# A STUDY OF BACTERIAL AGENTS ASSOCIATED WITH DIARRHOEAL CASES IN THE FEDERAL CAPITAL TERRITORY, ABUJA.

**BY**

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**(B.Sc., AIMLS, M.Sc)**

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# CERTIFICATION

This is to certify that the Research work for this thesis and the subsequent preparation of this thesis by OSUOCHA, CALLISTA CHINYERE (UJ/PGMS/97/10498) was carried out under my supervision.

Professor E.I. Ikeh (PhD, FIMLS) SIGNATURE.............................

(Supervisor) DATE......................................

Dr. (Mrs) L. H. Nimzing SIGNATURE..............................

(Acting H.O.D) DATE.......................................

# DECLARATION

I, hereby, declare that this work is the product of my own research efforts; undertaken under the supervision of Professor E. I. Ikeh and has not been presented elsewhere for the award of a degree or certificate. All sources have been duly distinguished and appropriately acknowledged.

CALLISTA CHINYERE ASAMOLE-OSUOCHA UJ/PGMS/97/10498

# DEDICATION

This work is dedicated to

#### MY FATHER

and

#### MY HUSBAND

My father nurtured my academic ambitions and my husband sustained it. Thanks both of you and GOD BLESS.

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#### LIST OF ABBREVIATIONS

APW - Alkaline Peptone Water

APFLP - Amplified Fragment Length Polymorphism

BPW-VCC - Buffered Peptone Water supplemented with vancomycin, cefsulodine and cefixime

CDC - Centres for Disease Control

CT-SMAC - Cefixime-Tellurite Sorbitol MacConkey agar DCA - Deoxycholate citrate agar

EC-IMS - Enrichment culture followed by immunomagnetic separation EaggEC - Enteroaggregative E. coli

EHEC - Enterohaemorrhagic E. coli

EIEC - Enteroinvasive E. coli EPEC - Enteropathogenic E. coli ETEC - Enterotoxigenic E. coli FCT - Federal Capital Territory HC - Haemorrhagic colitis

HUS - Haemolytic uraemic syndrome IMS - Immunomagnetic separation LEE - Locus of enterocyte effacement mTSB - Modified Tryptone soybroth

MLEE - Multilocus Enzyme Electrophoresis

NLF - Non – Lactose Fermenters NSF - Non – Sorbitol Fermenters PCR - Polymerase Chain Reaction

PFGE - Pulsed Field Gel Electrophoresis QC - Quality Control

RAPD - Random Amplified Gel Electrophoresis SF - Selenite F broth

SMAC - Sorbitol ManConkey agar

STEC - Shiga – Toxin producing E. coli

TCBS - Thiosulphate Citrate Bile salts Sucrose agar TTP - Thrombotic Thrombocytopenic Purpura VTEC - Verocytotoxin-producing E. coli

#### ABSTRACT

One hundred and six faecal samples were analyzed to detect the sporadic involvement of E. coli O157:H7, other Enterohaemorrhagic E. coli and common bacterial pathogens in enteric infections within the Federal Capital Territory, Abuja. Enrichment culture in modified peptone water followed by immunomagnetic separation (IMS) with magnetic beads coated with an antibody against Escherichia coli O157 was used in addition to Direct culture on Sorbitol MacConkey agar and Cefixime-Tellurite Sorbitol MacConkey agar for the isolation of E. coli O157: H7. For the identification of non-O157 STEC among the Non-sorbitol fermenting E. coli isolated, PCR for virulence markers was used. Verocytotoxicity Assay was used to detect free faecal toxin in the stool samples for the identification of other Enterohaemorrhagic E. coli (EHEC) infections. Routine methods were used for the isolation of common bacterial pathogens and for identification of isolates. Antimicrobial susceptibility tests were carried out on the isolates using the disc diffusion technique while interpretation of zone sizes was done using the NCCLS interpretative chart. Stool Microscopy for ova or cyst of parasites was also done using the formol ether sedimentation technique. No E. coli O157:H7 was isolated. However, free faecal toxin was demonstrated in 16 (15.09%) of the 106 faecal samples analyzed indicating the involvement of non-O157 VTEC in diarrhoeal diseases in the Federal Capital Territory, Abuja. None of the non-sorbitol fermenting E. coli was found to be STEC. Enteric pathogens isolated from the study include V. cholerae (1.9% of the faecal sample), S. typhi (1.9%), S. paratyphi B (0.95%), S. paratyphi C (0.95%), unidentified Shigella species (0.95%), Shigella dysenteriae A1 (1.9%), Shigella flexneri (4.7%) Entamoeba histolytica (0.95%), Hookworm (0.95%), Schistosoma mansoni (0.95%) and Candida spp (6.6%). Sorbitol MacConkey agar was found to be more sensitive than the Deoxycholate Citrate agar and Selenite F enrichment broth routinely used for the isolation of Shigella flexneri. Thus, for optimal isolation of Shigella flexneri, the inclusion of MacConkey agar among the media used for routine cultures is recommended. V. cholerae isolates were 100% sensitive to tetracycline and ciprofloxacin, Shigella species were 100% resistant to tetracycline and 100% sensitive to ciprofloxacin. The Salmonella isolates were 100% sensitive to ciprofloxacin while showing varied degrees of resistance to tetracycline, chloramphenicol and trimethoprim-sulphamethoxazole. These results show the need to promote rational use of antibiotics in FCT. The results, also, suggest that the incidence of non-O157 VTEC involvement in diarrhoeal diseases may be quite common and should be considered as an agent in diarrhoeal illnesses especially among children.

# CHAPTER ONE INTRODUCTION

#### BACKGROUND OF THE STUDY

* + 1. **Diarrhoeal Disease and its Prevalence**

Diarrhoeal diseases of the bowel make up a veritable augean stable of entities. Microbiologic agents cause many; others arise in the setting of malabsorptic disorders and idiopathic inflammatory bowel disease (Crawford, 1999).

A precise definition of diarrhoea is elusive, given the considerable variation in normal bowel habits. An increase in stool mass, stool frequency or stool fluidity is perceived as diarrhoea by most patients (Crawford, 1999). For many individuals, this consists of daily stool production in excess of 250 gm, containing 70 – 95% water. More than 14 litres of fluid may be lost per day in severe cases of diarrhoea (i.e the equivalent of the circulating blood volumes). Diarrhoea is often accompanied by pain, urgency, perianal discomfort, and incontinence. Low-volume, painful, bloody diarrhoea is known as dysentery (Crawford, 1999).

Diarrhoeal disease is a common cause of morbidity and mortality, with worldwide distribution and is of significant public health concern (Snyder & Merson, 1982;

Ling & Chen, 1993). It is one of the commonest illnesses of children and one of the major causes of infant and childhood mortality in developing countries (Bern, Martins, de Zoysa, and Glass, 1992) of which Nigeria is one. The magnitude of the problem cannot be overemphasized with an estimated 1,000 million episodes and

3.3 million deaths (range 1.5 – 5.1 million) occurring each year among under 5 year

olds (Bern et. al., 1992). In the general population, there are an estimated 4 billion episodes of diarrhoeal diseases largely foodborne and waterborne resulting in over

2.2 million deaths (Murray & Lopez, 1996; WHO, 2000).

Diarrhoeal disease caused by microbiologic agents is principally a foodborne and waterborne illness. Foodborne and waterborne illnesses are leading global health problems, accounting for more morbidity and mortality than tuberculosis and Malaria (Besser, Beebe, and Swaminathan, 2003).

#### Principal Mechanisms of Diarrhoea

Diarrhoea is a disease of the gastrointestinal tract (Baron, Peterson and Finegold, 1994). The principal mechanisms of diarrhoea, one or more of which may be operative in any one patient according to Crawford, 1999 are as follows:

Secretory diarrhoea: Net intestinal fluid secretion leads to the output of greater than 500 ml of fluid stool per day, which persists, on fasting.

This is grouped into 3:

* + - 1. Infectious**:** Viral damage to mucosal epithelium. Agents include rotavirus, Norwalk virus, and enteric adenovirus.
      2. Infectious: Enterotoxin mediated. Bacterial agents such as Vibrio Cholerae, Escherichia coli, Bacillus cereus, and Clostridium perfringes cause this.
      3. Neoplastic: Tumor elaboration of peptides, serotinin, prostagladins, villous adenoma in distal colon (non-hormone mediated); Excess laxative use.

Osmotic diarrhoea: Excessive osmotic forces secreted by luminal solutes lead to output of greater than 500 ml of stool per day, which abates on fasting.

This includes: disaccharide (lactase) deficiences, lactulose therapy (for hepatic encephalopathy, constipation), prescribed gut lavage for diagnostic procedures, antacids (MgSO4 and other magnesium salts), and primary bile acid malabsorption.

Exudative diseases: Mucosal destruction leads to output of purulent, bloody stools, which persists on fasting. Stools are frequent but may be small or large volume. This could be as a result of damage to mucosal epithelium caused by infectious agents such as Shigella, Salmonella, Campylobacter, and Entamoeba species, idiopathic inflammatory bowel diseases, and typhlitis (neutropenic colitis in the immunosuppressed).

Malabsorption: Improper absorption of gut nutrients produce voluminous, bulky stools with increased Osmolarity combined with excess stool fat. The diarrhoea abates on fasting. The following conditions are involved: defective intraluminal digestion, primary mucosal cell abnormalities, reduced small intestinal surface area, lymphatic obstruction, and infectious: impaired mucosal cell absorption caused by Giardia lamblia.

Deranged motility: Improper gut neuromuscular function may produce highly variable patterns of increased stool volume; other forms of diarrhoea must be excluded. This includes decreased intestinal transit time: surgical reduction of gut length, neural dysfunction including irritable bowel syndrome; hyperthyroidism, diabetic neuropathy, carcinoid syndrome; decreased motility (increased intestinal transit time); small intestinal diverticula; surgical creation of a blind intestinal loop; and bacterial over growth in the small intestine.

#### AGENTS OF GASTROINTESTINAL INFECTIONS

Pathogenic mechanisms of agents of gastrointestinal infections

Aetiological agents of gastrointestinal infection are known to cause disease in only four ways (Baron et. al., 1994):

* + - 1. By producing a toxin, that affects fluid secretion, cell function or neurologic function (secretory, bacterial).
      2. By growing within or close to intestinal mucosal cells and destroying them, thus disrupting function (secretory, viral).
      3. By invading the mucosal epithelium causing cellular destruction and occasionally invading the bloodstream and going on to systemic disease (exudative, bacterial and parastic).
      4. By adhering to intestinal mucosa, thus preventing the normal functions of absorption and secretion (malabsorption – parasitic).

Viral aetiological agents

Symptomatic human infections are caused by several distinct groups of viruses such as rotavirus, enteric adenoviruses, caliciviruses, astroviruses and Norwalk viruses/small round structured viruses (SRSV) (Crawford, 1999).

Rotavirus is the most common and accounts for an estimated 140 million cases and 1 million deaths worldwide per year. The target population is children 6 to 24 months of age but young infants and debilitated adults are susceptible to symptomatic infection (Crawford, 1999).

Of the 2,751 food borne disease outbreaks reported to CDC from 1993 to 1997, 56 (4.2%) were caused by viruses (Besser et. al., 2003). Outbreaks caused by viruses are probably greatly underestimated due to lack of widespread testing

capability (Besser et. al., 2003). Of the 691 outbreaks associated with drinking water reported to CDC between 1971 and 1998, 53 (7.7%) were caused by viruses.

Over 60% patients with AIDS have serologic evidence of Cytomegalovirus (CMV). The rate of acute CMV disease rises sharply as the CD4 lymphocyte count falls below 100 cells/mm3 (Smith, Lane and Gill, 1988). CMV is the most common colonic pathogen in patients with AIDS. Smith et. al. (1988) found CMV to be one of the pathogens in 45% of patient with severe diarrhoea.

Parasitic aetiological agents

Parasites are also among the aetiologic agents of gastrointestinal diseases. Some of these are Entamoeba histolytica, Giardia lamblia, Cryptosporidium parvum, Cyclospora cayetenesis, Trichinella species, etc. Parasites caused 19 (0.7%) out of the 2,751 food borne disease outbreaks reported to CDC from 1993 to 1997 (Besser et. al., 2003). Of the 691 outbreaks associated with drinking water reported to CDC between 1971 and 1998, 32 (4.6%) were caused by parasities.

Common infectious organisms known to cause disease in AIDS patients but not usually thought to be gastrointestinal pathogens, including Pneumocystis carini and Toxoplasma gondii have been reported to cause colonic disease (Bellomo, Perlman, and Kaminsky, 1992; Pauwels, Meyohas and Eliaszewica, 1992).

Chemical aetiological agents

Some of these, as per Besser et. al (2003) report, include marine toxins (ciguatoxin), scombroid toxin (histamine), paralytic or neurotoxic shellfish poison, pufferfish tetrodotoxin, heavy metals (antimony, cadmium, copper, iron, tin, zinc), monosodium glutamate (MSG), mushroom toxins (shorter-acting toxins and longer- acting toxins). Chemicals were reported to have caused 148 (5.4%) of the 2,751

food borne disease outbreaks reported to CDC and 79 (11.4%) of 691 outbreaks associated with drinking water.

Diarrhoea of unknown aetiology

Many pathogens can cause diarrhoea. The major offenders vary with the age, nutrition and immune status of the host as well as the environment (living conditions, public health measures); and special predispositions, such as hospitalization, wartime dislocation, or foreign travels. In 40 to 50% of cases, the specific agent cannot be isolated (Crawford, 1999). Out of the 2,751 foodborne disease outbreaks reported to CDC from 1993 through 1997; 1,873 (68.1%) were of unknown aetiology while 336 (48.6%) of 691 outbreak associated with drinking water were also of unknown aetiology (Besser et. al., 2003).

Fungal aetiological agents

In AIDS patients, disseminated histoplasmosis is uncommon, occuring in less than 0.5% of cases. When it does occur, it can involve the gastrointestinal tract in up to 75% of cases. There are reports in which the colon has been the reason for presentation and is the primarily affected organ (Clarkson, Bonacini, and Peterson, 1991). Symptoms of gastrointestinal histoplasmosis are non-specific and include diarrhoea, weight loss, fever and abdominal pain (Heneghan, Li, and Petrossian, 1993). Involvement can occur throughout the entire intestinal tract but most commonly occurs in the terminal ileum and caecum (Clarkson et. al., 1991; Heneghan et. al., 1993). C. albicans colitis has also been described in an HIV- infected individual who presented with a 3-month history of watery diarrhoea, weight loss and abdominal pain (Jayagapol & Cervia, 1992).

Bacterial aetiological agents

According to Thomson and Miller (2003), bacterial aetiology of diarrhoea is grouped into two:

* + - * 1. Infectious Diarrhoea i.e. disease caused by ingestion of bacterium followed by tissue invasion, toxin production or other pathogenic mechanism. Implicated organisims include: - Salmonella spp (many biovars), Shigella Spp, Campylobacter jejuni/coli, Campylobacter spp, EHEC serotypes, Clostridium difficile, Vibrio spp. Aeromonas spp, Pleisiomonas shigelloides, Yersinia enterocolitis; E. coli toxigenic, invasive and effacing strains, Listeria monocytogenes, Clostridium perfringens, Bacillus cereus.
        2. Ingestion of preformed toxin: Disease is caused by ingestion of preformed toxin. Implicated organisms are Staphylococcus aureus, Bacillus cereus and Clostridium botulinum.

Diarrhoeal illness induced by bacteria as reported by Crawford, (1999), is caused by a variety of bacterial species and several pathogenic mechanisms such as:

Ingestion of preformed toxin, present in contaminated food (Major offenders are Staphylococcus aureus, Vibrio and Clostridium perfringes).

Infection by toxigenic organisms, which proliferates within the gut lumen and elaborate an enterotoxin.

Infection by enteroinvasive organism, which proliferates, invades and destroys mucosal epithelium cells.

In the case of infection, key bacterial properties are:

1. Ability to adhere to the mucosal epithelial cells and replicate.
2. The ability to elaborate enterotoxins
3. The capacity to invade.

The ingested organism must adhere to the mucosa in order to produce disease; otherwise, they are swept away by the fluid. Adherence of enterotoxigenic organisms such as E. coli and Vibrio cholerae is mediated by plasmid-coded adhesins. These proteins are expressed on the surfaces of the organism, sometimes in the form of fimbriae or pili. Adherence is also dependent on plasmid-coded proteins but their form is not known. Adherence causes attachment of the apical enterocyte membrane with destruction of the microvillus brush border and changes in the underlying cell cytoplasm.

Bacterial enterotoxins are polypeptides that cause diarrhoea. Some enterotoxins cause intestinal secretion of fluid and electrolyte without causing tissue damage; this is accomplished by binding of the toxins to the epithelial cell membrane, entry of a portion of the toxin into the cell, and massive activation of electrolyte secretion accompanied by water e.g. cholera toxin, E. coli heat-labile and heat-stable toxins. Leukocytes are absent from the stool in these patients. A second group of enterotoxins are cytotoxins; exemplified by shigatoxin and toxins produced by enterohaemorragic E. coli.

Bacterial Invasion: Both enteroinvasive E. coli and Shigella posses a large virulence plasmid that confers the capacity for epithelial cell invasion, apparently by microbe stimulated endocytosis. Bacterial invasion is followed by intracellular proliferation, cell lysis, and cell-to-cell spread. Salmonella quickly pass through intestinal epithelial cells via transcytosis with minimal epithelial damage. Entry into the lamina propria leads to a 5 to 10% incidence of bacteremia. Yersinia

enterocolitica penetrate the mucosa and multiplies within the peyers patches and regional lymph nodes.

Antibiotic associated diarrhoea (Baron et. al., 1994)

Most cases of antibiotic associated diarrhoea are preceded by antibiotic prophylaxis or therapy with Beta-lactam agents. Clindamycin is most frequently implicated, although any antimicrobial agent may be causative. The primary aetiological agent is Clostridium difficile. Rare cases may result from Clostridium perfringens type C and other Clostridia as well as Staphylococcus aureus.

Known pathogens of gastrointestinal diseases

Known pathogens account for only about 18% (38.3 million) of the acute gastroenteritis cases that occur each year in the United States. Of these 36% (13.6 million cases) are attributable to foodborne transmission (Mead et. al., 1999). Common causes foodborne disease include a wide range of infectious agents (bacterial, viral, and parasitic) as discussed above. Below is a table summarizing these agents of gastrointestinal diseases (Vidyya Medical News service, 2005).

**Table 1. Recognized Bacterial, Viral, and Parasitic Agents that commonly cause Foodborne Diseases (Diarrhoea)**

Bacterial Agents\* Parasitic Agents† Viral Agents

Bacillus cereus Cryptosporidium parvum Caliciviruses (including Norwalklike viruses)

Campylobacter species Cyclospora cayetanensis Hepatitis A virus

Clostridium botulinum Entamoeba histolytica Rotavirus

Clostridium perfringes Giardia lamblia Other viruses (eg, astroviruses, adenoviruses

Enterotoxigenic Escherichia coli (ETEC)‡ Isospora belli Listeria monocytogenes Toxoplasma gondii

Plesiomonas shigelloides Trichinella spiralis Salmonella species

Shiga-toxin-producing Escherichia coli

(STEC, including O157:H7)‡ Shigella species Staphylococcus aureus Vibrio cholerae

Vibrio parahaemolyticus Vibrio vulnificus Yersinia enterocolitica

\*Other bacterial agents are uncommon causes of foodborne disease; examples include group A streptococccus, Bacillus anthracis (causes gastrointestinal anthrax), Brucella abortus, Francisella tularensis (may be transmitted via contaminated water), Coxiella burnetii, and Leptospira interrogans (transmitted via contaminated water), Mycobacterium bovis and Mycobacterium tuberculosis can be transmitted via consumption of contaminated milk.

†Other parasitic agents also cause foodborne disease including nematodes and cestodes.

‡Other types of diarrhoeagenic E. coli include enteroinvasive E. coli (EIEC), enteropathogenic E. coli (EPEC), and enteroaggregative E. coli (EaggEC).

#### LIMITATIONS TO THE STUDY

Cost was a very strong limiting factor. In as much as the number of samples, worked on met the calculated sample size, it would have been beneficial to do more samples particularly in the search for E.coli O157:H7. As a result, a population– based survey for E.coli O157:H7 is recommended.

#### PREAMBLE

Usual gastrointestinal pathogens routinely sought for in FCT, Abuja, Nigeria and in the country at large are Salmonella and Shigella species. Inclusion of a less frequently encountered pathogen in any given environment in the diagnosis of gastrointestinal disease should be considered when epidemological and possibly, clinical factors suggest an increased likelihood (Thomson & Miller, 2003). The routine use of selective media for these agents or bacteria that have very low prevalence rate or incidence is not justified (Thomson & Miller, 2003). Periodic surveys of one’s community to establish which pathogens are most common is recommended (Thomson & Miller, 2003).

Thus, this study is basically a survey to see the involvement of E. coli 0157: H7 and other enterohaemorrhagic E. coli in diarrhoeal diseases in FCT-Abuja, Nigeria while excluding the possible involvement of Salmonella/Shigella species in such cases. It is actually a study of bacterial agents associated with diarrhoeal cases in the Federal Capital Territory, Abuja with special reference to Escherichia coli O157:H7 and other enterohaemorrhagic Escherichia coli. Any other bacterial pathogen sought was included when clinical factors suggest an increased likelihood. In addition, search for parasitic involvement in all the diarrhoeal case chosen was

also done, as this is part of the routine protocol of diagnosing diarrhoeal disease in our environment.

#### AIMS AND OBJECTIVES

This study is aimed at:

1. Determininig the occurrence of E. coli O157:H7 in diarrhoeal illnesses in the FCT, Abuja.
2. Assessing their involvement in haemorrhagic colitis and the haemolytic uraemic syndrome within the territory.
3. Determining the prevalence of other Enterohaemorrhagic E. coli infections in diarrhoeal cases in the FCT.
4. Determining the involvement or otherwise of other common bacterial pathogens in those diarrhoeal cases.
5. Determining the antibiotic susceptibility pattern of E.coli O157:H7 isolates and

other bacterial pathogens that may be isolated.

