern of haemagglutination produced by MRHA *E. coli* strains isolated from diarrhoeagenic calves or children are not produced by *E. coli* from their normal flora. Therefore MRHA was very specific and reliable indicator for the presence of the colonization factor which is an important attribute to virulence associated with ETEC. Because of this, *E. coli* producing MRHA should be tested for enterotoxigenicity. Hence screening for MRHA is considered the first step towards the identification and characterization of ETEC. The test was very simple and easy to perform. However techniques used for the demonstration of the production of LT or ST and their toxigenicity are laborious, cumbersome, time-consuming and require steady supply of laboratory animals. This means that it would require maintenance of a fully fledged animal house with
facilities for bleeding and surgical intervention. On the other hand assay for toxigenic strains also require specialized set up of tissue culture (TC media, fetal calf serum, CO₂ incubator and laminar air flow cabinet) and skilled technical manpower. The whole procedure is costly and probably beyond the scope of most routine laboratories in developing countries.

In *vitro* immuno diagnostic test, Elek test was employed with a view to simplify the diagnosis of ETEC in the culture plates. The test gave variable results but no improvement for the reproducibility of the results was obtained.

From the results obtained in this study using the above techniques the incidence of ETEC in dairy farms varies from 22.5% (Jebel Toria farm) to 8.7% (Hitaana farm) and for human was about 4.2%.

**Recommendation:**

1. As only 13% and 4.2% of the aetiology of diarrhoeal diseases in calves and children was respectively attributed to ETEC in this study, a team work should investigate the other possible
causes of diarrhoeal diseases viz viruses, other enteropathogen, cryptosporidum etc.

2. To study the extend and nature of naturally acquired immunity to ETEC to elucidate the relationship between serum level of immuno globulins and mortality from diarrhoea in calves and children.

3. To introduce a simplified method for the identification of virulence factors or Ags for example:

a. Direct identification of LT and ST in stool using ETISA. ST might be coupled to a protein carrier to produce an antiserum against it preferentially using monoclonal antibody technology.

b. Direct identification of LT and ST genes using oligonucleotide probes.

c. Direct identification of adhesion factor using monoclonal antibodies.

d. Identification of ETEC serotypes for epidemiological purposes.
4. Newborn calves should receive colostrum within the first 40h of birth. Veterinarian should instruct their clients on the importance of good management practice of the herd to reduce calf-scour and to institute oral and/or parenteral rehydration fluid to compensate fluid and electrolyte loss and to correct acidosis whenever needed.

5. The national health care delivery programme should work in concert with WHO and Diarrhoeal Disease Programme, for the control and reduction of mortality and the sequelae of acute diarrhoea as well as morbidity and to promote research to develop better strategies to achieve those goals. Long-term objective must focus on improving water supplies, sanitation health care facilities and promotion of breast-feeding.
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