The final objective of the study is, therefore, to determine the importance of Enterohaemorrhagic E. coli as part of the spectrum of diarrhoeal diseases in the Abuja environment.

# CHAPTER TWO LITERATURE REVIEW

## SALMONELLA

#### Description of the Genus

Salmonellae are Gram-negative, motile bacteria that belong to the family enterobacteriaceae (Bopp, Brenner, Fields, Wells, and Strockbine, 2003). They are non-sporing and with the exception of S. typhi, non-capsulate (Cheesborough, 2000).

* + 1. **Taxonomy of *Salmonella***

DNA hybridization studies have shown all Salmonellae to be genetically identical (Cheesborough, 2000). Thus, two species are currently recognized in the genus Salmonella: Salmonella enterica and Salmonella bongori (formally sub-species V) (Reeves, Elvis, Heiba, Plikaytis, and Farmer III, 1989).

Salmonella enterica has been subdivided into six subspecies.

* + - 1. S. enterica sub sp. enterica designated sub-species I
      2. S. enterica sub sp. salamae designated sub-species II
      3. S. enterica sub sp. arizonae designated sub-species IIIa
      4. S. enterica sub sp. diarizonae designated sub-species IIIb
      5. S. enterica sub sp. huntane designated sub-species IV
      6. S. enterica sub sp. indica designated sub-species VI

The species of medical importance is S. enterica sub.sp enterica. Sub-species I strains are usually isolated from human and warm-blooded animals. Subspecies II, IIIa, IIIb, IV and V are usually isolated from cold-blooded animals and the environment (rarely from humans) (Boppl et. al., 2003).

#### Nomenclature for *Salmonella* and Distribution of Serotypes

The World Health Organisation (WHO) collaborating center for Reference and Research on Salmonella, located at the Pasteur Institute in Paris, France, designates serotypes (serovars) belonging to Salmonella enterica sub sp. enterica (sub-species I) with a name which is related to the geographical place where the serotype was first isolated (Popoff, Bockmuhl, Brenner, and Gleesling, 2001). The serotype name is written in roman (not italicized) letters, and the first letter is a capital (for e.g, Salmonella serotype {Ser.} Typhimurium or Salmonella Typhimurium).

Serotypes belonging to other subspecies are designated by their antigenic formular following the sub-species name (for eg, S. enterica subsp salamae ser. 50:z: e, n, x or Salmonella serotype II 50:z: e, n, x). The National Salmonella Reference Laboratory at CDC uses this nomenclature, with the minor deviation of using the term “serotype” instead of “serovar” and strongly encourages its use because it communicates the appropriate taxonomic relationship of the more than 2,500 antigenically distinct members of the two species (Brenner, Villar, Angulo, Tauxe, and Swaminathan, 2000; Bopp et. al., 2003).

Currently, there are 2,501 Salmonella serotypes (Popoff et. al., 2001). Most of these serotypes, including Salmonella, serotype Typhi, belong to subspecies 1 (1,478 recognized serotypes) and are found in O groups A, B, C, C2, D and E. The two

most commonly isolated serotypes in the United States are Salmonella serotypes Typhimurium and Enteritidis (CDC, 2001; Bopp et. al., 2003).

Serotypes belonging to subspecies II (498 serotypes), IIIa (94 serotypes), IIIb (327 serotypes), IV (71 serotypes), VI (12 serotypes) and S. bongori (21 serotypes) are found primarily in O groups O11 (F) through 067 (the higher O groups) (60, 62, 63, 92). The genus “Arizona” was incorporated into the genus Salmonella as subspecies IIIa, containing the monophasic strains, and subspecies IIIb containing the diphasic strains (Rhode, 1979: Bopp et. al., 2003).

#### Pathogenicity

Salmonella organisms cause mainly enteric fever (Typhoid and Paratyphoid) with bactaeraemia. This is caused by Salmonella serotype Typhi (the most serious type) and Salmonella serotype Paratyphi A, Paratyphi B and Paratyphi C (Cheesborough, 2000).

Typhoid fever is a serious blood stream infection that is common in the developing world. However, it is rare in the United States where an estimated 800 cases with fewer than five deaths occur each year; more than 70% of US cases are related to foreign travel (Mead et. al., 1999). Typhoid fever typically presents with a sustained debilitating high fever and headache, without diarrhoea. Illness is milder in young children (non-specific fever) (Miller, Hohmann, and Pegues, 1995). Humans are the only reservoir and may be healthy carriers. Typhoid fever typically has a low infectious dose (<103) and a long, highly variable incubation period (1 to 6 wks). It is transmitted through person-to-person contact or feacally contaminated food and water (Bopp et. al., 2003).

A syndrome similar to typhoid fever is paratyphoid fever caused by “paratyphoidal” strains of Salmonella: Salmonella serotypes Paratyphi A, Paratyphi B and Paratyphi C. These serotypes according to Bopp et. al., (2003) are rare in the United States.

Others are diarrhoel disease (enterocolitis) and bacteremia (Cheeseborough, 2000). Strains of non-typhoidal Salmonella usually cause an intestinal infection accompanied by diarrhoea, fever and abdominal cramps that often last one-week or longer (Miller et. al., 1995; Hohman, 2001). Less commonly, non-typhoid Salmonella can cause localized infections (eg osteomyelitis or urinary tract infections or bacteremia especially in immunocompromised persons (Bopp et. al., 2003).

#### Epidemiology of *S*almonellosis

Persons of all ages are affected, with the incidence being highest in infants. Salmonella is ubiquitous in animal populations and human illness is usually linked to foods of animal origin (Bopp et. al., 2003).

Salmonellosis is generally acquired through contaminated food and water, although cross-contamination in hospitals and institutions occur, as does occasional transmission by medications, diagnostic agents, platelet transfusions and contaminated fiberoptic instruments (Maloney & Guerrant, 1992; Hook, 1990). Salmonellosis also, is transmitted by direct contact with animals, by non-animal foods, by water and occasionally by human contact (Bopp et. al., 2003). Each year, an estimated 1.4 million cases of illness and 600 deaths are caused by non-typhoidal salmonellosis in the United States (Mead et. al., 1999). The majority of cases occur

in sporadic individuals; with less than 10% occurring in a recognized outbreak. In less than 20% of these outbreaks, there is a specific vehicle presumptively identified (Duguid & North, 1991).

Information on sources of human salmonellosis generally, comes from the epidemiologic study of such outbreaks (Maloney & Guerrant, 1992). About 50% of determined outbreaks are caused by poultry and poultry products especially eggs, 13% by meats such as pork, lamb and beef, and 4% by dairy products such as raw and powdered milk (Hook, 1990).

Disease caused by Salmonella serotype Enteritidis in the United States is part of an expanding global pandemic (Bopp et. al., 2003). The proportion of reported Salmonella isolates in the United States that were Salmonella serotype Enteritidis increased from 5% in 1976 to a peak of 26% in 1994 (CDC, 1997). From 1993 to 2000, State and territorial health departments reported 40% Salmonella serotype Enteritidis outbreaks to CDC. In these outbreaks, the dominant location was a commercial venue (e.g restaurants, delicatessans, and cafeterias). Eighty percent (80%) of identified vehicles were foods containing raw or lightly cooked shell eggs (Bopp et. al., 2003).

The WHO collaborating center for Enteric Phage Typing developed a phage- typing system that is used internationally to monitor Salmonella serotype Enteritidis phage types (Hickman-Brenner, Stubbs, and Farmer III, 1991). Although, phage type 8 is currently the most common type among isolates from outbreaks in the United States, Salmonella serotype Enteritidis type 4 infections were first identified in California and other western states during 1993 and 1994 and have spread rapidly to other regions of the United States (Passaro et. al., 1996).

A strain of Salmonella serotype Typhimurium phage type DT 104 resistant to ampicillin, chloramphenicol, streptomycin, sulfonamides, and tetracycline (ACSST,) has emerged in the United Kingdom and the United States as the predominant strain of this serotype; it comprised 28% of Salmonella serotype Typhimurium isolates in 2,000 (CDC, 2,000). In 1996, the first outbreak of pentadrug-resistant DT 104 infection in the United States was reported (CDC, 1997) and since then according to Bopp et. al., (2003) outbreaks have occurred each year.

#### Isolation Procedures

Enrichment

Maximal recovery of Salmonella from faecal specimen is obtained by using an enrichment broth, although isolate from acutely ill persons is usually possible by direct plating of specimen (Bopp et. al., 2003). Enrichment broth for Salmonella are usually highly selective and inhibit certain serotypes of Salmonella, particularly Salmonella serotype Typhi. The three selective enrichment most widely used to isolate Salmonella from faecal specimens are tetrathionate broth, tetrathionate broth with brilliant green and selenite broth (Bopp et. al., 2003).

Selenite broth may also be used for the recovery of Salmonella serotype Typhi and Shigella, although its value as enrichment for the latter has not been clearly established (Bopp et. al., 2003).

Plating media

Many differential plating media, varying from slightly selective to highly selective, are available for the isolation of Salmonella from faecal specimens (Bopp

et. al., 2003). Media of low selectivity include MacConkey agar (MAC) and Eosin Methylene Blue (EMB). Media of intermediate selectivity include xylose lysine deoxycholate (XLD) agar, Deoxycholate Citrate Agar (DCA), Salmonella -Shigella (SS) agar and Hektoen Enteric (HE) agar. Highly selective media include Bismuith sulfite agar, the preferred media for the isolation of Salmonella serotype Typhi and brilliant green agar. Bismuth sulfite, XLD and HE all have H2S indicator systems, which are helpful for the detection of lactose-positive Salmonella strains (Bopp et. al., 2003). Most laboratories today use HE or XLD because these media may also be used for the isolation of Shigella (Bopp et. al., 2003). DCA can also be used for the isolation of both Salmonella and Shigella (Cheesborough, 2000).

Screening procedures

A latex agglutination test has been described for screening for Salmonella from SEL enrichment broth (Metzler & Nachamkim, 1988). This kit can also be used to screen individual colonies from primary plates. These suspect colonies may be innoculated into a screening medium such as KIA or TSI. On KIA or TSI, most Salmonella strains produce a K/AG H2S positive reaction, indicating that glucose is fermented with gas and H2S production (Bopp et. al., 2003).

Isolate may also be identified by a battery of biochemical tests or by slide agglutination with antiserum for Salmonella O groups A, B, C, C2, D and E. Isolates suspected of being Salmonella serotype Typhi should be tested serologically with Salmonella VI and O group D antisera (Bopp et. al., 2003). If the biochemical reactions for a particular isolate are not characteristic but Salmonella antigens are found, the cultures should be plated on MAC or EMB to obtain pure cultures, tested

with a complete set of biochemical tests or forwarded to a reference laboratory (Bopp et. al., 2003).

#### Identification

An isolate is confirmed as Salmonella when the O group has been determined and biochemical identification has been completed (Bopp et. al., 2003).

Biochemical identification**:** Suspect colonies from one of the differential plating media mentioned above can be identified biochemically as Salmonella sp with traditional media in tubes or commercial biochemical systems (Bopp et. al., 2003).

Serotyping: Salmonella sp are serotyped according to their O (somatic) antigens, Vi (capsular) antigen and H (flagella) antigen (Brenner & McWhorter-Muslin, 1998). Serotype can be expressed as a name or as the antigenic formular. The antigenic formular of Salmonella serotype are listed in the kauffman-white scheme and are expressed as follows: O antigen (s), Vi (when present): H antigen(s) (phase1): H antigen(s) (phase 2, when present) for instance the antigenic formular of Salmonella serotype Typhimurium is 4, 5, 12:1:1,2 (Bopp et. al., 2003).

#### Antimicrobial Susceptibilities

Antimicrobial therapy is not recommended for uncomplicated Salmonella gastroenteritis, and routine susceptibility testing of faecal isolates is not warranted for treatment (Bopp et. al., 2003). Determination of antimicrobial resistance patterns is often valuable for surveillance purposes and may be performed periodically to monitor the development and spread of antimicrobial resistance among Salmonella isolates (Bopp et. al., 2003). Treatment with an appropriate

antimicrobial agent can be crucial for patients with invasive Salmonella and typhiod infections and the susceptibilities of these isolates should be reported as soon as possible (Lee, Puhr, Mahoney, Bean, and Tauxe, 1994). The untreated case mortality rate for typhiod fever is > 10%. When patients with typhiod fever are treated with appropriate antibiotics, the rate should be < 1%.

## SHIGELLA

#### Description of the Genus

Shigellas are Gram negative, non-sporing and non-capsulate rods. (Cheesborough, 2000). The genus is made up of non-motile bacteria that conform to the definition of the family Enterobacteraceae (Ewing, 1986).

* + 1. **Taxonomy of *Shigella***

Based on antigenic structure and Biochemical reactions Shigella organisms are divided into 4 subgroups. (Cheeseborough, 2000; Bopp et. al., 2003). These subgroups have been historically treated as species. They are:

Sub-Group A - Shigella dysenteriae contains 15 distinct serotypes.

Sub-Group B - Shigella flexneri contains 8 related serotypes (with serotypes 1 to 5 being subdivided into 11 subserotype).

Sub-Group C - Shigella boydii contains 19 distinct serotypes. Sub-Group D - Shigella sonnei contains one serotype.

From a genetic stand point, the 4 species of Shigella and E. coli represent a single genomospecies with > 75% nucleotide similarity by DNA-DNA reassociation studies (Brenner, Faming, Skerman, and Falkow, 1972). Using a genetic definition

for species, the four species of Shigella would be regarded as serologically defined anaerogenic biotypes of E. coli (Bopp et. al., 2003).

* + 1. **Nomenclature of *Shigella***

The current Nomenclature of Shigella is maintained largely for medical purposes because of the useful association of the genus epithet with the distinctive disease (Shigellosis) caused by these organisms (Bopp et. al., 2003).

#### Pathogenicity

Since the late 19th century, Shigella organisms have been recognized as the causative agents of bacillary dysentry (Acheson & Keusch, 1995). The organism is able to resist gastric acidity, allowing a very small inoculum (as few as 10 organism) to cause disease (Baron et. al., 1994). Shigella causes bloody diarrhoea (dysentry) and non-bloody diarrhoea. Shigellosis often begins with watery diarrhoea accompanied by fever and abdominal cramps but may progress to classic dysentry with scant stools, containing blood, mucus and pus (Bopp et. al., 2003). Blood stream infections can occur but are rare. All four subgroups of Shigella are capable of causing dysentry but S. dysenteriae serotype 1 has been associated with a particularly severe form of illness thought to be related to its production of shiga toxin (Bopp et. al., 2003). Infection can also be assymptomatic particularly infection with S. sonnei strains. Although these organisms are very important as causes of gastrointestinal infections, they rarely cause others types of infections (Bopp et. al., 2003).

Complications of Shigellosis include Hemolytic Uremic Syndrome (HUS), which is associated with S. dysenteriae 1 infection, and Reiter chronic arthritis syndrome,

which is associated with S. flexnerii infection (Acheson & Keusch, 1995). The identification of Shigella species is important for both clinical and epidemiological purposes (Bopp et. al., 2003).

#### Epidemiology of Shigellosis

Humans and other large primates are the only natural reservoirs of Shigella species (Bopp et. al., 2003). They are not disseminated in nature (Baron et. al., 1994). Shigellosis occurs primarily in children although the incidence is not as high as in Salmonellosis (Besser, Lett, and Weber, 1994).

Most transmission is by person-to-person spread, but infection is also caused by ingestion of contaminated food or water (Bopp et. al., 2003). Shigellosis is most common in situations in which hygiene is limited (eg child care centers and other institutional settings). In populations without running water and indoor plumbing, shigellosis can become an endemic problem. Sexual transmission of Shigella among homosexuals also occurs (Bopp et. al., 2003). There is a reported annual estimate of

164.7 million episodes of shigellosis throughout the world. Out of this, 163.2 million occur in developing countries with 1.1 million deaths (61% involving children under

5 years) (Kotloff, 1999). In the United States, an estimated 450,000 cases of shigellosis occur each year, with 70 deaths (Mead et. al., 1999). Up to 20% of all United States, cases of shigellosis are related to international travel. S. sonnei causes most infections in the United States and other developed countries. S. flexnerii is second most common group (Bopp et. al., 2003).

In developing countries, depending on region, about 60% infections were found to be due to Shigella flexnerii, 15% to S. sonnei, 6% to S. boydii and 6% to S.

dysenteriae (30% of cases were Shigella dysenteriae Sd 1). Shigella dysenteriae Sd 1 causes major epidemics of bacillary dysentry in developing countries with high loss of life among young children (over 6 months) (Cheesborough, 2000). Since, 1979, a prolonged S. dysenteriae 1 epidemic has affected Southern Africa, and major epidemics have occurred in other parts of Africa, in Asia, and in Central America (Bopp et. al., 2003). Infection with S. dysenteriae is associated with high rates of morbidity and mortality in developing countries particularly when antimicrobial resistance or its misdiagnosis as amoebiasis makes appropriate treatment problematic (Bopp et. al., 2003).

A recent compilation of data from Thailand and Nepal showed that Shigella

organisms and enteroinvasive E. coli (EIEC) accounted for 36% to 59% of the cases of bloody diarrhoea and 15% to 27% of all cases of diarrhoea (Swansonetti, 1992).

#### Isolation Procedures

Enrichment and plating media.

There is no reliably effective enrichment media for all Shigella isolates, but Gram-Negative (GN) broth and selenite broth are frequently used (Bopp et. al., 2003). Salmonella-Shigella agar (SS) should be used with caution because it inhibits the growth of some strains of S. dysenrteriae 1 (Bopp et. al., 2003).

Screening procedure

Shigella strains appear as lactose- or xylose- non-fermenting colonies on the isolation media described above. S. dysenteriae I colonies may be smaller on all of

the media, and these strains generally grow best on media with low selectivities (e.g MAC) (Bopp et. al., 2003).

Suspect colonies are innoculated on kligers iron agar (KIA) or triple sugar iron agar (TSI). Shigella species characteristically produce an alkaline slant and an acid butt; do not produce gas or H2S. The motility, urea and lysine decarboxylase tests (all are negative for Shigella) can be used to further screen isolate before doing serologic testing. Isolates that react appropriately with the screening biochemical tests should then be identified with a complete set of biochemicals with automated systems or self-contained commercial kits. Confirmation requires both biochemical and serotype identification (Bopp et. al., 2003).

#### Identification

Biochemical

Shigella and inactive E. coli (anaerogenic or lactose non-fermenting) are frequently difficult to distinguish by routine biochemical tests. S. dysenteriae and S. Sonnei are biochemically distinct. S. flexnerii and S. boydii are often biochemically indistinguishable, so that serologic grouping is essential (Bopp et. al., 2003).

Serotyping

Serologic testing is essential for the identification of Shigella. Serologic identification is typically performed by slide agglutination with polyvalent somatic (O) antigen grouping sera followed by testing with monovalent antiserum for specific serotype identification (Bopp et, al., 2003).

Subtyping

A variety of methods has been used to subtype Shigella, including colicin typing, plasmid profiling, restriction fragment length polymorphism analysis, pulsed field gel electrophoresis and Ribotyping (Bopp et. al., 2003).

Serodiagnostic tests

Several serodiagnostic assays based on different antigens possessed by Shigella have been described. These assays are practical only in research settings and are not currently used for the diagnosis of infection in individual patients (Bopp et. al., 2003).

#### Antimicrobial Susceptibilities

Infections by Shigella are treated with antimicrobial agents. All isolates should undergo susceptibility testing because of the widespread antimicrobial resistance among Shigella strains. In certain areas of Africa and Asia, S. dysenteriae 1 strains are resistant to all locally available antimicrobial agents, including nalidixic acid but are still susceptible to the fluoroquinolones (Sack, Rhaman, Yunus, and Khan, 1997). The following antimicrobial agents are currently recommended by WHO for treatment of Shigella dysenteriae A1 and possibly, for the treatment of other infections with Shigella species as well. They are Ampicillin, trimethoprim- sulfamethoxazole, Nalidixic acid, privmecillinam, ciprofloxacin, norfloxacin and

enoxacin (CDC, 1999).

WHO recommends that the selection of antimicrobial treatment should be based on recent susceptibility testing of Shigella dysenteriae A1 strains from the area or near by areas if Sd 1 is new to the area (CDC, 1999). It goes further to

state that for developing a treatment policy, the antimicrobial agent chosen should be effective against at least 80% of local Shigella dysenteriae A1 strains, be given by mouth, be affordable and be available locally or able to be obtained quickly. Unfortunately, according to the report, resistance of sd 1 to Ampicillin and trimethoprim-sulphamethoxazole has become widespread. Nalidixic acid, formerly used as a “back up” drug to treat resistant shigellosis, is now the drug of choice in most areas, but resistance to it has appeared in many places, Pivmecillin (amdinocillin pivoxil) is still effective for most strains of sd 1 but may not be readily available. Fluoroquinolones (i.e ciprofloxacin, norfloxacin, enoxacin) should be considered only if sd 1 isolates are resistant to Nalidixic acid. Fluoroquinolones are often costly and may not be readily available (CDC, 1999).

Currently, Sd1 strains are often resistant to ampicillin, trimethoprim- sulfamethoxazole, metronidazole, streptomycin, tetracycline, chloramphenicol, and sulfonamides. In addition, although Sd 1 may be susceptible to some antimicrobial agents in vitro, the drug may have no documented efficacy in vivo. Examples of such agents are nitrofurans (e.g nitrofurantion, furazolidone), aminoglycosides (e.g gentamicin, Kanamycin), first- and second-generation cephalosporins (e.g cephalexin, cefamadol), and amoxicillin (CDC, 1999).

## VIBRIO

#### Description of the Genus

Vibrios are small straight, slightly curved or comma-shaped Gram-negative rods 0.5 to 0.8 µm in width and 1.4 to 2.6 µm in length. They are motile with monotrichous or multitrichous polar flagella when grown in liquid media. Some

species produce numerous lateral flagella when grown on solid media. They do not require vitamins or amino acid. Na+ stimulates the growth of all Vibrio species, and Na+ is an absolute requirement for most species. They are facultative anaerobes and oxidase positive. They ferment D-glucose, producing acid but rarely gas, they reduce nitrate to nitrite and they grow on thiosulphate-citrate bile salts sucrose (TCBS) medium (Farmer III, Janda, and Birkhead, 2003).

V. cholerae is highly motile with a distinctive rapid to and fro-movement, which has been likened to swarming gnats. Vibrio motility is best-seen using dark-field microscopy but can also be seen using light microscopy (Cheesborough, 2000).

#### Taxonomy

Vibrio is classified under the family vibrionaceae. It is the type genus of the family. Twelve Vibrio species occur in human clinical specie. Among them, Vibrio cholerae is the most important specimen in the genus (Farmer III et. al., 2003). It is now divided into 3 major Subgroups: V.cholerae O1, V. Cholerae O139, and V. cholerae non-O1 (Farmer III et. al., 2003).

Although, no genetic basis exists for a division, V. cholera have been divided into two biovars or biotypes: Classical and eltor (formerly EL Tor) (Barron et al, 1994). The eltor biovar is voges-proskaeur positive, agglutinates chicken Red blood cells and is resistant to 50 iu disk of polymyxin whereas the classical biovar yields the opposite reactions (Baron et. al., 1994). The eltor biotype is responsible for most V. cholerae O1 cholera while the classical is confined to India and Bangladesh (Cheesborough, 2000).

The V.cholera O1 is also divided into 3 serotypes Inaba, Ogawa and Hikojima. The Hikojima serotype is rare, it possesses characteristics of both Inaba and Ogawa serotypes (Cheesborough, 2000). Other species of Vibrio of clinical importance include V.mimicus, V. parahaemolyticus, V. vulnificus (V. vulnificus Biogroups 2 and 3), V. fluvialis, V. furnissi, V. Hollissae, V. damsela, V. alginolyticus, V. metschnikovii,

V. cincinnatiensis and V. harveyi (or V.carchariae) (Farmer III et. al., 2003).

#### Pathogenicity

Twleve Vibrio species occur in human clinical specimens. All except V. furnissii are apparently pathogenic for humans (Daniels, Evans, and Griffin, 2000; Farmer III et. al., 2003). Vibrio usually cause diarrhoea or extraintestinal infections but some such as V .cholerae cause both (Farmer III et. al., 2003). Vibrios are often isolated from blood, wounds of arms and legs, infected eyes and ears, and the gall bladder, but they are rarely reported from patients with meningitis, pneumonia and infection of the reproductive organs or urinary tract. (Farmer III et. al., 2003).

V. cholerae causes cholera which is endemic in about 80 countries (Cheeseborough, 2000). It is the most important species in the genus Vibrio. It has caused many epidemics of cholerae and millions of death. V. cholerae O1 and V. cholerae O139 (Bengal) both cause epidemic cholera. Other serogroups of V. cholerae cause diarrhoeal disease but not epidemic cholera (Cheeseborough, 2000).

The pathogencity of diarrhoea due to V. cholerae O1 is well understood. A large number of organisms (108 organism in some human volunteer experiments) is ingested, and some cells survive the acid pH of the stomach and pass into the small

intestine. There, they multiply and produce cholera toxins, which results in massive fluid loss (Farmer III et. al., 2003).

Originally, it was thought that V. cholerae 01 did not occur in aquatic environment unless they had been contaminated with faeces from patients with cholera. However, recent indigenous cases of V. cholerae O1 in the United States and Australia (Wachsmuth, Blake, and Olsvik, 1994), along with ecological studies in areas where cholera is endemic, have suggested that there may also be a free-living state (Farmer III et. al., 2003).

Treatment for the most severe cases of cholera is intravenous therapy with large volumes of a balanced salt solution, which restores water and electrolyte balance and prevents acidoses. Antibiotic resistance is becoming more common in V. cholerae, particularly with strains circulating in the developing world (Wachsmuth et. al., 1994; Janda, 1998; Farmer III et. al., 2003).

V. cholerae O1

V. cholerae serogroup O1 is the organism responsible for seven pandemics of Cholera (1816-1817, 1829, 1852, 1863, 1881, 1889 and 1961 to the present). In patients with severe cholera or “cholera gravis”, there is massive diarrhoea with large volumes of “rice water stool” (Clear fluid with flakes of mucus) passed painlessly. The amount of fluid can be a litre or more per hour. In 4 to 6 days, this would amount to over twice the body weight. There is usually vomiting and little desire to eat. If left untreated, the patient becomes prostrate with symptoms of severe dehydration, electrolyte imbalance, painful muscle cramps, watery eyes, loss of skin elasticity and anuria (absence of urine excretion). Death can occur quickly

after the onset of symptoms because of the severe dehydration (Farmer III et. al., 2003). Interestingly, there is a correlation between human blood types and susceptibility to V. cholerae infection. In areas where cholera is endemic, many individuals will have either a mild diarrhoea or only asymptomatic colonization of the intestine. This pattern of mild disease has been much more common in the seventh Cholera pandemic caused by the eltor biogroup of V. cholerae (Farmer III et. al., 2003).

V. cholerae O139

V. cholerae serogroup O139 (Synonym: V. cholerae O139 Bengal) is a relatively new organism that causes epidemic cholera (Albert, 1994). The symptoms are typical of cholera but the organism does not react in V. cholerae O1 antisera or in O2 – 138 antisera. Thus, it was named O139 (Farmer III et. al., 2003). Unlike V. cholerae O1, strains of O139 produce a capsule (Weintraub et. al., 1994) as do some strains of V. cholerae non-O1 (Morris Jr., 1994). It has been speculated that the emergence of this strain is the beginning of the Eighth Cholera pandemic (Farmer III et. al., 2003).

V. cholerae non-O1

V. cholerae non-O1 strains (synonym: ‘nag vibrios”; non-agglutinating vibrios, “non-cholera vibrios; V. cholerae “non-O1, non-O139’; and V. cholerae non-O1, 139) do not agglutinate in O1 or O139 antisera but are otherwise typical strains of V. cholerae in their biochemical reactions (Farmer III et. al., 2003). They usually do not produce cholera toxin but can produce other toxins (Honda, Arits, Takeda, Yoh,

and Miwaten, 1985; Farmer III et. al., 2003). They can cause a severe, cholera-like disease, but they are usually isolated from patients with mild diarrhoea, extraintestinal infections, seafood, and the environment (Farmer III et. al., 2003). V.cholerae non-O1 strains have also caused septicaemia in patients with cirrhosis or other underlying diseases (Morris Jr, 1994). Strains have also been isolated from ears, wounds, the respiratory tract and urine (Farmer III & Hickmann-Brenner, 1992; Morris Jr, 1994).

Other pathogenic VIbrio species

Other Vibrio species can cause a spectrum of illness. They can cause diarrhoea similar to V.cholerae non-O1 in most of its clinical, epidemiological and ecological aspects e.g V.mimicus. (Farmer III et. al., 2003).

V. parahaemolyticus causes gastroenteritis with nausea, vomiting, abdominal cramps, low-grade fever and chills. The diarrhea is usually watery but can sometimes be bloody. The disease is usually mild and self-limiting but can be fatal. The fatality rate was 7% in the first outbreak. Rehydration is usually the only treatment needed, but in some severe cases the patient will require hospital admission. Antimicrobial therapy may be beneficial (Farmer III et. al., 2003).

V. vulnificus is associated with two disease syndromes: primary septicaemia and wound infection (Blake, Merson, Weaver, Hollis, and Heushin, 1979). V. vulnificus biogroup 2 was originally isolated from diseased eels, but in 1995, Amaro and Biosca isolated it from a human wound infections. Bisharat et. al. (1999) who isolated it from patients with wound infections and bacteraemia, described V.vulnificus biogroup 3 in 1999. Cases have been limited to Israel and were acquired from exposure to live fish (tilapia) grown in aquaculture. V. fluvialis causes

sporadic cases of diarrhea world wide and was also implicated in a large outbreak of diarrhoea in Bangladesh (Huq, Alam, Brenner, and Morris, 1980; Farmer III et. al., 2003).

V.furnisii has a clear association with diarrhoea but an unproven etiological role. V.hollisae causes sporadic cases of diarrhoea. V.damsela was isolated from wound infections of damselfish off the California coast and from human wound infections. V.alginolyticus has been isolated from infections of soft tissues, wounds, the ears and occasionally the eyes. V.metschiokoni as been reported to have caused peritonitis and bacteraemia in a patient with an inflamed gall bladder. V.cincinatiensis was first reported from a patient with bacteraemia and meningitis. Subsequently, it has been isolated from faeces (intestine), the ear, a leg wound, animals and water V.carchariae has been isolated from a human wound infection following a shark bite (Farmer III et. al., 2003). V.carchariae is almost identical to V.harveyi in phenotype and the type strains of the two species are 88% related by DNA – DNA hybridization (Pederson et. al., 1998). The 2 organisms also have identical or almost identical 16s or RNA sequences. The evidence seems conclusive that the 2 species are (subjective) synonyms V.harveyi is the older name, thus it has priority (Pederson et. al., 1998; Farmer III et. al., 2003).

* + 1. **Epidemiology of *Vibrio* species**

Members of the genus Vibrio are natural inhabitants of sea water (Baron et. al., 1994). Thus, they are primarily aquatic and the species distribution is usually dependent on the temperature, Na+ concentration, nutrient content of the water and plants and animals present (Wachsmuth et. al., 1994). All organisms in this group

are able to grow in media containing increased salt concentrations and with the exception of V.cholera and V. mimicus (the non-halophilic Vibrios) require sodium chloride for growth. The sodium ion-requiring members are called halophilic Vibrios (Baron et. al., 1994). A number of Vibrios have been reported to exist in the Natural environment (e.g the ocean) in a dormant state. These are refered to as viable but non culturable” (VNC). According to this hypothesis, vibrios are alive but cannot be cultured when plated onto common plating media. This VNC state is not universally accepted and if it is true, its role in Vibrio ecology and pathogenicity is unclear (Farmer III et. al., 2003).

V.cholerae serogroup O1, of the eltor biogroup is the organism responsible for

seven pandemic of cholera (1816-1817, 1829, 1852, 1863, 1881, 1889 and 1961 to the present) (Farmer III et. al., 2003). The current seventh cholera pandemic started in Indonesia in 1961, spread rapidly to Bangladesh, India, Iran, Iraq and in 1970 to West Africa from where it spread to East, Central and South Africa. In 1991, cholera reached Peru and has now spread throughout South and Central America. In 1997-1998, 80% of the global total of cholera cases was reported from countries in the Horn of Africa due to exceptionally heavy rain and floods (Cheesborough, 2000).

Originally, it was thought that V.cholerae O1 did not occur in aquatic environments unless they have been contaminated with faeces from patients with cholera. However, recent indigenous cases of V.cholera O1 infection in the United States and Australia (Wachsmuth et. al., 1994) along with ecological studies in areas where cholera is endemic, have suggested that there may also be a free-living state (Farmer III et. al., 2003).

V. cholerae serogroup O139 emerged in October, 1992 in Bengal Madras India, and by 1994 had spread to many Asian countries and had been imported into industrialized countries (Farmer III et. al., 2003). Cholera due to V.cholerae O139 has been reported in South East Asia, the Far East China, Saudi Arabia and eleven countries in South Asia (Cheesborough, 2000). It has been speculated that the emergence of this strain is the beginning to the eighth cholera pandemic (Farmer III et. al., 2003).

Food borne out breaks and sporadic cases due to V. haemolyticus occur worldwide and are usually associated with the consumption of raw or contaminated seafood. In Japan, V. parahaemolyticus is an extremely important diarrhoeal agent, causing 50 to 70% of the cases of foodborne enteritis, usually associated with the consumption of raw fish or shellfish. Cross-contamination after the food is cooked is another important mechanism of spread. Out breaks are not common in the United States (Farmer III et. al., 2003). Recently, a pandemic clone of serotype 03:K6 has emerged (Okude et. al., 1997). Strains of this serotype also caused an unusually high proportion of V. parahaemolyticus foodborne disease outbreaks in Taiwan (from 1996 to 1999), suggesting something unusual in the organism’s ecology, epidemiology, or virulence (Chiou, Hsu, Chiu, Wang, and Chao, 2000; Farmer III et. al., 2003).

Cases of infection by V.vulnificus Biogroups 2 and 3 have been limited to Israel

and were acquired from exposure to live fish (tilapia) grown in aquaculture (Farmer III et. al., 2003). V. fluvialis causes sporadic cases of diarrhoea world wide. It was also implicated in a large outbreak of diarrhoea in Bangladesh (Huq et al, 1980). V. furnisii is widespread in the aquatic environment and is more common in estuaries

(Farmer III et. al., 2003). V. damsela isolates have also been recovered from marine, fish, sewage, oysters and a wound on a racoon (Farmer III et. al., 2003).

#### Isolation Procedures

Extra intestinal specimens are collected and processed without special attention to Vibrio species. The collection, processing and isolation of Vibrio species from extra intestinal specimens (e.g. blood and wounds) do not typically present a pattern in the clinical laboratory because most Vibrio strains are found as the only pathogen. In addition, most pathogenic Vibrios grow well on common plating media such as MacConkey agar and blood agar designed for the isolation of gram-negative bacilli (Farmer III et. al., 2003).

Stool specimen should be collected early before the patient has received any antimicrobial agents and inoculated on isolation plates with minimal delay. If delay is expected stool sample should be placed in semisolid transport medium of Cary and Blair, which maintains the viability of vibrios up to 4 weeks (Farmer III et. al., 2003). Alkaline peptone water is also an excellent transport and enrichment medium for V.cholerae (Cheesborough, 2000). Routine cultures for Vibrios are not common even in areas of high prevalence. It is important therefore, that physicians alert the laboratory when Vibrio infection is suspected by relaying relevant information in the clinical history of the patient.

Specimens are enriched in alkaline peptone water at 36oC. They are subcultured at 8 hours and again at 16 to 24 hours. Thiosulphate Citrate Bile Salt Sucrose agar is the most widely used medium for isolating Vibrio strains from human clinical specimens and the environment. Its main advantage is that it increases a

laboratory worker’s awareness of suspect Vibrio colonies. Its use will result in the detection of Vibrio isolates that otherwise would be missed (Farmer III et. al., 2003). Cultures of Vibrio grow well on blood agar where they may be beta-hemolytic (V.cholerae non-O1 and some V.cholerae O1 strains of the eltor biotype), alpha- hemolytic (V.vulnificus and many others), or non-haemolytic. Vibrio strains usually grow well on MacConkey agar (sometimes with a reduced plating efficiency) and will appear as colorless (Lactose-negative) colonies. Vibrio cultures often do not grow well on more selective plating media for enteric organism. Oxidase testing can be done on colonies from blood agar and on lactose-negative colonies from selective media. However, Lactose-positive colonies from selective media can give a false-

negative oxidase reaction (Farmer III et. al., 2003).

Direct detection of V.cholerae O1 in faeces

There are two methods of detecting V.cholerae O1 from faeces:

* + - 1. Agglutination of V.cholerae O1 antigens present in high numbers (often 106 -108 organisms/stool) in rice water stools from patients with cholera (Jesudaon, Thangaveli, and Lalitha, 1984).
      2. Microscopic immobilization test, which detects the rapid loss of motility of V.cholerae O1 cells in the presence of 01 antiserum as observed with a microscope (Farmer III et. al., 2003). Antibodies to V.cholerae O139 could also be included in both of these assays (Farmer III et. al., 2003).

#### Identification

The first and most important step in identifying a Vibrio strain is to suspect that it is a member of the genus. For Vibrio identification by standard tube tests, NaCl should be added to several biochemical test media because the commercial formulas do not include NaCl. Otherwise, halophilic Vibrios will not grow or will grow poorly and give negative reactions in tests that should be positive. Fortunately, commercial media for most of the biochemical tests are formulated to contain 0.5 to 1.0% NaCl.

Serotyping and serodiagnosis

Serotyping schemes are available for several of the Vibrio species but complete serotyping is done in only a few specialized reference laboratories. However, clinical laboratories should maintain V.cholerae O1 and O139 antisera so that these two important pathogens can be identified completely and reported immediately (Farmer III et. al., 2003).

#### Antimicrobial Susceptibility Testing

Pathogenic Vibrio species usually grow well on Mueller-Hinton broth and agar. Antibiotic resistance is rare in Vibrio compared to Enterobacteriacae. In most cases, resistance is probably intrinsic to the species rather than acquired through plasmid transfer or antibiotic exposure. The main exception is that strains of V.cholerae apparently become resistant through exposure to antibiotics. In cholera outbreak, resistance can be acquired by the acquisition of R-factors (Farmer III et. al., 2003).

Antimicrobial therapy is helpful, although not essential, in treating cholera patients. Antimicrobial agents reduce the duration of illness, the volume of stool, and

the duration of shedding of Vibrios in faeces (CDC, 1999). When antimicrobial agents are used, it is essential to choose one to which the organism is susceptible. Antimicrobial agents recommended by WHO for treating cholera patients include tetracycline, doxycycline, furazolidone, trimethoprim-sulfamethoxazole, erythromycin, or chloramphenicol. ciprofloxacin and norfloxacin are also effective (CDC, 1999). Because antimicrobial resistance has been a growing problem in many parts of the world, the susceptibility of V.cholerae O1 strains to antimicrobial agents should be determined at the beginning of an epidemic and be monitored periodically (CDC, 1999). It should be noted that disc diffusion tests is not recommended for use in testing doxycycline and erythromycin because the results of these drugs are frequently inaccurate for V.cholerae O1 and O139 strains. However, the tetracycline disc test can be used to predict the likely susceptibility of isolates to doxycycline (CDC, 1999).

## ESCHERICHIA

#### Description of the Genus

The genus Escherichia is composed of motile or non-motile bacteria that conform to the definitions of the family Enterobacteriaceae (Ewing, 1986). There are five species in this genus: Escherichia blattae, E.coli, E.fergusonii, E.hermani and E.vulnaris. The type species is E.coli (Bopp et. al., 2003).

#### Pathogenesis

Of the five Escherichia species, E.coli is the species usually isolated from human specimens. It is part of the bowel flora of healthy individuals. However, certain strains may cause extra intestinal and intestinal infections in

immunocompromised as well as healthy individuals. Urinary tract infections, bacteremia, meningitis and diarrhoeal disease are the most frequent clinical syndrome and are caused primarily by a limited number of pathogenic colonies of E.coli (Bopp et. al., 2003). E. hermanii and E.vulnaris are most often obtained from wound infections but have also been isolated from infections at other body sites, while E.fergusonii is most frequently identified from human faeces (Bettelheim, 1992). E.blattae, which is a commensal organism of cockroaches, is not recovered from human specimen (Bopp et. al., 2003).

* + 1. **Diarrhoeagenic *E.coli***

There are at least four categories of recognized diarrhoeagenic E.coli, Enterotoxigenic E.coli (ETEC), Enteropathogenic E.coli (EPEC), Enteroinvasive E.coli (EIEC), and Shiga toxin producing E.coli (STEC) (also refered to as Enterohaemorrhagic E.coli [EHEC]). The clinical significance of several other groups of putative diarrhoeagenic E.coli, including Enteroaggregative E.coli (Eagg. EC) is unclear (Bopp et. al., 2003).

ETEC

ETEC is an important cause of diarrhoea in developing countries, particularly

among young children. It produces heat labile E.coli enterotoxin (LT), heat-stable E.coli enterotoxin (ST), or both LT and ST. (Nataro and Kapper, 1998). ETEC is also a frequent cause of travelers’ disease. Ten US outbreaks were reported to the Centers for Disease Control and Prevention (CDC) from 1995 to 2001, while only 15

outbreaks occurred during the preceeding 25 years (Dalton, Mintz, Wells, Bopp, and Tauxe, 1999).

The most prominent symptoms of ETEC illness are diarrhoea and abdominal cramps, sometimes accompanied by nausea and headache but usually with little vomiting or fever (Dalton et. al., I999). Although, ETEC is usually associated with relatively mild watery diarrhoea, illness in recent ETEC outbreaks has been notable for its prolonged duration (Bopp et. al., 2003).

EPEC

In the past, EPEC strains were defined as certain E.coli serotypes that were

epidemiological associated with infantile diarrhoea but did not produce enterotoxins or shiga toxins and were not invasive (Bopp et. al., 2003).

EPEC serotypes typically show a distinct pattern of localized adherence to Hela and Hep-2 cells. They also demonstrate actin aggregation in the fluorescent actin stain test, which correlates with the attaching and effacing lesion in vivo (Nataro and Kaper, 1998). Due to the lack of simple diagnostic methods for EPEC, few laboratories attempt to identify these organisms (Bopp et. al., 2003).

EPEC infections are rare in developed countries but are recognized cause of infantile diarrhoea in the developing world (Bopp et. al., 2003). The symptoms of severe, prolonged and non-bloody diarrhoea, vomiting and fever in infants or young toddlers are characteristic of EPEC illness (Nataro & Kaper, 1998). Infection with EPEC has been associated with chronic diarrhoea, sequelae may include malabsorption, malnutrition, weight loss and growth retardation (Bopp et. al., 2003).

EIEC

EIEC strains invade cells of the colon and produce a generally watery but

occasionally bloody diarrhoea by a pathogenic mechanism similar to that of Shigella. EIEC is very rare in the United States and is less common than ETEC or EPEC in the developing world (Nataro & Kaper, 1998). EIEC strains, like ETEC and EPEC strains, are associated with only a few characteristic serotypes. Three large outbreaks of diarrhoea caused by EIEC have been reported in the United States (Nataro & Kaper, 1998).

Putative diarrhoeagenic E.coli

EAggEC, which exhibits a specific pattern of aggregative adherence to Hep-2 cells in culture, has been associated with diarrhea in children in Chile, Persistant diarrhoea in children in Mexico and Kenya, and bloody diarrhea in Children in India (Nataro & Kaper, 1998; Bopp et. al., 2003). These organisms may also have a role in chronic diarrhoea among human immunodeficiency virus infected patients (Polotsky, Nataro, Kotler, Barret, and Orenstein, 1997). EaggEC was isolated from children with diarrhoea during an outbreak in Japan (Bopp et. al., 2003).

Diffusely adherent E.coli (DAEC) strains, which exhibit a diffuse pattern of

adherence to Hep-2 cells, have been implicated as causes of diarrhoea in some studies but not others (Nataro & Kaper, 1998). Little is known about their associated clinical syndrome, epidemiology, and pathogenic, mechanisms. In a retrospective case-control study, the majority of children infected with DAEC strains had watery diarrhoea without blood or faecal leukocytes. In one study, DAEC

infections were significantly associated with diarrhoea in children 1 to 5 years of age but were not associated with illness in infants (Bopp et. al., 2003).

Cytotoxic necrotizing factor (CNF)-producing E.coli strain produce a toxin that induces morphological alterations (multinucleation) and death in tissue cultures. Two forms have been described: CNF 1 and CNF2. CNF 1-producing strains were originally detected in infants with enteritis and were latter detected in humans with extra intestinal infections (Caprioli et. al., 1987). Most CNF 1-producing strains are also hemolytic, although the toxin is distinct from haemolysin (Caprioli et. al., 1987). CNF-2 producing strains have been isolated from animals with diarrhoea. The role of these strains in human diarrhoea disease has not been definitively determined (Nataro & Kaper, 1998).

Cytolethal distending toxin (CLDT) - producing E.coli strains produce a heat- labile factor that induces cytotonic and cytotoxic changes in Chinese hamster ovary cells similar to those caused by LT (Johnson & Lior, 1988). This factor does not affect Y-1 cells. The results of one study in Bangladesh suggested that CLDT- producing E.coli strains are not associated with diarrhoea, but other studies are needed to establish their status as aetiologic agents (Albert et. al., 1996; Bopp et. al., 2003).

#### Isolation Methods for ETEC, EPEC, EIEC and the Putative Diarrhoeagenic *E.coli*.

Method for the identification of ETEC, EPEC, EIEC and the putative diarrhoeagenic E.coli are generally available only in reference or research settings. Public health and reference laboratories usually examine specimens for these

pathogens only when an outbreak has occurred and specimen are negative for routine bacterial pathogens (Bopp et. al., 2003).

ETEC should be considered a possible etiologic agent of watery diarrhoea for which no pathogen has been identified (Dalton et. al., 1999). EPEC should be considered a possible pathogen in outbreak of severe non-bloody diarrhoea in infants or young toddlers, particularly in nursery or day care settings. EIEC should be considered a possible etiologic agent in outbreaks of diarrhoea (bloody or non- bloody), when routine bacterial agents are ruled out (Bopp et. al., 2003).

Faecal specimens should be plated on a differential medium of low selectivity (e.g. MAC). Between 5 and 20 cells mostly lactose fermenting but with a representative sample of non-fermenting colonies should be selected and innoculated onto a non-selective agar. These colonies are then screened for virulence-associated characteristics appropriate to the pathogen being sought (Bopp et. al., 2003).

#### Screening Procedures for ETEC, EPEC and EIEC Strains

ETEC or EPEC strains cannot be distinguished from other E.coli strains by biochemical screening technquies. Many EIEC strains are non-motile and fail to decarboxylate lysine, however, some EIEC strains are motile or lysine positive (Bopp et. al., 2003). Use of commercial antisera to the classical EPEC somatic (O) and capsular (K) antigens yields many false-positive results. Further testing with H (flageller) antisera would reduce the number of false-positive reports. However, this type of testing is not practical for the average clinical microbiology laboratory (Bopp et. al., 2003).

#### Antimicrobial Susceptibilities (ETEC, EPEC, EIEC and other Diarrhoeagenic *E.coli* strains)

Treatment with appropriate antibiotic can reduce the severity and duration of symptoms of ETEC infection (Nataro & Kaper, 1998). Antimicrobial resistance, particularly to tetracycline is common among ETEC strains isolated from outbreaks in the United State (Dalton et. al., 1999). Antibiotic treatment may be helpful for diarrhoea caused by EPEC (Nataro & Kapper, 1998). Most EPEC strains associated with outbreaks are resistant to multiple antimicrobial agents. Little information about the efficacy of antimicrobial treatment or the prevalence of resistance is available for EIEC or other putative diarrhoeagenic E.coli strain (eg EaggEC); but determination of antimicrobial susceptibility pattern may be helpful in establishing whether isolates are associated with an outbreak (Bopp et. al., 2003).

* 1. ***ESCHERICHIA COLI* O157:H7 AND OTHER ENTEROHAEMORRHAGIC**

## ESCHERICHIA COLI

A few studies have demonstrated the occurrence of sporadic cases of haemorrhagic colitis caused by E.coli O157:H7 from stool samples submitted to hospital laboratories (Grandsen, Damm, Anderson, Carter, and Lior, 1986). Prevalence studies of E.coli O157:H7 in the United States have been done (Macdonald et. al., 1988). Studies implicating some other serotypes of Escherichia coli in some cases of haemorrhagic colitis have also been carried out in various parts of the world.

However, no literature of studies on this group of organisms conducted in this country is available to me. Thus, this study is aimed primarily at these groups of organisms hence the elaborate literature review on them.

* + 1. **History of *Escherichia coli* O157:H7**

Escherichia coli serotype O157:H7 was first reported in 1970 as a cause of piglet enteritis in Ireland (Furowitz & Orskov, 1972). Isolations of serogroup O157 in cases of piglet enteritis have also been made in Canada, the United States, France, Germany and Yugoslavia (Hockin & Lior, 1986).

Evidence that E.coli O157:H7 is a human pathogen is relatively new. In Canada, the laboratory for disease control first identified it in a case of bloody diarrhoea in 1978. Between then and 1982, isolation, were made in another five cases from different parts of Canada. All the isolates were negative for invasiveness and for heat-labile and heat-stable enterotoxins (Hockin & Lior, 1986).

Two nearly simultaneous report, brought E.coli O157:H7 into prime focus. In the first, Karmali, Steele, Petric, and Lim (1983) at the Hospital for sick children in Toronto identified cytotoxin-producing E.coli of various serotypes including E.coli O157:H7 in the stools of children with haemolytic uraemic syndrome (HUS). One week after the publication of their article, Riley et. al.,(1983) at the centers for disease control reported their findings in an investigation of an outbreak of bloody diarrhoea, a disorder they termed haemorrhagic colitis. There was an epidemiologic association between the consumption of hamburgers and the development of haemorrhagic colitis. The two outbreaks of severe bloody diarrhoea occurred in 1982 and were associated with the same fast food restaurant chain. It led to the

identification of the same strain of E.coli that expresses O antigen 157 and H-antigen 7 (Riley et. al., 1983). Subsequently, this strain was shown to belong to a category of E.coli that produce toxins that are similar to shiga toxins of Shigella dysenteriae and distinct from previously described E.coli heat-stable and heat-labile toxins (Armstrong, Hollingsworth and Glenn-Morris Jnr, 1996).

In the decades, since its initial description (Riley et. al., 1983), E.coli O157:H7 has emerged as a major cause of both sporadic and outbreaks of diarrhoeas in North America (Boyce, Swerdlow, and Griffin, 1995). At that time, little was known about the pathophysiology, epidemiology or clinical sequelae of infection with E.coli O157:H7 (Boyce et. al., 1995). Since that report, several studies have shown that infection with E.coli O157:H7 is responsible for most cases of the haemolytic uremic syndrome, which is a major cause of acute renal failure in children (Karmali et. al., 1985; Tarr et. al., 1990; Bitzen et. al., 1991).

In addition, it has been discovered that infection with this organism is a common cause of bloody and non-bloody diarrhoea in North America (Boyce et. al., 1995). Verocytotoxin-producing Escherichia coli (VTEC) strains are now fully recognized as a major cause of both haemorrhagic colitis (HC) and the haemolytic uraemic Syndrome (HUS); conditions with high morbidity and mortality (Chapman & Siddons, 1996). In North America, also, research has indicated that E.coli O157:H7 is the cause of 85% - 95% of cases of haemolytic uraemic syndrome and that non- O157 shiga-toxin-producing E.coli are responsible for another 5% - 10% (Griffin, 1995).

In the years, since the discovery of this pathogen, in the United States and other developed world, E.coli O157:H7 has become increasingly prominent. It is

estimated to cause about 20,000 infections and as many as 250 deaths each year in the United States (Consensus conference statement, 1995). A more recent report, gave an estimate of 73,000 cases of illness and 60 deaths caused by 0157 STEC, annually in the United States (Mead et. al., 1999). In 1994, the CDC reported 30 separate outbreaks of this pathogen (CDC surveillance data). Public interest in E.coli O157:H7 grew enormously in the wake of a large multi state outbreak in the Western United States in early 1993, which resulted in more than 700 illnesses and four deaths (Bel, Goldoft, and Griffin, 1994).

* + 1. **Nomenclature of Enterohaemorrhagic *Escherichia coli***

E.coli serotype O157:H7 is designated by its somatic (O) and flagella (H) antigens (Boyce et. al., 1995). It produces large amounts of toxins called verocytotoxins first detected because of their toxic effects on Vero cells in tissue culture or shiga - like toxins (now called shiga toxins) because they are similar to or homologous to the cytotoxins) produced by Shigella dysenteriae (Stockbine et. al., 1986; Karmali et. al., 1995).

Other strains of E.coli also produce these toxins. The strains of E.coli that produce these toxins are known interchangeably as verocytotoxin-producing E.coli (VTEC) or as shiga-toxin producing E.coli (STEC) formerly shiga-like toxin producing

E. coli (Armstrong et. al., 1996) and Enterohaemorrhagic Escherichia coli (EHEC) (Griffin & Tauxe, 1991). The use of EHEC as a synonym for all shiga-toxin producing E.coli is discouraged because it implies that all E.coli that contain stx genes can cause bloody diarrhoea (Tarr & Neil, 2001). Also, STEC category of diarrhoeagenic E.coli are referred to according to the toxin that these organisms produce i.e STEC

rather than EHEC, because the essential genetic features that define organisms capable of causing HC and HUS are not clear (Bopp, Brenner, Wells, and Strockbine, 1999; Bopp et. al., 2003). Although, strains of VTEC may belong to many serotypes, the majority of severe human infections are caused by strains of serogroup O157 and to a lesser extent O26 and O111 (Griffin & Tauxe, 1991). In some countries, non-O157 STEC strains, particularly E.coli serotypes O111: NM and O26:H11 are more commonly isolated than O157 STEC, although most outbreaks and cases of HUS are attributed to O157 STEC (Griffin, Mead and Sivapalasingam, 2002; Bopp et. al., 2003). In the United States, E.coli O157:H7 is the most frequently isolated STEC but increasing non-O157 STEC are identified as causes of outbreaks of sporadic illnesses (Bopp et. al., 2003). At the Centers for Disease Control and Prevention (CDC), E.coli reference laboratory, 72% of all non-O157 STEC isolates received between 1983 and 2,000 belonged to eight serogroups (O26, O111, O103, O121, O45, O145, O165 and O113) (Bopp et. al., 2003).

Haemorrhagic diarrhoea and the haemolytic uraemic syndrome (HUS) have also been reported with serotypes other than O157:H7 in South America and Ontario and Quebec (Cordovez, Brado, and Maggi, 1992; Karmali et. al., 1985). Most laboratory methods for the detection of O157:H7 STEC do not detect non-O157 STEC, as a result, the numbers of documented infections with serotypes other than O157:H7 or O157: NM are probably underestimated. Serotype O103:H2 is not often isolated from humans but has been identified as a cause of diarrhoea in rabbits (Rondeau & Peraldi, 1996).

Enterohaemorrhagic E.coli produces two main shiga toxins, types 1 and 2. In contrast to the Shiga-toxin gene of Shigella, the E.coli genes encoding shiga toxins 1

and 2 are located not on the bacterial chromosome but on bacteriophages that infect the bacteria (Rondeau & Peraldi, 1996). Shiga toxin 1 is nearly identical to the principal extracellular toxin produced by Shigella dysenteriae Serotype 1 (Tarr & Neil, 2001); Stx2 is 58% and 58% identical to Stx1 at the nucleotide and amino acid levels respectively (Rondeau & Peraldi, 1996).

In a study in France, strains of E.coli O103:H2 were isolated from stools of 69 children with the haemolytic uraemic syndrome. Comparison of the human and rabbit strains suggested a common origin but found no evidence of horizontal transmission between the two species (Marian-Kurkdjiani, Denamur, and Milton, 1993). An unusual case of the haemolytric uraemic syndrome in a six-year old girl caused by E.coli O103:H2 has also been reported (Tarr et. al., 1996).

Although, over 100 serotypes of E.coli that produce shiga-like toxins have been isolated from humans, not all these serotypes have been shown to cause illness (Boyce, Swerdlow, and Griffin, 1995). 60 serotypes have been implicated in diarrhoeal diseases, and several non-O157: H7 serotypes have been implicated as the cause of foodborne outbreaks and HUS in the United States, Europe, and Australia (Fey, Wickert, Rupp, Safranek, and Hinrichs, 2000). Studies from Canada, Europe, Argentina, and Australia suggest that non-O157: H7 STEC infections are as prevalent, or more so, than O157: H7 infections. (Fey et. al., 2000). However, from both a clinical and public health standpoint, E. coli O157:H7 is by far the most important serotype (Boyce et. al., 1995).

#### Epidemiology

Incidence

In the United States and infact, all over the world, few laboratories culture stool specimen routinely for E.coli O157.H7, resulting in the actual incidence of infection with this organism being unknown (Boyce et. al., 1995). A prospective population-based study conducted in the Seattle area in 1985 and 1986 reported an incidence rate for E. coli O157:H7 of 8 infections per 100,000 persons per year. Incidence rates for other confirmed enteric infections reported by them are as follows: Campylobacter – 50 infections per 100,000 persons per year, Salmonella – 21 infections per 100,000 persons per year, and Shigella – 7 infections per 100,000 persons per year (MacDonald et. al., 1988). Based on this figure, E.coli O157:H7 is estimated to cause 21,000 infections in the United States annually.

In this study, and other US and Canadian studies, comparing the rates of isolation of E.coli O157:H7 and other bacterial enteric pathogens, E.coli O157:H7 has generally been isolated more frequently than Shigella (MacDonald et. al., 1988; Pai et. al., 1988). The isolation rate of E.coli O157:H7 is particularly high among patients with bloody diarrhoea. Canadian studies have reported isolation rates of 15% to 39% in specimens of bloody stools (Pai, Gordon, Sims, and Bryan, 1984; March & Ratnam, 1986).

The number of outbreaks of E.coli O157:H7 infection reported to the Centers for Disease Control and prevention (CDC) has increased in recent years from 4 in 1992 to 30 in 1994 (Boyce et. al., 1995) and may be more by now. This increase can be attributed in part to increased reporting of and screening of E.coli infection by laboratories (Boyce et. al., 1995). The organism is therefore, a more common

pathogen in the United States than is generally recognized and the diagnosis should be considered for patients with suspected enteric infections (MacDonald et. al., 1988). E.coli O157:H7 may infect persons of any age, but children less than 10 and the elderly have the highest frequency of extra intestinal complications and deaths (Tarr & Neil, 2001).

Geographic and seasonal factors

E.coli O157:H7 has been isolated in many parts of the world including Europe, Asia, Africa and South America (Griffin, 1995). However, its prevalence in these areas is unknown. Infections with E.coli O157:H7 have been reported most frequently in Canada and the United States (Griffin & Tauxe, 1991). Sporadic cases and outbreaks are reported more frequently in Northern States than in the Southern States of the United States and whether this variation reflects, the true distribution of this organism is unknown (Boyce et. al., 1995). E.coli O157:H7 infections are more common in warmer months than in colder months with a peak incidence from June through September (Pai et. al., 1988). This seasonal variation may reflect the ecology of the organism, variation in the consumption of ground beef or some other factors (Boyce et. al., 1995). In most countries, E. coli O157:H7 is the predominant serotype, but a large number of other serotypes have been incriminated in sporadic cases and outbreaks and there appears, also to be geographic differences in serotype prevalence (Boyce et. al., 1995).

Reservoirs of Enterohaemorrhagic E.coli

The intestinal tracts of domestic animals, particularly cows have been identified as the major reservoirs of E.coli (Mariani-Kurkdjiani et. al., 1993). Studies from the

United Kingdom show that the incidence of these strains in the intestinal flora of apparently healthy cattle can be high (Chapman et. al., 1994).

The usual route of human contamination is the ingestion of unpasteurized milk or cooked meat from these animals (Mariani-Kurkdjiani et. al., 1993). Thus, outbreaks of food borne EHEC diseases have occurred throughout the world following consumption of contaminated raw or uncooked ground beef products. Infections have likewise occurred through drinking of contaminated raw (unpasteurized) milk (Karch, 1996). EHEC have also been isolated from a wide range of foodstuffs such as dried salami (CDC, 1994). Moreover, EHEC O157 strains survive food conservation procedures (drying, cold storage and acidity) traditionally thought to eliminate or reduce survival of enteric bacteria (Feng, 1995). This resistance to food-handling procedures suggests that these organisms may survive in nature and could be more widespread than has been suspected (Karch, 1996). Indeed the occurrence of O157 in drinking water (Swerdlow et. al., 1992) and in recreational water sources (Keene et. al., 1994) represents real danger.

As noted earlier, O157 STEC colonizes dairy and beef cattle; not surprisingly, therefore, ground beef has caused more O157 STEC outbreaks than any other vehicle of transmission (Griffin et. al., 2002). Other known vehicles of transmission include raw-milk, sausage, roast beef, unchlorinated municipal water, apple cider, raw vegetable and sprouts (alfalfa and raddish) (Bopp et. al., 2003).

E.coli O157:H7 and cattle

Ever since the first human outbreak linked to ground beef consumption in 1982, a bovine reservoir of E.coli O157:H7 has been suspected (Armstrong et. al.,

1996). Before that time, neither the US department of Agriculture (USDA), Animal Laboratories nor the Pennsylvania state University Veterinary Research Laboratory had ever detected this serotype in any of its samples (Riley et. al., 1983). Since then, numerous studies in several countries have shown that this organism is present in the gastrointestinal tract of varying percentages of cattle (Armstrong et. al., 1996). Although, many studies of the prevalence of E.coli O157:H7 in cattle have been published, the large variety of screening techniques in use limits the ability to compare them (Armstrong et. al., 1996). As might be expected, studies using very sensitive techniques have found the highest prevalence. Nonetheless, examining differences between sub-groups within studies, have shown some consistent patterns (Armstrong et. al., 1996). Thus, despite the limitations of the prevalence data, three generalizations can be made (Armstrong et. al., 1996): -

1. E. coli O157:H7 can be isolated from the faeces of both healthy and sick cattle and estimates of the prevalence in North America and European cattle range from less than 1% to almost 10%.
2. E.coli O157:H7 has a wide geographic distribution in the United States. In one large survey, it was found in 63% of feedlots (National Animal Health monitoring System, 1995).
3. E.coli O157:H7 is more common in calves than in older cattles.

The last conclusion is supported by one of the earliest surveys of cattle herds performed in Washington State and Wisconsin, which showed a higher prevalence of this organism in heifers and calves (17 of 604) than in adult cattle (1 of 664) (Wells, Shipman, and Greene, 1991).

Subsequent studies have consistently shown that young animals have the highest prevalence rates although the youngest animals show relatively low rates, perhaps reflecting a lack of contact with other animals shedding this organism (Armstrong et. al., 1996). The relatively high prevalence in young animals is consistent with the fact that calves when infected experimentally with this bacterium shed the organism for a longer period than older cattle (Cray & Moon, 1996). A similar pattern has been noted in humans. Young children shed E.coli O157:H7 longer than adults (Pai et. al., 1984; Pai et. al., 1988; Belongia et. al., 1993). The longer duration of shedding seen in calves, compared with older cattle, is not unique to E.coli O157:H7. E.coli strains in general are shed more frequently and in higher numbers in calves compared with adult cattle (Armstrong et. al., 1996).

Most studies have examined the prevalence of E.coli O157:H7 in cattle by measuring faecal shedding (Armstrong et. al., 1996). It is possible that the principal location of this organism in these animals is not the distal gastrointestinal tract but rather, the rumen (Armstrong et. al., 1990). The fact that this organism is relatively acid-tolerant may represent an adaptation to the acid environment of the rumen, and the fact that this organism can be found in other ruminants such as sheep and deer, supports this hypothesis (Armstrong et. al., 1996). If this is the case, then, faecal shedding may be a poor measure of prevalence in these animals (Armstrong et al, 1996).

Role of E.coli O157:H7 in bovine population

E.coli O157:H7 is generally not pathogenic in adult cattle. Many studies have found this organism in the faeces of healthy cattle, indicating that it is usually a

harmless commensal while some other studies have found the organism in the faeces of calves with diarrhoea, suggesting that it may cause diarrhoea in young animals (Armstrong et. al., 1996).

In a study, experimental infection of 17 calves was followed by transient diarrhoea in four. The cause of the diarrhoea could not be definitively attributed to E.coli O157:H7 because under normal circumstance such animals show high incidence in diarrhoea (Cray & Moon, 1996). In the same study, all of the 12 adult cattle infected with the organism remained healthy. Both the calves and the adults showed asymptomatic shedding of this organism, though the calves shed it for a longer duration (Armstrong et. al., 1996). Data indicate that the disease only occurs in animals fed a particular low grade of raw meat (4 - D” meat, obtained from dead, dying, disable or diseased cattle). It was found that the organism could be isolated from this meat and that the affected dogs shed E. coli O157:H7 temporarily after developing the disease (Armstrong et. al., 1996).

Other animal reservoirs of E.coli O157:H7

A survey of a single flock of sheep in Idaho using methods sensitive for E.coli O157 found the prevalence of faecal shedding varied from zero percent in November to 31% in June (Kudva, Hatfield, and Hoode, 1996). In the United Kingdom, a survey of 700 sheep at a slaughterhouse found the organism in the faeces of 18 (2.6%), a rate lower than that of cattle at the same site (Chapman, Siddons, and Harkin, 1996). Two other surveys found only non-O157 STEC-producing E.coli strains in various farm animals in England (Wray, Mclaren, and Carroll, 1993) and in Germany (Beutin, Gelar, and Steinruck, 1993).

An outbreak of the haemolytic uremic syndrome in Northern Italy was linked to contact with chicken coops (Tozzi, Nicolini and Caprioli, 1994). Although, natural infection of chicken with E.coli O157:H7 has never been demonstrated, young chicks (I day old) can be experimentally infected with this organism (Beery & Doyle, 1985; Schoeni & Doyle, 1994) and will shed it in their faeces and the surface of their eggs for up to 11 months (Schoeni & Doyle, 1994). The susceptibility of chicken to colonization by E.coli O157:H7 drops significantly during the first 3-days of life (Stavric, Buchanan, and Glaeson, 1993). So far, this serotype has been isolated from two asymptomatic dogs, a deer and one horse as well as from environmental sources, including stable flies (Stomoxys calcitrans) and drinking water. The two isolates from deer matched isolates from cattle slowing the same pasture. None of 106 rodent samples was positive (Hancock, Besser, and Rice, 1995; Rice, Hancock, and Besser, 1995; Armstrong et. ai., 1996). Though E.coli O157:H7 is probably not pathogenic in cattle, recent studies at Kansas State University indicate that it may be the cause of idiopathic cutaneous and renal glomerular vasculopathy (or “Alabama rot”) a disease very similar to the haemolytic uremic syndrome which is only known to affect greyhounds (Armstrong et. al., 1996).

E. coli O157:H7 in food Infectious dose

The low concentrations of E.coli O157:H7 found in food responsible for outbreaks have confirmed the suspicion from epidemiological studies that the infectious dose of the organism is small (Armstrong et. al., 1996). The Hamburger patties implicated in the large multistate outbreak in 1993, for example, had fewer

than 700 organisms before they were cooked and probably contained fewer by the time of consumptions (Griffin, 1995). An investigation into food borne outbreaks show that illness may occur after ingestion of less than 100 organisms (Willshaw et. al., 1994). Another investigation of an outbreak due to the consumption dry-cured salami (CDC, 1994) estimated the infectious dose to be fewer than 50 organisms; some of the cases had probably consumed fewer than five organisms (Griffin, 1995). One study showed that when pre-school children with EHEC O157 diarrhoea attended day-care centers, transmission rates were between 3% to 38% (Median 22%) (Belongia et. al., 1993). Other studies showed similar transmission rates in household members of infected individuals (Rowe, Orbine, Lior, Wells, and Melanie, 1993). Although conclusive data are lacking, these findings taken together are evidence of the fact that a low dose of O157 is sufficient to transmit the disease (Karch, 1996). In addition, the ease with which E.coli O157:H7 is spread from person-to-person suggests that as with Shigella, the infectious dose is small (Boyce et. al., 1995).

The low infectious dose attributes of E. coli O157: H7 dominates almost all

mitigation strategies as well as public health and Infection control considerations. It also contributes to the propensity of this pathogen for person-to-person spread. Such transmissions account for 10% of overall cases during a food-borne outbreak. This low infectious dose is particularly problematic in considerations of food safety, particularly, for foods that are not cooked and are eaten raw (Tarr & Neill, 2001).

E.coli O157: H7 in beef products

A survey of retail meats collected from stores in the Madison, Wisconson Area, found the organism on several types of meat: One of 147 ground beef (0.7%), three of 257 poultry samples (1.6%) and four (2.0%) of 200 lamb samples tested positive for E.coli O157:H7 (Doyle & Schoenii, 1987). In the same study samples collected from around the Calgary area, Alberta Canada analyzed by the same methods showed an even higher prevalence of E. coli O157:H7 - five of 17 ground beef samples (31%), one of 14 pork samples (7%) but none of six poultry samples (Doyle & Schoeni, 1987). So many surveys of E. coli O157:H7) in beef products have been done extensively using techniques that are not very sensitive (Armstrong et. al., 1996). Four of such studies designed primarily to detect shiga-toxin producing E. coli all tested negative for E. coli O157:H7 and found non-O157 Shiga toxin- producing E. coli to be common (Armstrong et. al., 1996).

One large-scale survey of beef products using an appropriately sensitive method of E.coli O157:H7 detection found 3 out of 5,000 tests to be positive (Armstrong et. al., 1996). The reason for this low prevalence is not clear. It is possible that there are regional differences in the prevalence of food contamination (Armstrong et. al., 1996). It is also possible that the prevalence is relatively high in areas such as Western Canada where the incidence of human disease is relatively high (Waters et. al., 1994) and that the prevalence is low in areas like the Southern United States where few outbreaks have been reported and the reported incidence is low (Armstrong et. al., 1996). It is possible, also that reductions in rates are related to efforts on the part of the meat industry to reduce the rate of contamination of E. coli O157:H7 in ground meat products (Armstrong et. al., 1996).

In September 2005, the first national food-related outbreak of shiga toxin- producing Escherichia coli in the Netherlands was investigated (Doorduyn et. al., 2006). Evidences suggest that the outbreak was related to the consumption steak tatare, a raw beef product (Doorduyn et. al., 2006).

E. coli O157:H7 in milk

Milk is the only other food product that have been systematically tested for E. coli O157:H7. A study of 1,021 filters from milk processors found 20 positive for shiga toxin-producing E. coli (Clarke, McEwen, and Gannon, 1989). The assay used was insensitive to E.coli O157:H7 and therefore none was found to be present. In a second study, using a more sensitive assay, E.coli O157:H7 was found to be present in 10% of 115 samples of raw milk examined (Padhye & Doyle, 1991).

E. coli O157:H7 in other foods

Unrefridgerated sandwiches (Carter et. al., 1987), potatoes (Morgan et. al., 1988), apple cider (Besser, Lett, and Weber, 1993); mayonnaise (Keene, Mc Anulty, and Williams, 1993), cantaloupe (Corvallis, 1993) and lettuce (Griffin, 1995), have all been suspected or implicated as vehicle in outbreaks of E. coli O157:H7. In such cases involving non-bovine foods, cross-contamination by beef or contamination with bovine faecal material has often been suspected (Armstrong et. al., 1996).

E.coli O157:H7 in water

There have been reported outbreaks of E.coli O157: H7associated with drinking water. The largest occured in late 1989 in a Missouri town with

unchlorinated water supply resulting in 243 cases with four deaths (Swerdlow et. al., 1992; Armstrong et. al., 1996). Drinking water that was probably contaminated with bovine faeces was implicated in outbreaks in both Scotland (Dev, Main, and Goward, 1991) and South Africa (Isaacson, Carter, and Effler, 1993). Another outbreak in Japan was caused by well water contaminated from an unknown source (Akashi, Joh, and Tsuji, 1994; Armstrong et. al., 1996). In two of these outbreaks, E. coli O157 was isolated from the water (Isaacson et. al., 1993; Akashi et. al., 1994; Armstrong et. al., 1996).

Swimming-associated outbreaks have been reported in Oregon (Keene et. al., 1994), New York (Ackman, Birkhefad, and Root, 1995); Illinois and Wisconsin (Amstrong et. al., 1996). Illness was presumed to be due to swallowing small amounts of water during swimming (Armstrong et. al., 1996). The initial sources of contamination in any of these outbreaks were not identified. However, speculation has it that before these outbreaks, a child with E. coli O157:H7 infection may have contaminated the swimming area (Keene et. al., 1994; Ackman et. al., 1995).

#### TRANSMISSION

Food borne-transmission in outbreaks

Much of the data on the transmission of E.coli O157:H7 have been obtained from investigations of outbreaks. The majority of outbreaks have resulted from transmission of organism through the consumption of beef, most commonly ground beef (Griffin et. al., 1991). The largest reported outbreak in North America affected over 700 persons (4 of whom died) in four western states and was traced to under cooked hamburgers from a fast-food restaurant chain (Bel et. al., 1994; Griffin,

1995). E. coli O157: H7 is present in the intestines in about 1% of healthy cattle (Griffin & Tauxe, 1991). Beef may be contaminated during slaughter and the process of grinding beef may transfer pathogens from the surface of the meat to the interior. If the beef is improperly cooked, the bacteria can survive and be ingested since ground beef may include meat from many carcasses, a small number of infected animals can contaminate a large supply of ground beef (Boyce et. al., 1995).

Vehicles or modes of transmission implicated in outbreaks of E. coli O157:H7 in the United States up to and including 1994 are all foods, ground beef, all beef products and milk, drinking water or swimming associated, person-to-person (i.e. no food identified), unknown sources (Armstrong et. al., 1996). Of all these ground beef is the vehicle responsible for the largest portion (58%) of food borne E. coli O157:H7 outbreaks (Armstrong et. al., 1996).

Attempts to confirm the sources of these outbreaks by testing meat samples have been hampered by the fact that the product has often been completely consumed before the outbreak is recognized and investigated: Nonetheless, in at least six ground-beef associated outbreaks E coli O157:H7 has been isolated from the implicated meat (Armstrong et. al., 1996). In three of these outbreaks, molecular sub-typing was performed, which confirmed that the meat isolate matched the outbreak isolate.

It is only in a single instance that trace back succeeded in identifying the farm from which the infected animal originated. In this case, which occurred in New Jersey, the family of the infected patient had purchased one side of a cow from a small produce. Ground beef in the family freezer contained 500 - 1000 cfu/g of E.coli O157. The other side of the cow was traced and was found to contain E. coli

O157: H7 with the same pulse-field gel electrophoresis pattern but a lower concentration of 100 cfu/g (Armstrong et. al., 1996). In another outbreak, trace back uncovered six cattle colonized with E.coli O157:H7, though the strain differed genetically from the outbreak strain (Armstrong et. al., 1996). It is not surprising that trace back has failed to reveal outbreak strain in animal reservoirs, several strains can be found in a single herd of cattle and cattle shed E. coli O157:H7 intermittently. More importantly, trace back is hampered by modern production methods in which meat from large numbers of cattle go into any given lot of ground beef (Armstrong et. al., 1996).

Even in foodborne out breaks in which beef is not implicated, contamination with cattle faeces is often suspected (Armstrong et. al., 1996). In an outbreak linked to apple cider consumption (Besser et. al., 1993), it was found that the apples had been taken from the ground of an orchard next to a cow pasture. Though environmental and bovine faecal cultures were negative in this study, they were obtained 2 months after the outbreak.

Cow’s milk has been documented as a vehicle for E.coli O157:H7 infection. Two cases of paediatric haemolytic uremic syndrome investigated by the CDC in 1986 were the first to be linked to raw-milk consumption (Martin et. al., 1987). The milk in these cases came from two separate farms, both of which had heifers that tested positive for E.coli O157:H7. One of the 23 raw-milk specimens collected from the farms at a latter date also tested positive (Wells et. al., 1991). There are many other reported outbreaks linked to raw milk (Borczyk, Karmali, and Lior, 1986; Chapman et. al., 1993; Wright, Chapman, and Siddons, 1994). Pasteurized milk

(Upton & Coia, 1994) and Yoghourt (Morgan, Newman, and Hutchinson, 1993) have also been implicated in outbreaks.

Food-borne transmission in sporadic E. coli O157: H7

Though the source of E. coli. O157: H7 in sporadic infections (i.e. cases not associated with a known out break) is less clear, existing data show that transmission from bovines play an important role as well (Armstrong et. al., 1993). Uncontrolled studies of sporadic E.coli O157:H7 have noted ground beef (Pai et. al., 1984; Waters, Sharp, and Dev, 1994), rare ground beef (Ostroff, Kobayashi, and Lewis, 1989), pasteurized milk (Ostroff et. al., 1989; Waters et. al., 1994) as possible vehicles of infection, though lack of controls in these studies precluded implicating these sources. E. coli O157:H7 infection has also been associated with consumption of ground beef in a non-commercial setting such as Picnic or “special event”, drinking of well water (Le Saux, Spika, and Friesesn, 1993); swimming, handling animal faeces and close contact with a person with diarrhoea (Ries, Griffin, and Greene, 1993).

Studies comparing E.coli O157:H7 strains from cattle with those from sporadic human infections have shown varying amounts of overlap. In a study in Washington State, 22 bovine and 50 sporadic human isolates were compared by plasmid typing, bacteriophage lambda restriction fragment length polymorphism and shiga toxin production (Pavos, Tarr, and Kim, 1993). Forty-three separate strains were identified among the seventy-seven isolates.

Only three of these were found in both humans and cattle. Of the human islolates 10% (5 of 50) were strains also found in cattle. Of the bovine isolates, 27%

(6 of 22) were strains also found in humans. Conflicting results were reported in a British study in which 96 cattle isolates and 63 sporadic human isolates were compared by phage-typing, plasmid typing and shiga toxin-production (Chapman & Siddons, 1994). Forty-one different strains were identified out of 159 isolates. Of the human isolates, 94% (59 of 63) were strains also found in cattle, although only 26% (10 of 39) of cattle isolates were found in humans.

Direct transmission from bovines to humans

Two instances of apparent direct transmission of E. coli O157:H7 from bovines to humans have been documented (Renwick, Wilson, and Clarke, 1993; Synge, Hopkins, and Riley, 1993). In Canada, in 1992, a 13-month-old child became ill with E.coli O157:H7 after having direct prolonged contact with calves on his family’s farm (Renwick et. al., 1993). Two calves on the farm tested positive for E. coli O157:H7 of the same phage type and shiga toxin type as the child. It was noted that the family did not drink unpasteurized milk and had not consumed undercooked ground beef. A similar case occurred in Scotland in which a 15-month-old child living adjacent to a farm became ill with E coli O157:H7 (Synge et. al., 1993). One of the 84 dung samples on the farm yielded E.coli O157: H7 of the same phage type and the same unusual plasmid profile as the human isolate. It was speculated that the family dog, which frequently roamed the farm, might have acted as a vector in the case. Though direct transmission from cattle to humans may occur as purported in these cases, such transmission appears to be rare (Armstrong et. al., 1996).

Other modes of transmission have also been documented including transmission from an unchlorinated water supply (Swerdlow et. al., 1992),

transmission to persons swimming in a faecally contaminated lake (Keene et. al., 1994) and secondary transmission from person to person (Spika et. al., 1986; Pavia, Nichols, and Greene, 1990; Belongia et. al., 1993; Bel et. al, 1994).

Person-to-person transmission Secondary transmission in outbreaks

Secondary transmission after point source outbreak of E.coli O157 is common. An example is the 1988 outbreak in Minnesota linked to under cooked harmburger paties served at a Junior high school (Belongia et. al., 1991). Thirty-two cases were reported, including a 12-year-old student whose mother ran a day-care centre. One week after this student’s illness, two of the children in the day-care centre developed haemorrhagic colitis and were cultured positive for E.coli O157:H7. So many other instances of secondary transmission from person to person have been reported (Carter, Borczyk, and Carlson, 1987; Belongia et. al., 1993; Besser et. al., 1993; Bel et. al., 1994; CDC, 1994; Keene et. al., 1994; Upton & Coia, 1994).

Since asymptomatic cases can occur in outbreaks, there has been concern that persons with such infections could unwittingly spread their infection to others (Armstrong et. al., 1996). The existence of such asymptomatic cases during outbreaks has been well demonstrated in family members and other close contacts of persons with haemolytic uraemic syndrome or asymptomatic E. Coli O157:H7 (Pai et. al., 1984). As with other secondary cases, cases due to transmission from asymptomatic individuals are few in number but highlight the importance of preventing food borne and other common source out breaks of E.coli O157:H7 (Armstrong et. al., 1993)

Person-to-person transmission in day-care centre

Person-to-person transmissions are of special importance in day-care centres, where it may be the primary mode of transmission (Armstrong et. al., 1996). In this context, data are very comparable to those obtained for Shigella another enteric pathogen with a low infectious dose which is known to be an important cause of day-care centre outbreaks (Weisman, Schmarler, and Weiter, 1974). Evidence of continuing person-to-person transmission was found in all nine day-care facilities surveyed in Minnesota in which children with the infection had been identified (Belongia et. al., 1993). Exclusion of all the children from the facility until two or more cultures tested negative in six of these facilities resulted in the cessation of transmission at each facility. In another day-care centre outbreak cohorting ill children stopped transmission until they were culture negative (Armstrong et. al., 1996). This is an approach that has also proven successful in the control of shigellosis (Armstrong et. al., 1996). The ease with which E. coli O157: H7 is spread from person to person also suggests that, as with Shigella, the infectious dose is low (Boyce et. al., 1995).

Incubation period and duration of shedding

The typical incubation period is three to four days, although incubation may be as short as one day or as long as eight days (Griffin, 1995). Incubation periods longer than eight days may represent secondary spread (Boyce et. al., 1995). It is a common mistake for patients infected with this pathogen and their physicians to suspect that infection was acquired from food consumed within a few hours before the onset of diarrhea. Illnesses caused by E.coli O157:H7 does not result from

ingestion of a preformed toxin, as for example, in food poisoning caused by Staphylococcus aureus or Bacillus cereus toxins hence the incubation period is almost more than 24 hours (Tarr & Neil, 2001).

For unknown reasons, the duration of excretion of E.coli O157:H7 varies with age. In one study of sporadic cases, 53% of children under five years of age had positive stool cultures, three weeks after the onset of the illness as compared with 8% of older children and adults (Pai et. al., 1988). Although, assymptomatic infection and prolonged carriage may occasionally occur, E. coli O157:H7 is not part of the normal flora in the human body. The organism was not isolated from stool specimens obtained from 530 healthy controls (Griffin, 1995). As with enteric pathogens such as Salmonella typhi, long-term asymptomatic carriage (greater than 1 year) of E.coli O157:H7 has never been demonstrated (Armstrong et. al., 1996). Though long-term carriers may not exist, infected individuals especially children, can shed the organism for several weeks after the onset of their illness (Belongia et. al., 1993; Karch, Russman, and Schmidt, 1995; Pai et. al., 1998).

A study of transmission of E. coli O157:H7 in day care centres in Minnesota

(Belongia et. al., 1993) found the mean duration of shedding to be 17 days for the 24 children studied. One child, who had been treated with amoxicillin, excreted the organism for 62 days. A Second study of 53 children infected with E.coli 0157:H7 (Karch et. al., 1995) found the median duration of shedding to be 13 days with one child shedding the organism for 124 days. Children often shed this organism intermittently (Belongia et. al., 1993; Karch et. al., 1995) and without symptoms (Karch et. al., 1995).

These studies may overestimate the average duration of shedding because: -

1. They involve only children, who shed the organism longer than do adults.
2. They do not include data from children who had very short durations of shedding or who were asymptomatic and not diagnosed as having E. coli O157: H7.

Another method of examining the duration of shedding is to look at the isolation rate of E. coli O157 in persons who develop diarrhoea after a known exposure to the organism or to look at this isolation rate after diarrhoea-associated hemolytic uremic syndrome. These studies show that the rate of E. coli O157: H7 isolation from stool falls to 50% by 6-8 days after the onset of symptoms (Pai et. al., 1984; Spika et. al., 1986; Tarr et. al., 1990). In one study of post diarrhoea hemolytic uremic syndrome (Tarr et. al., 1990), the isolation rate was 100% for those stools collected within 2 days of the onset of diarrhoea. This dropped to 92% at 3-6-days and 33% after 6 days.

#### Epidemiological Investigations of Verocytotoxin-producing

***Escherichia coli* by Molecular Epidemiology**

The knowledge of the occurence and the transmission routes of E.coli O157:H7 are limited. Therefore, molecular epidemiology is an important tool in augmenting the knowledge of the behaviour of the bacteria and the possibility to control spread from animal to man (Aspan, Lohikari Eriksson and Gurarsson, 2002).

When faced with an outbreak in humans, two questions must be answered: -

1. How to trace the infection back to its sources and
2. How to compare EHEC from different sources (China et. al., 2001).

The different EHEC from different sources can be compared:

* + At the bacterial level using phenotypic assays such as complete serotyping, biotyping, hemolysin production, tellurite and antibiotic resistance profile.
  + At the molecular level on the whole bacterial cell by multi locus enzyme electrophoresis (MLEE), pulse-field gel electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD).
  + Compare their virulence-associated properties such as VT variants, the VT-encoding phages, the LEE (Locus of enterocyte effacement, the EHEC plasmids).

Typing of VTEC

Bacterial typing systems are described as those capable of identifying strains accurately and reproducibly at different times and in different laboratories. Fingerprinting methods are those suitable only for grouping or excluding isolates in small-scale or local studies of bacterial transmission. For outbreak, investigations screening methods are needed to provide rapid results at the level of field investigation. Further studies are frequently required to confirm and extend the results of those screening tests (Smith, Willshaw, and Cheast, 2002).

Several methods have been used for the typing and fingerprinting of VTEC. These methods can be broadly categorized as follows (Smith et. al., 2002) Phenotypic: Based on expressed properties such as somatic and flagella antigens (O: H serotyping); susceptibility to bacteriophages (phage-typing); resistance to antimicrobials (antimicrobial and heavy metal resistance tests); enzyme activity; enterohemolysin production.

Genotypic: Based on specific molecular characteristics of the chromosomal, plasmid or phage DNA such as VT tests and VT gene typing including DNA sequencing; plasmid DNA analysis; Genomic DNA; Restriction fragment length polymorphism (RFLP) including Ribotyping; Pulsed filed gel electrophoresis (PFGE); Polymerase chain reaction (PCR) – based techniques including amplified fragment length Polymorphism (AFLP); DNA sequencing; Attaching and effacing ability

Serotyping

This is based on the identification of the somatic “O” antigens and flagella “H” antigens (Lior, 1994). The scheme at present identifies O antigens 1 to 173 and H antigen 1 to 56. Results of serotyping have shown that VTEC associated with human disease belong to a large number of different sterotypes (Pierard, Steves, Moriau, Lior, and Lauwers, 1997; Beutin, Zimmerman, and Gleies, 1998; Caprioli & Tozzi, 1998).

Serotyping together with further characterization is very helpul in attempts to evaluate the role of non-O157 VTEC. Outbreaks have been caused by several of these sterotypes for example, O26:H11, O103:H2, O104:H21, O111: H-, O145: H- and O? H19 (Smith et. al., 2002). Another application of serotyping information is the development of specific tests. For example in Australia, magnetic beads were coated with an O111 antiserum for detection of E.coli O111 in the mettwurst- associated outbreak (Paton, RatCliff, and Doyle, 1996).

Phage typing for E.coli O157

The scheme was developed in Canada and published in 1987 (Ahmed, Bopp, Borczyk, and Kasatiya, 1987). The scheme uses 16 phages and now identifies more than 80 phage types. The use of phage typing has been very helpful in many epidemiological studies. However the majority of strains isolated in England and Wales fall into a small number of types such as phage type 2, so it is necessary to use the results of phage-typing in combination with other techniques (Smith et. al., 2002). This phage-typing scheme is not usually used for VTEC of serogroups other than O157 but can show whether other serotypes have a common phage lysis pattern (Strockbine, Wells, Bopp, and Barret, 1998).

Resistance to antimicrobial and heavy metals

Resistance to antimicrobial drugs is a useful epidemiological marker for VTEC (Farina, Goglio, Conedera, Minelli and Caprioli, 1996). Studies on O157 VTEC isolates in England and Wales in 1997 have shown that 23% were drug resistant but only 2% were resistant to four or more drugs. In combination with other methods, the patterns of resistance to antimicrobial and heavy metals may serve as useful epidemiological markers (Smith et. al., 2002).

Verocytotoxin typing

Verocytotoxins belong to a family with two major types VT 1 and VT 2 (Scotland & Smith, 1997). Variants of VT 1 and VT 2 have been reported with most variation seen in the VT2 gene. These variants can be detected by PCR (Pierard et. al., 1997), Oligonucleotide probes based on sequences within the B subunit gene or

by DNA sequencing (Smith et. al., 2002). For O157 VTEC the types of VT genes detected so far are VT1, VT2 and VT2c. In the case of non-O157 VTEC a wider range of VT genes has been identified and primers have been developed from VT 2/OX3a sequences in order to identify these sequences in non-O157 VTEC (Pierard et. al., 1997).

Plasmid DNA

This method is limited in its application to studies of VTEC and particularly E.coli O157. Plasmid analysis of O157 VTEC can sometimes be used to examine strains in outbreaks and sporadic cases of infection (Smith et. al., 2002). However, virtually all O157 VTEC strains carry a plasmid of about 90 KB and only a proportion of strains carry additional plasmids (Chapman et. al., 1993).

Genomic DNA

The restriction fragment length polymorphism (RFLP) method has been used in several outbreak investigations caused by O157 VTEC but can clearly be applied to non-O157 VTEC (Samadpour, 1995). It involves hybridization of genomic DNA digested with restriction enzymes with probes for vt genes. This allows identification of fragments carrying these genes. The use of each enzyme alone did not provide sufficient discrimination for use in epidemiological studies but with two enzymes, the combination of patterns provided high sensitivity. The use of Eco R 1 and PVUII and the two probes gave 83 patterns in a study of 165 isolates (Samadpour, 1995).

Restriction fragment length polymorphism analysis using a vt phage as probe

VT sequences are carried on lambdoid bacteriophages in many human strains of O157 VTEC. Labelled DNA of a VT 2 - encoding phage has been used as a probe in hybridization tests with genomic DNA. So far, this method has only been used to examine O157 VTEC (Willshaw et. al., 1994).

Restriction fragment length polymorphism analysis using phage λ as a probe

This approach was developed because of the observed homology between λ and the bacteriophage encoding VT production (Samadpouret. al., 1993). They used the technique to examine strains in a large O157 VTEC outbreak in the Western United States that occurred in 1993 and it was concluded that this method is useful for epidemiological analysis of O157 VTEC. Non-O157 VTEC can also be analysed by this method and the results showed considerable diversity (Smith et. al., 2002).

Ribotyping

Ribotyping has been widely used as a subtyping method for many different bacterial genera. Ribotyping systems have been developed for E.coli including some studies of VTEC. The conclusion from these studies was that ribotyping provided a useful method for discrimination of non-O157 VTEC (Smith et. al., 2002). Martin, Tyler, Tyler, Khakhira, and Johnson, 1996, published a Study of O157 VTEC strains. They evaluated ribotyping in comparison with phage typing, VT genotyping and PFGE. A total of 121 O157 VTEC strains was examined. The isolates belonged to different phage types, VT genotypes and could also be subdivided using PFGE.

However, with ribotyping 54 strains examined gave identical patterns. Hence, it was concluded that ribotyping was not useful for discriminating VTEC isolates.

Pulsed field gel electropheresis

In this technique, genomic DNA is digested with infrequently cutting restriction enzymes. PFGE was first reported for O157 VTEC by Bohm and Karch (1992) and since then a large number of reports have shown how the technique can be used for epidemiological investigations (Smith et. al., 2002). There are also several reports of subtyping of non-O157 VTEC belonging to O serogroups (Smith et. al., 2002). Several enzymes were evaluated and it was concluded that xba 1 provided the best discrimination and AvrII could be used to help to interpret minor differences found with xbaI.

PCR based techniques

Several PCR based methods have been evaluated as faster alternatives to PFGE (Tyler, Wong, Tyler, and Johnson, 1997). Examples are arbitrarily primed PCR (AP-PCR), Random Amplified Polymorphic DNA (RAPD) and the use of repetitive sequences (e.g. REP, ERIC). Tyler et. al., 1997 in a review of such methods considered that these techniques have significant limitations for “typing” but may be useful in local settings, for example, investigation of institutional outbreaks.

AFLP (Amplified Fragment Length Polymorphism)

There have been recent reports of studies of AFLP with VTEC of serogroup O157 and non-O157 (Chen, Yee, and Weller, 1997; Zhao, Mitchell, and Mong, 1997).

Preliminary results suggested AFLP has the potential to provide a high degree of discrimination of VTEC strains (Chen et. al., 1997). Among 42 non-O157 VTEC of human and animal origin, AFLP patterns differed between serotypes and strains within a serotype isolated from the same location had the same unique pattern. For two of the serotypes, strains were distinguishable by AFLP but not by PFGE (Chen et. al., 1997).

The Use of Virulence Properties of VTEC other than VT Production in the Characterization of VTEC Strains

The ability of certain VTEC, including all E.coli O157 VTEC, to adhere intimately to intestinal epithelium, resulting in the effacement of the microvillius surface is considered an important virulence property of these orgamisms. The locus of enterocyte effacement (LEE region) encodes the properties required for this attaching and effacing (AE) ability (McDaniel, Jarvis, Donnenberg and Kaper, 1995). The eae gene is used as a probe to examine VTEC. It encodes a 94KDa protein termed intimin. eae gene is present in all O157 VTEC and certain other VTEC (Pierard et. al., 1997). VTEC can be tested for the presence of eae and other genes in the LEE region and the eae genes can be subdivided using specific primers (Abu- Bobie et. al., 1998). There is also heterogeneity in the point of insertion of the LEE region in different serogroups (Weiler, McDonald, Whittam, and Kaper, 1997).

Another property associated with some VTEC is that of haemolysin production. O157 VTEC and some VTEC strains of other serogroups produce a novel type of haemolysin termed enterohaemolysin (Pierard et. al., 1997). The gene encoding this property can be detected with DNA probe CVD4/9 or by PCR.

The methods for typing O157 VTEC are well developed but require standardization on an International basis. For non-O157, VTEC Serotyping provides an internationally recognized and standardized scheme that has been demonstrated to be very helpful in the characterization of such strains (Smith et. al., 2002). However, it is limited in application because the full scheme is only employed in certain reference centers (Smith et. al., 2002).

Several studies of non-O157 VTEC have now shown the value of tests for virulence factors such as attaching and effacing ability and the production of enterohaemolysin. Use of DNA probes or PCR for the detection of eae or E–hly provides a practical means of testing although some strains do not express the genes detected (Smith et. al., 2002).

At present, a combination of methods appears to provide the most informative approach to the typing of VTEC. For O157 VTEC the combination of phage typing, VT typing (including VT 2 gene subtyping) and PFGE provides suitable methods applicable to epidemiological investigations. For non-O157 VTEC, the use of serotyping, VT gene analysis and tests for virulence factors has proved very helpful. For analysis that is more detailed these tests can be complemented by ribotyping or PFGE (Smith et. al., 2002).

#### Pathogenesis

Enterohaemorrhagic Escherichia coli has a novel mechanism of pathogenesis involving the expression of adhesion factors called intimins, and toxins such as shiga-like toxins (SLT’S, Verocytotoxin) and EHEC-haemolysin (Karch, 1996). The organisms apparently colonize the intestine after being ingested in contaminated

food or transmitted by direct person-to-person contact and they produce at least three different virulence properties as noted above (Zimmerhackl, 2000):

* + - 1. Intimin – a protein necessary for their attachment to the intestinal wall.
      2. Hemolysin – a protein that affects the growth of other bacteria and that may also hemolyse human cells.
      3. Shiga toxin (also referred to as verotoxin and shiga-like toxin) that induces micro-vascular damage, hemorrhagic colitis and the hemolytic uremic syndrome (Rondeau & Peraldi, 1996).

Like all diarhoeagenic E.coli strains, EHEC need first to colonize the intestinal musosa. Intimin an outer membrane protein encoded by eae is responsible for the tight association of pathogen with the host cell (Oswald et. al., 2002). They produce a characteristic histopathological feature known as the “attaching and effacing” (A/E) lesion by subverting intestinal epithelial cell function (Frankel et. al., 1998). This striking phenotype is characterized by effacement of microvilli and intimate adherence between the bacteria and the epithelial cell function (Moon, Whipp, Argenzio, Lewis, and Gianella, 1998). Marked cytoskeletal changes including accumulation of polymerized actin, are seen directly beneath the adherent bacteria (Knutton, Baldwin, Williams and McNeigh, 1989).

A/E lesion formation is governed by a pathogenicity island called the locus of enterocyte effacement (LEE). The LEE was first described in EPEC E2348/69 (McDaniel et. al., 1995) but is also present in EPEC and EHEC strains and in other bacterial species such as Hafnia alvei and Citrobacter rodatium (formerly C. freundi biotype 4280). The LEE from EPEC E 2348/69 contains 41 genes organized into three major regions with known function (Elliot et. al., 1998). Similar organization

was observed in the LEE from EHEC O157:H7 strain EDL 933 which presents 13 additional genes belonging to a putative p4 family prophages (Perma et. al., 1998). The central region contains the eae (for E.coli attachment effacement) gene encoding a 94 to 97 KDa outer membrane protein known as intimin (Jerse, Yu, Tall, and Kaper, 1990). This protein mediates close contact between the bacteria and the target cell, following interaction with its Translocated receptor Tir (Translocated intimin receptor) the gene for which is located upstream of eae. Tir has been identified initially as a 90 KDa tyrosine phosphorylated protein from the target cell membrane and was called Hp 30. The role of intimin in human disease was demonstrated by studies in human volunteers, who ingested an isogenic eae null mutant of EPEC E 2348/69 (Donnenberg et. al., 1993a). Using animal models, intimin was shown to be similarly required for EHEC O157:H7 to intensively colonize the intestine and cause diarrhoea and A/E lesions in piglets (Donnenberg et. al., 1993b). The presence of four distinct intimin subtypes intimin α, intimin β, intimin γ and intimin δ has been described (Adu-Bobie et. al., 1998). Oswald et. al., 2002 described the nucleotide sequence of a fifth intimin type ε.

Other virulence characteristics

The cardinal virulence trait of E.coli O157:H7 has been considered for its ability to produce stx 1, stx 2 or both. Almost all strains from North America have gene that encodes stx 2; approximately two thirds have a gene that encodes stx 1. Clinical isolates that contain the gene encoding stx 1 but lack the gene encoding stx 2 or that do not produce stx 2 are rare (Tarr & Neill, 2001). The shiga toxin genes are contained on separate bacteriophages (Strockbine et. al., 1986).

E. coli O157:H7 have the ability to produce the attaching and effacing (AE) lesion on epithelial cells to which they adhere, by way of a bacterial surface molecule called intimin, encoded by eae. The AE lesion is similar to the lesion induced by enteropathogenic E.coli and in gnotobiotic pig model of E.coli O157:H7 infection, eae is important for causing intestinal lesions. E.coli O157:H7 with an intact eae are better able to induce neurologic lesions in gnotobiotic piglets than E.coli O157:H7 in which eae has been disrupted. It is plausible that the attaching and effacing lesion permits increased intestinal absorption of toxin in these infected animals (Tarr & Neill, 2001).

Additional, putative virulence characteristics of E.coli O157:H7 are being, reported at an accelerating rate. The 92 KB pair plasmid common to all E.coli O157:H7 contains open reading frames encoding several molecules that are plausible virulence factors, including enterohaemolysins, related to other E. coli haemolysins, which is itself a cytotoxin, and a homologue of cytotoxin B of Clostridium difficille (Tarr & Neill, 2001). The bacteriophage that contains the gene that encodes stx 2 also contains an open reading frame encoding a putative serine/threonine kinase. A novel adherence-conferring molecule, Iha, has been described, suggesting that there are intimin-independent adherence mechanisms in E.coli O157:H7 (Siegler, Griffin, Barrette, and Strockbine, 1993). It is hoped that knowledge from the entire sequence of E.coli O157:H7 will shed light on the mechanisms that distinguish the virulence of E.coli O157:H7 from many other E.coli that contain stx genes that are not pathogen or those that are considerably less pathogenic (Tarr & Neill, 1996).

Mechanism of action

The mechanism by which E.coli O157:H7 causes diarrhoea and the haemolytic uremic syndrome are not completely understood (Boyce et. al., 1995). The organisms adhere to mucosal surfaces and produce these shiga-like toxins, which may act both locally and systemically on the gut mucosa. The toxins bind specifically through their B-subunits to glycolipid receptors identified as ceramide trihexoside (globotriogylceramide) (Wadell, Cohen, and Lingwood, 1990). The toxins then enter the cell through clathrin-coated pits and their A subunits are dissociated from their B subunits and proteolytically cleaved. The activated A subunit inhibit protein synthesis by inactivating ribosomal subunits, which block the elongation of peptides and leads to cell death (Rondeau & Peraldi, 1996).

The shiga toxins can initiate apoptosis in endothelial and epithelial cells in animals. The syndrome in these animals does not completely resemble the hemolytic uremic syndrome in children, but in baboons, parenteral administration of shiga toxin results in endothelial and epithelial cell damage in the intestine that is similar to the damage that occurs in patients with the hemolytic uremic syndrome. In studies involving the intravenous injection of shiga toxin 1 in rabbits, Zoja, Corn and Farina, 1992, demonstrated that micro vascular lesions appeared in the central nervous system, colon and lungs where endothelial cells express shiga-toxin receptors. Unfortunately, endothelial cells of the rabbit kidney do not express ceramide trihexoside, so they cannot be used as a model for the haemolytic uraemic syndrome.

Conversely, Obrig et. al., 1993, showed that in human cells, the basal levels of ceramide trihexoside were approximately 50 times higher in renal micro vascular

endothelial cells than in umbilical vein endothelial cells in culture. They also showed that 1 ppm shiga toxin reduced protein synthesis and the viability of renal endothelial cells by 50%, whereas umbilical vein cells were not even affected by much larger concentration (> 1nm) (Obrig et. al., 1993). Glomerular endothelial cells from infants (children less than two years old) but not from adults have recently been reported to express shiga toxin receptors, suggesting that the greater frequency of the hemolytic uremic syndrome in children could be related to glomerular expression of ceramide trihexoside early in life (Lingwood, 1994).

The sequence of events that follows the injury of renal endothelial cells by shiga toxin in vivo is not well understood, mainly because of the lack of an animal model of the hemolytic uremic syndrome. Pathological studies of renal biopsy specimens from patients with the syndrome have provided diagnostic and prognostic information (Rondeau & Peraldi, 1996). These studies have also revealed that platelets accumulate in this disorder, because of their adhesion to the subendothelium and activated endothelial cells and that fibrin is deposited indicating local generation of thrombin. There is indirect evidence of the activation of the functional thrombin receptor of renal endothelial cells (Rondeau & Peraldi, 1996). In addition, up-regulation of plasminogen–activator inhibitor type 1 and the receptor of urokinase type plasminogen activator within the renal capillaries indicates an activated phenotype of endothelial cells during the repair process (Rondeau & Peraldi, 1996). Indeed, local fibrinolysis and regeneration of endothelial cells usually lead to complete or partial recovery of renal function after shiga-toxin-induced hemolytic uremic syndrome. However, precise control of hypertension is essential to

prevent secondary vascular lesions induced by sheer stress (Rondeau & Peraldi, 1996).

The case reported by Tarr et. al., 1996 in which the hemolytic uremic syndrome occurred after a urinary tract infection and a few previous reports show that shiga toxin can enter the circulation through the inflammed urinary mucosa or pyelonephritic lesion, even if the strain responsible does not have the urovirulence traits usually expressed by E.coli strains that cause pyelonephritis. This route of entry would have been more firmly established if stool analysis had been negative for shiga toxin, thereby, excluding the possibility, of a simultaneous intestinal infection with serotype O103:H2, O157:H7 or both.

#### Pathophysiology

Apart from the advances made in identifying EHEC showing the likely involvement of SLT’s, little progress has been made in understanding the pathophysiological origins of the predominant clinical features, including the dramatic haemotological and renal changes. Haemodynamic alterations are probably responsible for severe colonic tenesmus, suggested by the earlier term “ischemic colitis” as well as for the acute renal failures and central nervous system disturbances (Karch, 1996). Increased urinary excretions of endothelin and lipid mediators, including platelet activating factors and prostaglandins, has been reported but it is not known whether these agents are the cause or effect of the pathophysiological changes (Karch, 1996).

The histologic pattern of human colonic injury caused by E.coli O157:H7 infection is similar to the pattern that characterized Clostridium difficile colitis, which

is caused by a locally acting toxin (Griffin, Olmstead, and Petras, 1990). This similarity suggests that shiga-like toxin plays a part in colonic injury (Boyce et. al., 1995). Colonic vascular damage by shiga-like toxin may allow lipopolysaccharide and other inflammatory mediators to gain access to the circulation thus initiating the hemolytic uremic syndrome (Boyce et. al., 1995). This possibility is supported by the finding that among persons with E.coli O157:H7 infection, the hemolytic uremic syndrome appear to be more likely to develop in those with bloody diarrhea than in those with non-bloody stools (Carter et. al., 1987).

#### Immunity

Patients with E.coli O157: H7-associated HUS have a brisk and early antibody- response to the O157 lipopolysacharide antigen (Chart et. al., 1991). This is useful in establishing the cause of an episode of HUS or of hemorrhagic colitis if culture confirmation of E.coli O157:H7 infection has not been obtained. An episode of E.coli O157:H7 infection does not provide lasting immunity to subsequent infection. There have been reports of a second infection in the same individual (Tarr & Neil, 2001).

So far, the only potential E.coli O157:H7 adherence factor that has been demonstrated to play a role in the intestinal colonization in an animal model is the intimin encoded by the eae gene. It is also similarly required for EHEC O157:H7 to intensively colonize the intestines, cause diarrhoea, and A/E lessions in calves and to cause colonic edema and A/E lesions in piglets (Donnenberg et. al., 1993b). These results suggest that anti-intimin vaccines might interfere with EHEC infections. Such vaccines could help reduce the levels of ETEC in cattle and thus reduce the number of EHEC infection in humans. However, additional research is needed if an intimin-

based broad-spectrum vaccine against A/E - positive bacterial pathogens is sought because firstly there are many EHEC carried by domestic animals of a variety of serotypes and a great variety of non-O157 serotypes have been reported from human disease around the world. Removal of E.coli O157 producing intimin γ, without tackling the problem of the non-O157 serogroups producing other intimin subtypes would create a greater niche for these and probably make the problem worse. Secondly, lack of correlation between levels of intimin antibodies in serum and disease severity does not support the hypothesis that an immune response to intimin provides protection against subsequent disease (Donnenberg et. al., 1998). Thirdly, the existence of intestinal adherence factors distinct from intimin is suggested by the isolation of stx–producing E.coli strains of serotypes other than O157:H7 that lack the eae gene but are still associated with bloody diarrhoea or HUS in humans (Morabito et. al., 1998).

Voravuthikunchai, Chaowana, Perepat, Lida, and Honda (2005) conducted a study in Thailand to determine whether the presence of a humoral immune response to O157 lipopolosaccharide (LPS) could be a reason for the lack of disease associated with infection by these strains in people with diarrhoea in that country. An IgM response to O157 LPS was found in 12% of 332 serum samples obtained from healthy blood donors and patients with diseases unrelated to diarrhoea. In the case of an IgG response, 23% of these same samples showed a positive response to O157 LPS (Voravuthikunchai et. al, 2005). Similar findings have been reported in Mexico by Navarro et. al. (2003). In the same study, Navarro et. al. (2003), reported that rabbit antiserum raised against O6, O114, and O157 LPSs has a homologous and a heterologous bactericidal capacity against strains with these LPSs, and that

serum samples obtained from herds of cattle in different parts of Mexico showed a similar response to that found in humans, which explains why the local animals are seldom shown to be colonized by O157:H7 strains (Navarro et. al., 2004; Navarro et. al., 2005).

#### Clinical Manifestations

Enterohaemorrhagic Escherichia coli (EHEC) causes a variety of clinical conditions ranging from near asymptomatic carriage of the organism with or without mild abdominal symptoms to severe haemorrhagic colitis and haemolytic uraemic syndrome (HUS) (Karch, 1996). The organism can cause asymptomatic infection, non-bloody diarrhoea, bloody diarrhoea (haemorrhagic colitis), the haemolytic uraemic syndrome, thrombocytopenic purpura and death (Griffin et. al., 1988).

The illness typically begins with severe abdominal cramps and non-bloody watery diarrhoea, which may become grossly bloody by the 2nd or 3rd day of illness. About half the patients have nausea and vomiting (Boyce et. al., 1995). Other infectious causes of diarrhoea must be considered in making the diagnosis. Unlike most bacterial enteric diseases, E.coli O157:H7 infection is usually characterized by low-grade fever or the absence of fever (Griffin, 1995). This feature, may lead clinicians to suspect non-infectious causes such as inflammatory bowel disease, ischemic colitis, or in children intussusceptions. Because the abdominal pain and tenderness may be severe, appendicitis or another acute conditions requiring surgery may be the initial diagnosis, leading to exploratory laparatomy (Boyce et. al., 1995).

Stool from patients with E.coli O157:H7 infection are sometimes described as “all blood and no stool” imitating gastrointestinal haemorrhage. Alternatively, the diarrhoea may be minimally streaked with blood or non-bloody. In reported outbreaks, the frequency of bloody diarrhoea has ranged from 35% – 90% (Swerdlow et. al., 1992; Bel et. al., 1994). Thus, although bloody stools are common with E.coli O157:H7 infection, the diagnosis may be considered in patients with non- bloody diarrhoea as well.

Edema and submucosal haemorrhage in the ascending and transverse colon may be demonstrated by a thumb-printing pattern on barium enema examination (Riley et. al., 1983). At endoscopy, the colonic mucosa appears edematous and hyperemic, sometimes with superficial ulcerations. Pathological findings include infectious or ischemic patterns of colony injury, usually in a patchy distribution and sometimes with fibrin micro-thrombin (Griffin et. al., 1990). Pseudo-membranous colitis may be present making it difficult to distinguish between Clostridium difficile pseudomembranous colitis and infection with E.coli O157:H7 (Griffin et. al., 1990).

Symptoms of infection with E.coli O157:H7 usually subside in about one week, with no obvious sequelae. However, the haemolytic uraemic syndrome develops in about 6% of patients (Griffin, 1995) and is usually diagnosed 2 - 14 days after the onset of diarrhea (Karmali et. al., 1985).

#### Complications of Infection with Enterohaemorrhagic *E. coli*

Two major sequelae of infection with EHEC are known: -

Haemolytic uraemic syndrome (HUS): - This syndrome is characterized by

* Haemolytic anaemia: - Haematocrit less than 30% with evidence of microangiopathy on peripheral blood smear.
* Thrombocytopaenia: - Platelet count less than 150,000 platelets/mm3
* Renal Insufficiency: - Creatinine greater than upper limit of age for normal.

It occurs in about 6.5 (±2.8 SD) days after the onset of diarrhoea with day 1 of illness being considered the first day of diarrhoea.

There are at least four major causes of the syndrome: -

* Bacterial and viral infections: - which is responsible for the epidemic form.
* Hereditary Factors: - which are responsible for familial forms in particular recurrent or atypical haemolytic uraemic syndrome.
* Systemic Conditions: - such as systemic lupus erythematosus, cancer, transplant rejection, glomerulonephritis and pregnancy.
* Exposure to toxins or potential toxins such as cyclosporin, tacrolimus, mitomycin and radiation (Zimmerhackl, 2000).

Among children, the chief cause of the haemolytic uremic syndrome is infection with enterohaemorhagic strains of Escherichia coli. Originally, a variety of serotypes was described as causing the syndrome (Zimmerhackl et. al., 2000). Though most cases of post-diarrhoeal hemolytic uremic syndrome among children in the Northern Hemisphere have been caused by E.coli O157:H7, there is concern about the emergence of cases associated with infections with other strains of E. coli that are more difficult to detect (Zimmerhackl et. al., 2000). Predictors of the severity of the haemolytic uraemic syndrome include an elevated white-cell count, a severe

gastrointestinal prodrome, anuria, early in the course of illness, and an age less than two years (Boyce et. al., 1995).

The extra renal complications usually associated with severe HUS involve the central nervous system (seizure, coma), the cardiovascular system (hypertension, myocarditis), the colon and rectum (gangrenous necrosis or colonic perforation), as well as exocrine or endocrine functions of the pancreas (e.g. diabetes mellitus) (Karch, 1996). The mortality rate is 3% to 5% and about 5% of patients who survive have severe sequelae such as end-stage renal disease or permanent neurologic injury (Boyce et. al., 1995). Recent reports have highlighted the multi system nature of the syndrome (Boyce et. al., 1995).

Thrombotic thrombocytopenic purpura (TTP)

TTP has the clinical features of the haemolytic uraemic syndrome, although the renal injury is typically less severe and neurologic involvement is often more predominant with HUS (Boyce et. al., 1995). Few cases are preceded by a diarrhoeal prodrome. However, post-diarrhoeal thrombocytopenic purpura is probably the same disorder as the haemolytic uraemic syndrome (Boyce et. al., 1995). Differentiation of EHEC-associated HUS and thrombotic thrombocytopenic purpura (TTP, Moschwitz’s syndrome) based on clinical symptoms alone may be impossible. The similarity of the major clinical and pathogical features may indicate a common-host-derived mediator, the expression of which can be triggered by different causes (Karch, 1996). However, not all deaths of patients with E.coli O157:H7 infection are due to haemolytic uraemic syndrome. In reports on US outbreaks, seven of 19 patients who died did not have the haemolytic uraemic

syndrome or thrombocytopenic purpura. All seven were elderly (Boyce et. al., 1995).

Other post-infection medical complications

There are 2 important surgically treatable complications of E.coli O157:H7 and of HUS that usually become apparent in the post - HUS phase:

Biliary lithiasis: This occurs in 10% of children with HUS. It is presumed that the pigment load during the acute episode of hemolysis causes biliary sludging that progress to gall stones.

Colonic strictures: This can become manifest after HUS as abdominal pain and bloating. They often become apparent during the convalescent phase of HUS or within several weeks of discharge from the hospital (Tarr & Neil, 2001).

Other complications

Children and adults infected with E.coli O157:H7 can also have other surgically remediable complications, without HUS or preceding HUS including appendicitis and intussusception (Tarr & Neil, 2001). Careful and repeated abdominal evaluations are needed to ensure that these complications are not developing (Tarr & Neil, 2001).

#### Risk Factors that Determine the outcome of EHEC infections

Variability in the severity of infections has been noticed in outbreaks (Boyce et. al., 1995). Other risk factors, apart from age distribution also determine the outcome of EHEC infections (Karch, 1996).

Bacterial virulence factors seem unlikely, since all disease forms have been observed during a single outbreak involving the same strains. The relevance of potential host factors, such as the expression of Gb3 (pk blood group antigen) in target tissues (Lingwood, 1994) has still to be proven. The observation that markedly different clinical manifestations may even occur in monozygotic twins, make it unlikely that genetic factors play an important role (Karch, 1996). Thus, the question is whether it is simply the load of bacteria and/or shiga-like toxins, which determine the severity of EHEC diseases, or do unknown host factors have a decisive influence (Karch, 1996).

As early as 1986, Karch and coworkers performed an elegant in vitro experiment. They added trimethoprin-sulfamethoxazole to cultures of E.coli O157:H7 and found that these drugs increased the release of shiga toxins by the bacteria. The findings have been extended to other enterohaemorrhagic strains of E.coli and other antibiotics (Zimmerhackl et. al., 2000). The findings have also raised the possibility that antibiotic treatment of E.coli infections might actually increase the risk of the hemolytic uremic syndrome. Wong et. al. (2000) provided data that validates this concern. Specifically, children who received antibiotics (trimethoprim- sulfamethoxazole or beta-lactam) for diarrhoea caused by E.coli O157:H7 had a significantly higher risk of the haemolytic uremic syndrome than those who did not receive antibiotics (Wong, Jelacic, Habeeb, Watkins, and Tarr, 2000).

Bacteria might release preformed shiga toxins in response to injury (Wilshaw et. al., 1994). Treatment with antibiotics might give E.coli O157:H7 a selective advantage if these organisms are not as readily eliminated by this treatment as are the normal intestinal flora. Antibiotics could interfere with the metabolism of

enterohaemorrhagic E.coli. Several antimicrobial agents particularly, the quinolones, trimethoprim and furazolidone, are potent inducers of the expression of the shiga toxin 2 gene. Such an action could increase the level of toxin in the intestine and therefore, increase the amount of toxin absorbed into the circulation, thus increasing the risk of the haemolytic uremic syndrome (Zimmerhackl, 2000). Only estimates of the risk of patients with enterohaemorrhagic E.coli infections developing the haemolytic uremic syndrome are available (Zimmerhackl, 2000).

In their prospective study, Wong et. al. (2000), reported that the syndrome developed in 10 of the 71 patients (14%) studied. Zimmerhackl (2000) also reported a similar figure. Therefore, other factors in addition to the use of antibiotics must be involved. A deficiency of factor H and mutations in the factor H gene appear to increase the risk. Whether changes in the Von Willebrand factor (specifically, the presence of unusually large multimers of the factor or a deficiency of von-willebrand factor-cleaving protease as found in patients with thrombotic thrombocytopenic purpura are important is remaining to be seen (Zimmerhackl, 2000).

In conclusion, many factors influence the transition from a gastrointestinal infection with E.coli O157:H7 to the haemolytic uremic syndrome. The data of Wong et. al. (2000) support the theory that antibiotics have an important role to play in this progression. It remains to be determined whether the syndrome can be prevented by reductions in the absorption of toxins with receptor antagonists or toxin binders or non-specific antibiotics (Zimmerhackl, 2000).

#### Diagnosis

Clinical diagnosis

The diagnosis of E.coli O157:H7 infections should be considered in any patient with bloody stools or the hemolytic uremic syndrome (Boyce et. al., 1995). The diagnosis should also be considered in persons with non-bloody diarrhoea who may have been exposed to the organism – for example by consuming under cooked ground beef or unpasteurized milk. During an outbreak, physicians should be particularly alert to the possibility of milder cases (Boyce et. al., 1995).

Clinical presentation

The prototypical illness with E.coli O157:H7 consists of 24 to 48 hours of non- bloody diarrhoea that converts to frankly bloody stools (Ostroff et. al., 1989). The illness typically begins with severe abdominal cramps and non-bloody watery diarrhoea, which may become bloody by the 2nd or 3rd day of illness (Boyce et. al., 1995).

E.coli O157:H7 infections frequently can be distinguished by the history from inflammatory bowel diseases (Tarr & Neill, 2001). Most notably, E.coli O157:H7 infections present abruptly, usually in previously healthy hosts, with symptoms that have lasted no more than one week. Inflammatory bowel disease, in contrast, usually has a longer interval between the first symptom and the presentation (Schumacher, Ljungli and Sandstedt, 1995).

Prediarrhoeal herald signs of E.coli O157:H7 include a self-limited fever, abdominal pain, irritability, fatigue, headache, myalgias, and confusion. Vomiting can occur at any stage of the illness, and faecal incontinence can return to a child who has been toilet trained. Abdominal pain frequently is intense and is disproportionate

to findings on physical examination. The pain usually is spasmodic and intermittent in nature. Fever is reported by about one third of patients, but rarely is present when patients present to care, usually soon after the diarrhea becomes bloody (Tarr & Neill, 2001). The absence of a fever at the time of presentation tends to distinguish E.coli O157:H7 infections from other bacterial diarrheas (Wong et. al., 2001; Tarr & Neill, 2001).

The clinical presentations of EHEC infections is remarkably age dependent, severe colitis, reminiscent of flare ups of ulcerative colitis, or dysentery is the major disease phenotype observed in adults whereas children present more frequently with an uncharacteristic diarrhoea (Riley et. al., 1983).

Differential diagnosis

The intensity of the abdominal pain, lack of fever at the time of initial evaluation, and the colonic bleeding have led to mistaken diagnosis, usually of a non-infectious cause, in patients infected with E.coli O157:H7. In children, these considerations may include intussusceptions, inflammatory colitis, or, sometimes, appendicitis (Tarr & Neill, 2001). In adults, the most common mistake is the failure to recognize that diverticulus, cancer, hemorrhoids, ischemic colitis or bowel infarction does not cause colonic bleeding.

In either group, lack of consideration of an infectious cause might result in stool culture not being done. Delay in obtaining the stool sample or in requesting that E.coli O157:H7 be sought delays accurate diagnosis, subjects the patients to unnecessary and potentially risky diagnostic procedures, and allows for person-to- person spread of infection from an index case (Tarr & Neill, 2001).

Laboratory diagnosis Laboratory considerations

The timely performance of a complete stool culture is the most important component of diagnostic evaluation of acute bloody diarrhoea (Tarr & Neill, 2001). Rapid non-culture-based tests are available for the identification of E.coli O157:H7 in stool. Such tests are perceived to be less expensive. Although, these tests are specific and a positive result should be taken seriously, they lack sensitivity and cannot be used to replace a stool culture. Isolation of the organism after a positive rapid test is important to facilitate DNA finger printing by public health laboratories. Faecal leukocytes are present variably in E.coli O157:H7 infections; their presence or absence is an irrelevant diagnostic considerations (Tarr & Neill, 2001).

Isolation techniques

Since the recognition of E.coli O157 as an important human pathogen, a large number of methods have been designed specifically for the isolation of this serotype in clinical, food, animal and environmental specimens (Desmachelier et. al., 1998). Selective agars and enrichment broths are available. The selectivity is based on the specific phenotypic characteristics of most E.coli O157:H7 strains namely, lack of sorbitol fermentation, failure to produce β-glucuronidase, and resistance to antibiotic and other inhibitory agents, such as tellurite (Armstrong et. al., 1996). Immunologically based assays have been developed for the detection of STEC by detection of shiga toxin production and more specifically, for the detection of E.coli O157 based on detection of O157 LPS antigen. With E.coli O157 enzyme-linked immunosorbent assay (ELISA) kits, few cells can be detected following enrichment in

selective medium and can be isolated by immunomagnetic separation (Desmachelier

et. al., 1998).

An alternative approach is the detection of genes characteristic of enterohaemorrhagic E.coli (EHEC) or specific to the serogroup. Hybridization with DNA probes or amplification of specific gene fragments by PCR has been successfully used to detect virulence factors of EHEC such as the stx, eae and ehly genes.

Genes more specific for the O157 serotype have been identified. E.coli O157:H7 has been identified using a DNA probe and by PCR, both of which targeted a unique base substitution in the uid A gene encoding production of the enzyme β- glucuronidase. Also, a PCR which identifies a fragment of a gene encoding an outer membrane protein of E.coli O157:H7 and O55 has been designed (Desmachelier et. al., 1998).

Because of the potential clinical severity of infection due to E.coli O157, a rapid

response is required in outbreak investigations and case management. There is a need for sensitive, specific, and rapid method, which will alert the clinician and the microbiologist to the presence of E.coli O157 in clinical and other specimens. In addition, there is need for a sensitive, rapid and specific technique to identify pathogenic E.coli O157 in polymicrobial substances such as food and water, in which the number of pathogenic organisms may be low. The isolation and identification of E.coli O157 finally depend on the confirmation of E.coli and identification of the O157 antigen.

Desmachelier et.. al. (1998), therefore, designed a PCR specific for E.coli O157 by using chromosomal sequences that encode the enzymes necessary for the biosynthesis of the O157 lipopolysaccharide. The PCR was subsequently evaluated

by the analysis of milk, thus, the O157 rbf PCR was found to be a sensitive, specific, and rapid method for the confirmation of the O157 serotype and has potential as a screening test for evidence of the presence of E.coli O157 in faecal, food and environmental samples (Desmachelier et. al., 1998).

Isolation procedures for STEC

All stools submitted for culture of bacterial enteric pathogen should also be examined for O157 STEC (Guerrant et. al., 2001). Culture for non-O157 STEC is indicated for patients with HUS, bloody diarrhoea, or a history of bloody diarrhoea (Guerrant et. al., 2001), and should be considered for other patients with diarrhoea.

Since there is no selective isolation medium for non-O157 STEC, testing for shiga toxin in the stool is the best option for the laboratory to detect these organisms. Commercial enzyme linked immunosorbent assay is a sensitive means of detecting shiga toxin. Isolation and serotyping of STEC from faecal specimens that are positive by Enzyme Immunoassay (EIA) should always be attempted because serotype information is important for public health purposes and may help in clinical decisions (Bopp et. al., 2003).

Enrichment

The most widely accepted laboratory methods for E.coli O157 include: Enrichment protocols using modified trypticase soybroth (mTSB) containing novobiocin (Doyle & Schoenii, 1987) or Buffered peptone water supplemented with vancomycin, cefsulodime and cefixime (BPW-VCC) (Chapman et. al., 1994).

Plating from broth cultures, directly or following the application of Immunomagnetic separation (IMS) techniques (Wright et. al., 1994) is usually onto Sorbitol MacConkey agar (SMAC) with or without the addition of cefixime and tellurite (CT-SMAC) (Zadik et. al., 1993).

Immunomagnetic separation enhances the detection of O157 STEC from patients with HUS, patients presenting a longtime after the onset of illness, asymptomatic carriers or specimens that have been stored or transported improperly (Chapman & Siddons, 1996; Bopp et. al., 2003). IMS beads for O157, O11 and O26 are available commercially or laboratories may produce beads with other O-specific antibodies (Bopp et. al., 2003).

Plating media

Verocytotoxin-producing E.coli O157:H7 do not ferment sorbitol unlike 80% - 90% of human faecal flora and most other serogroup of E.coli. As a result, sorbitol Macconkey (SMAC) agar has proved useful for the isolation (March & Ratnam, 1986).

The formulation of Sorbitol MacConkey agar is identical to Macconkey Agar No. 3 except that lactose has been replaced with sorbitol. E. Coli O157 does not ferment sorbitol and, therefore, produces colorless colonies. In contrast, most E.coli strains ferment sorbitol and form pink colonies on SMAC. The colorless, sorbitol-negative colonies can then be assayed for the O157 antigen with the use of commercially available antiserum. E.coli O157:H7 was found to have a sensitivity of 100% and a specificity of 85% on SMAC (March & Ratnam, 1986). They recommend the medium as a simple inexpensive, rapid and reliable means of screening E.coli O157.

Improvements to SMAC medium have resulted in increased sensitivity for isolation of E.coli O157 strains from faecal samples (Chapman et. al., 1993; Zadik, Chapman, and Siddons, 1993). They added cefixime and potassuim tellurite to SMAC to improve the selectivity of the medium. The addition of potassuim tellurite selects serogroup O157 from other E.coli serogroups and inhibits Providencia spp and Aeromonas spp. Cefixime is inhibitory to Proteus spp. Rhamnose was also added to SMAC to enhance the discrimination of O157 STEC strains, which do not ferment Rhamnose (Zadik et. al., 1993).

Cefixime-tellurite is mostly used because of its sensitivity. However, it has been reported, that a few O157: NM strains fail to grow on CT-SMAC. Cefixime-Rhamnose is more expensive to prepare and therefore, is not as widely used as CT-SMAC.

Screening procedures for STEC strains

For the isolation of O157 STEC from SMAC, colorless (non-fermenting) colonies are tested with O157 antiserum or latex reagent (Bopp et. al., 2003). The MUG reaction (4-methylumbelliferyl-beta-D-glucuronide for detection of beta- glucuronidase activity), used in conjunction with sorbitol fermentation and O157 antiserum is helpful in screening for these strains from human specimens (Scotland, Cheasty, Thomas, and Bowe, 1991). MUG-positive, urease-positive O157 STEC strains have been isolated in the United States but are still rare (Bopp et. al., 2003).

For the recovery of STEC from stool specimens that tested positive for shiga toxins, SMAC should preferably be innoculated. If sorbitol-non-fermenting colonies are negative with O157 latex, then sorbitol-fermenting colonies and a representative sample of sorbitol-non-fermenting colonies may be selected for shiga toxin testing

(Bopp et. al., 2003). This is because most non-O157 STEC strains ferment sorbitol. Latex reagents and antisera for detecting certain non-O157 STEC serotypes are now available and could also be used to test colonies from shiga toxin-positive specimens or to serogroup shiga toxin positive isolates (Bopp et. al., 2003).

Virtually all O157 STEC and 60% to 80% of non-O157 STEC produce a characteristic E.coli haemolysin, referred to as enterohaemolysin (Ehly), which is distinct from the alpha-haemolysin produced by other E.coli strains (Beutin et. al., 1989). Washed sheep Blood agar supplemented with calcium (WSBA-Ca), is used as a differential medium for the detection of enterohaemolytic activity (Beutin et. al., 1989). Ehly-producing colonies can be differentiated from alpha haemolysin- producing colonies on WSBA-Ca because the latter are visible after 3 - 4 hours of incubation. After 3 - 4 hours, colonies are marked for the appearance of alpha- haemolysin and the plates are examined again after 18 - 24 hours (Bopp et. al., 2003). Incorporation of mitomyin C into the WSBA-Ca enhances the appearance of the Ehly haemolysis and increases the proportion of non-O157 STEC strains that exhibit this activity. Because many non-O157 STEC strains do not demonstrate the enterohaemolytic phenotype and because enterohaemolytic non-toxgenic strain have been reported, additional screening methods should be used in conjunction with WSBA-Ca medium (Bopp et. al., 2003). Presumptive STEC isolates should, therefore, be sent to a reference laboratory or a public health laboratory for further characterization (Bopp et. al., 2003).

Identification Biochemical identification

The basic microbiologic characteristics of E.coli O157:H7 do not differ from those of other E.coli. On standard lactose-containing MacConkey agar, E.coli O157:H7 produces a lactose-fermenting, non-mucoid colony that is indistinguishable from commensal faecal E.coli. It is indole positive and oxidase negative (Tarr & Neil, 2001).

Biochemical identification of presumptive O157 STEC isolates is necessary because other species may cross-react with O157 antiserum or latex reagents, including Salmonella O group N, Yersinia enterocolitica serotypes O9, Citrobacter freundii and E. hermanii. Special biochemical test (Cellobiose fermentation, growth in the presence of KCN) may be necessary to differentiate E. hermanii from E.coli, but because E. hermanii is rarely detected in stool specimens, use of these tests is not cost-effective for most laboratories (Bopp et. al., 2003).

Serotyping

The serologic classification of E.coli is generally based on the O antigen (somatic) and the H antigen (flagella) (Bettelheim, 1995). The O and H antigens of E.coli are stable and reliable strain characteristics. Although 175 O antigens and 53 H antigens are currently recognized, the actual number of serotype combinations associated with diarrhoea disease is limited (Bopp et. al., 2003). Determination of the O and H serotypes implicated in diarrhoea disease is particularly useful in epidemiological investigations. Even though, antigens for the tube agglutination tests are available from several manufacturers, most laboratories do not attempt complete E.coli serotyping because it is costly (Bopp et. al., 2003).

Serologic confirmation of O157 STEC

Confirmation of E.coli O157:H7 requires identification of H7 flagellar antigen. H7-specific antigen and latex reagents are commercially available (Bopp et. al., 2003). Isolates that are non-motile or negative for the H7 antigen should be tested for the production of shiga toxins or the presence of shiga toxin gene sequence (Bopp et. al., 2003). Approximately, 85% of O157 isolates from humans received by CDC are seotypes O157:H7, 12% are non-motile, and 3% are H types other than H7. E.coli O157: NM strains frequently produce shiga toxin and are other wise very similar to O157:H7, but no O157 strain from human illness with an H type other than H7 has been found to produce shiga toxins (Bopp et. al., 2003).

Virulence testing

STEC (primarily non-O157 STEC), ETEC, EPEC, EIEC and EaggEC (and other putative diarrhoeagenic E.coli strain) are identified by detection of their respective, virulence-ssociated factors (characteristic toxins, adherence or invasiveness). Techniques for virulence testing include bioassays (eg cell culture or in-vivo testing), immunologic methods (eg Immunoblotting or EIA), or the detection of gene sequences by DNA-based methods (eg PCR or colony blot hybridization). The laboratory’s capability for performing the different types of assays will guide its selection for appropriate tests (Bopp et. al., 2003).

Antimicrobial susceptibilities

Antimicrobial therapy for O157 STEC diarrhoea or HUS has not been demonstrated to be efficacious and safe (Griffin et. al., 2002). Consequently, the

antimicrobial susceptibility pattern is usually determined only for epidemiological studies.

Even though, E.coli O157 is widely considered sensitive to multiple classes of antibiotic (Griffin & Tauxe, 1991). Strains showing multiple resistances have been described in the USA (Swerdlow et. al., 1992). The presence of antibiotic resistance has been reported as an emerging phenomenon, since it was observed only among E.coli O157 strains isolated after 1988 (Farina et. al., 1996). Thus, until recently, E.coli isolate were almost uniformly sensitive to antimicrobial agents. However, since, the 1990s, O157 and other STEC strains have demonstrated slowly increasing levels of resistance to certain antibiotics particularly streptomycin, sulfonamides and tetracycline (Bopp et. al., 2003).

Interpretation and reporting of results

A presumptive diagnosis of an O157 STEC (Isolate positive for O157 antigen) or a non-O157 STEC (isolate positive for shiga toxin) infection should be reported to the clinician as soon as the laboratory obtains this result. It would be advisable to indicate on the report that non-O157 STEC strains cause diarrhoea and HUS. Clusters and outbreaks of STEC should be reported to public health authorities (Bopp et. al., 2003).

Additional laboratory tests

At the time of presentation of a patient with bloody diarrhoea and on learning that a patient has a positive E.coli O157:H7 culture, it is important to obtain results for a complete blood count, electrolytes, blood urea nitrogen (BUN) and creatinine determinations.

These values become the baseline determinations in monitoring the patient for the development of extraintestinal complications of E.coli O157:H7 infections, such as HUS. Urinalysis is not particularly helpful at this stage, especially if the serum creatinine concentration does not suggest renal insufficiency. Blood cultures may be done for other reasons, particularly if, Salmonellosis or Shigellosis are considered, but they almost never are positive in E.coli O157:H7 infections (Tarr & Neill, 2001).

Additional investigations

Children and adults infected with E.coli O157:H7 can have surgically remediable complications without HUS or preceding HUS including appendicitis and intussusceptions. Careful and repeated abdominal evaluations are needed to ensure that these complications are not developing. Abdominal imaging studies such as ultrasound or computed tomography scan are not routinely helpful but may be useful in selected clinical situations. Abdominal plain films are helpful in evaluating the gastrointestinal tract in children infected with E.coli O157:H7. Thumb printing of the mucosa as is seen in colonic ischemic state is suggestive of E.coli O157:H7 infection in children and possibly in adults who, otherwise, are not at risk of having ischemic colitis (Tarr & Neill, 2001).

#### Therapy (Treatment)

There is no specific treatment of EHEC infections. The diarrhoeal phase of the disease is self-limiting (Farina et. al., 1996).

Antimicrobial therapy

No benefit in terms of decreased symptoms, shortened illness duration or fewer complications has consistently been shown for antibiotic treatment of E.coli O157:H7 infections (Neill, 1998). Some retrospective studies have suggested that the haemolytic uremic syndrome is more likely to develop in patients treated with an antimicrobial agent such as trimethoprim-sulfamethoxazole than in those not receiving this treatment (Pavis et. al., 1990) but one study reported the opposite result (Cimolai, Carter, Morrison, and Anderson, 1990). In one prospective study of antimicrobial therapy (Proulx, Turgeon Delge, Lafleur, and Chicanel, 1992), trimethoprim-sulfamethoxazole had no significant effect on the course of gastrointestinal symptoms, the duration of excretion of the organism or the likehood of progression to the haemolytic uaremic syndrome. However, patients in the study began treatment at a mean of seven days after the onset of diarrhoea.

The use of trimethoprim-sulfamethoxazole in patients with E.coli O157:H7

infection has also been questioned based on a study showing that sub inhibitory concentrations of that agent increased the productions of shiga-like toxin in vitro (Karch et. al., 1986). In recent times, however, Wong et. al. (2000) provided a highly conclusive data that antibiotic treatment of children with E.coli O15:H7 infection increases the risk of the hemolytic uremic syndrome. The association was strong and independent of confounding variables such as objective indices of the severity of illness (Wong et. al., 2000).

The risk is greatest in children, but there are several published case series that suggest that antibiotics also might be contraindicated in adult E. coli O157: H7 infections. In a nursing home outbreak with E.coli O157:H7 in South Western Ontario, Canada, adults who received antibiotic for diarrhea or for other reasons

during the period when they ingested this pathogen were at a statistically increased risk of dying (Carter et. al., 1987).

There was no benefit accruing to the adults who were treated with antibiotics (mostly fluoroquinolones) in an outbreak of E.coli O157:H7 in Lanarkshire, Scottland in 1996. There was a higher complication rate (death or HUS) among the patients treated with antimicrobials although the difference did not achieve statistical significance (Tarr & Neill, 2001). In developed countries, where E.coli O157:H7 causes bloody diarrhoea more frequently than Shigella, the common practice of antibiotic administration to any patient with acute bloody diarrhoea is hazardous, unless, and until it is determined that the patient is infected with a pathogen that is treated appropriately by such agents (Tarr & Neill, 2001). There is no global “one size fits all” approach to this therapeutic dilemma; this is particularly well illustrated by the difficult decision-making process in Africa where both Shigella dysenteriae and E.coli O157:H7 can cause acute bloody diarrhoea, in the same population (Germain, Cunin, and Tedjouka, 1998; Tarr & Neill, 2001).

Physicians should not use antibiotics to treat other pathogens in the stool, unless and until it is confirmed that E.coli O157:H7 is not present. Co-infections (with Compylobacter jejuni and with Clostridium difficile) are possible and in such case the risk of antimicrobial use in E.coli O157:H7 infection outways the risks of temporary deferral of treatment for these other pathogens (Tarr & Neill, 2001).

Antitoxin therapy

Possible, future treatment of E.coli O157:H7 infections include orally administered toxin binding resins and antitoxin given intravenously (Griffin, 1995). In

vitro and animal data suggest the use of specific measures such as synthetic toxin binders or antibodies to oblate toxin effect. However, the use of an orally administered toxin binder, in humans, to prevent HUS failed to show significant benefit over placebo when administered to children infected with E.coli O157:H7 (Tarr & Neill, 2001).

Pain relief

Patients infected with E.coli O157:H7 are in considerable pain and present a compelling picture for relief of symptoms. Compounds such as antimotility agents, anticholinergic agents, or opoid narcotics that have the ability to slow intestinal peristalsis should not be used (Tarr & Neill, 2001).

Antimotility drugs are contraindicated in patients with bloody diarrhoea as one retrospective study showed that the hemolytic-uremic syndrome was more likely to develop in patients with E.coli O157:H7 infection who received anti-motility drugs than in patients not receiving these agents (Cimolai et. al., 1990). Another study demonstrated an increased severity of neurological manifestations in patients with the hemolytic uremic syndrome (Boyce et. al., 1995).

In several studies, the administration of these agents increased the risk of developing HUS or of having a complicated episode of HUS (Cimolai et. al., 1990; Bel et. al., 1994). Although antimotility agents might provide a temporary relief of symptoms, the use of such drugs was associated with a longer duration of bloody diarrhoea in children infected with E. coli O157:H7 (Bel et. al., 1994; Tarr & Neill, 2001).

Hospitalization

Patients with bloody diarrhoea or patients with intense pain should be admitted to the hospital. The need for hospitalization is based on several observations. Patients infected with E.coli O157:H7 often are in considerable pain and distress, and these symptoms appear to be worsened by oral intake, including the administration of fliuds intended to promote hydration (Tarr & Neill, 2001).

The most important medical complication of E.coli O157:H7 infection is HUS, which is a process that has prominent components of thrombotic and renal tubular injury (Tarr & Neill, 2001). Vascular expansion to counteract microvascular thrombi and to maintain organ perfusion and to maintain glomerular filtration rate and renal tubular flow is the principal measure that can be used to counteract the endothelial and renal tubular injury seen in E.coli O157:H7 infections (Tarr & Neill, 2001). During the week after the onset of diarrhoea, patients with documented infection should be monitored for signs and symptoms of the hemolytic uremic syndrome such as pallor and oliguria (Boyce et. al., 1995).

It is prudent to monitor patients at high risk for the syndrome (such as children less than 5 years old and the elderly) by means of peripheral-blood smears, blood counts and urinalysis during this period (Boyce et. al., 1995).

Management of the haemolytic uremic syndrome is supportive and includes, meticulous attention to fluid and electrolyte balance, dialysis is often necessary (Siegler, 1988). Other treatments include plasmapheresis, transfusion of fresh- frozen plasma and intravenous administration of immunoglobulin but their efficacy has not been proven (Siegler, 1988; Rossen et al, 1993).

Patients infected with E.coli O157:H7 are potential sources for the infection of others in the household. Approximately 10% of E.coli O157:H7 cases in outbreaks reflect secondary transmission (Bel et. al., 1994). Presumably, this is also the case in sporadic infections. Although nosocominal infections with E.coli O157:H7 can occur, infection control practices probably can be enforced better in hospitals. The committee on infectious diseases, American Academy of Pediatrics, 2000 recommends contact precautions for E. coli O157:H7 infections and it would be unreasonable to expect non-medical professional to comply with these requirements in the home (Tarr & Neill, 2001).

Fluid therapy

The degree of dehydration in patients infected with E.coli O157:H7 is easily underestimated. This is because the volume of stool produced by infected patients is not large, but evacuations are frequent. Many patients also have some degree of vascular injury and compelling leakage without or in advance of HUS, and such patients might not manifest cutaneous signs of dehydration. Vomiting and poor oral intake act in concert to dehydrate patients with E. coli O157:H7 infections, even without cholera-like purging (Tarr & Neill, 2001).

In view of the above, Tarr and Neill (2001), usually infuse isotonic crystalloid (normal saline, normal saline with 5% dextrose, or lactated Ringers solution) at maintenance volumes after administering a 20ml/kg initial fluid bolus. Hypotonic fluids frequently result in hyponatamia in E.coli O157:H7 infections. Potassium can be added to the fluid if the serum Potassium is normal or low (Tarr & Neill, 2001).

These recommendations are accompanied by several caveats: the patients underlying medical condition can tolerate high levels of salt infusion (a particular concern in the elderly or patient with underlying cardiopulmonary disease), and the patient is hospitalized in an institution where fluid overload can be assessed readily and managed. The blood pressure should be checked at least every 4 hours to monitor the development of hypertension (which may portend HUS), and electrolytes, BUN and serum creatinine should be checked daily or more frequently if needed to asses for electrolyte imbalance and renal insufficiency (Tarr and Neill, 2001). Urine output should be monitored closely although the use of urinary catheter is discouraged. Referral to a specialist experienced in managing HUS should be made at the earliest suggestion of renal insufficiency.

Patients who are receiving the aforementioned fluid regimen should be monitored in the hospital by following the complete blood count closely usually on a daily basis. A falling platelet count is the initial manifestation of microangiopathy and a rising platelet count heralds the end of this process and can be used to stop the aggressive infusion. Another blood count should be obtained within 24 hours of discharge to confirm that any microangiopathic process has abated. Similarly, if the platelet count obtained on learning that the culture is positive is higher than on presentation, it is unlikely that such a patient will develop severe microangiopathic sequelae, as long as these determinations are at least 5 days into illness (Tarr & Neill, 2001).

Treatment of Haemolytic Uraemic Syndrome

HUS is classified into two subgroups. Typical HUS usually occurs after a prodrome of diarrhoea (D+HUS), and atypical (sporadic) HUS (aHUS), which is not associated with diarrhoea (D-HUS). The majority of D+HUS worldwide is caused by Shiga toxin-producing Escherichia coli (STEC), type O157:H7 (Amirlak & Amirlak, 2006).

Currently, there are no specific therapies preventing or ameliorating the disease course. Although there are new therapeutic modalities in the horizon for D+HUS, present recommended therapy is merely symptomatic. Parenteral volume expansion may counteract the effect of thrombotic process before develpoment of HUS and attenuate renal injury. Use of antibiotics, antimotility agents, narcotics and non-steroidal anti-inflammatory drugs should be avoided during the acute phase. Prevention is best done by preventing primary STEC infection (Amirlak & Amirlak, 2006).

#### Convalescent Faecal Excretion of *E.coli* O157:H7

Some patients continue to excrete E.coli O157:H7 into the convalescent period even though most patients with HUS clear the infection from the stool within one week of the onset of diarrhoea. Because of the risk for transmission posed by convalescent faecal excretion of E.coli O157:H7, infected children should be excluded from settings where secondary transmissions can occur (such as day care centers) until they have multiple negative cultures. This consideration also applies to adult food handlers and health care providers (Tarr & Neil, 2001).

Relying on negative stool cultures to indicate that a child with prior E.coli O157:H7 infection no longer poses a risk for transmission of infection has an important caveat pertaining to it. However, all human excrement should be treated as a bioharzardous substance and be subjected to the same infection control measures regardless of whether or not an enteric pathogen is known to be present (Tarr & Neil, 2001).

#### 2.4.15 Recognition of and Response to Outbreaks

Improved detection of EHEC, or at least the O157 serogroup, in clinical samples and foodstuffs has potentially widespread public health implications (Zimmerhackl, 2000). The large number of outbreaks of EHEC infections and associated deaths in North America especially in the very young and elderly has had an impact on public health attitudes and led to major changes in regulations concerning food safety there (Karch, 1996).

Awareness of the fact that EHEC is a public health hazard has greatly increased among physicians, clinical microbiologists and consumers (Karch, 1996). The extensive movement of people and foodstuffs within and between European countries make EHEC infections a cross-border problem. A network of laboratories and clinical centers is needed to effectively monitor EHEC infections throughout Europe. This network could coordinate epidemiological studies, provide an early warning system and initiate preventive measures. Efforts to monitor EHEC infections at the National and European level would be greatly enhanced if EHEC-related illness such as HUS and hemorrhagic colitis and the isolation of the pathogen were notifiable by law in all countries (Karch, 1996). As the first link in the public health

chain, physicians can help to prevent E.coli O157:H7 infection by counseling patients about the risk of eating under cooked ground beef (Boyce et. al., 1995).

Any physician treating a patient with a probable or definite E.coli O157:H7 infection has an important public health role. First, the physician should ascertain if there are similarly affected contacts of the identified cases and should ensure that these people are evaluated accurately. Secondly, the treating physician must report to the appropriate public health unit the occurrence of an E.coli O157:H7 infection, just as for other reportable diseases and pathogens. E.coli O157:H7 infections should be reported immediately to the health department (including weekends) even if such urgent notification is not mandated because of the serious nature of these infections. The urgency is derived from the possibility that an individual patient may be the index case in an outbreak that warrants immediate investigation (Tarr & Neil, 2001).

Early notification efforts by gastroenterologists in at least 3 large E.coli O157:H7 outbreaks (Griffin, Ostroff, and Tauxe, 1988; Bel et. al., 1994; Cody, Glynn, and Farmer, 1999) initiated or facilitated critical public health interventions, preventing additional cases (Tarr & Neill, 2001). Early detection of cases can also prevent additional cases from occurring. In the large outbreak in January 1993 that affected persons in the Western United States, early detection and reporting of cases of E.coli O157:H7 infection and the hemolytic uremic syndrome resulted in a rapid recall of contaminated ground beef and prevented an estimated 800 infections (Bel et. al., 1994). Obtaining thorough histories of possible exposure is the first step in detecting a cluster of cases. Patients or their parents should be asked about the

consumption of undercooked ground beef or raw milk, attendance at day-care centers and exposure to other persons with diarrhoea.

In summary, there would appear little argument that the large outbreaks of E.coli O157:H7, which have occurred since the early 1980’s, represent a distinct, new phenomenon (Armstrong et. al., 1996). The emergence of these EHEC infections demonstrates clearly that we are still highly vulnerable to some of the microorganisms that share our environment (Karch, 1996). Although, significant progress has been achieved over the last decade, further basic, epidemiological and clinical research is needed to broaden understanding of the pathogenesis of EHEC- mediated disease and to develop specific forms of treatment and prevention (Karch, 1996).

# CHAPTER THREE MATERIALS AND METHODS

#### ETHICAL CONSIDERATIONS

Patients’ informed consent was sought by direct explanation of the study design and its purpose. This was done by clinicians when they were involved and by those assisting in filling the questionnaire (See Appendix1 for sample). For children, parents’ informed consent was sought in accordance to the recommendations of Rennie and Yank, (1997); Miller and Rosenstein,( 2002).

For the study conducted in National Hospital, Abuja, the approval of the Hospital Management was sought and obtained. This approval was gotten when the proposal for the study had to pass through the scrutiny of the Research Ethics committee of the Hospital after which the ethical clearance was received (See Appendix A3).

In addition, as part of ethical considerations, letters requesting for collaboration was written to the management of all the hospitals from which samples were collected. This was to make them aware that a study of this nature was gong on to avoid any possible problem at a latter date and to obtain their consent to collect samples from their patient (Appendix A2).

#### STUDY CENTRES

Abuja is the capital city of the Federal Republic of Nigeria with a population figure of 526,977 in 2003, estimate projected from the 1991 census figure (National Population Commission, (NPC), 2002).

Request for collaboration concerning sample collection was sent in writing to the management of various hospitals within the Federal Capital Territory. The response from those detailed to assist was poor. At the end, samples were obtained only from five hospitals within the territory namely: National Hospital, Abuja; Julius Berger Clinic, Gwarimpa; Zankli Medical Centre; Nyanya General Hospital; Asokoro District Hospital.

The research was conducted in the following laboratories: Microbiology Laboratory, Department of Medical Microbiology, National Hospital, Abuja, Nigeria; Haematology Laboratory, Department of Haematology/Blood Transfusion, National Hospital, Abuja, Nigeria; Virology Laboratory, Department of Virology, National Veterinary Research Institute (NVRI), Vom-Jos, Plateau State, Nigeria.

Samples were sent to the following laboratories for analysis: Epidemic Investigatons and Surveillance Laboratory, Foodborne and Diarrhoeal Diseases Laboratory Section, Centres for Disease Control and Prevention (CDC), Atlanta, Georgia, USA and National Reference Laboratory for Escherichia coli and Shigella, Foodborne and Diarrhoeal Diseases Branch, Centres for Disease Control and Prevention (CDC), Atlanta, Georgia, USA.

#### INCLUSION CRITERIA

Diarrhoeal stools with or without Blood. Diarrhoea was defined as passage of three or more loose or watery stools in a 24-hour period. Bloody diarrhoea, was defined as the presence of one or more loose or watery stools that were red streaked or pink (Spika et al., 1986).

#### SAMPLING TECHNIQUE

Non-probability sampling technique was used. The scheme of sampling did not involve elements of Randomization. Only those samples that met the inclusion criteria were chosen as representative of the population under investigation (Hassan, 1991).

#### DETERMINATION OF SAMPLE SIZE

Usually in determining sample size, there is a trade off between the desirability of a large sample and the feasibility of a small one (Hassan, 1991). The ideal sample is large enough to serve as an adequate representation of the population about which the researcher wishes to generalize, and small enough to be selected economically in terms of subjects’ availability, expense in both time and money, and the complexity of data analysis. Hence, there is no fixed number or percentage of subjects that determines the size of an adequate sample (Hassan, 1991).

Thus, the sample size is a function of three factors:

* + - E, the maximum allowable sampling error.
    - The number of standard error units associated with the level of confidence specified and
    - , The standard deviation of the population parameter of the trait of interest.

Depending on , the confidence level z would take the value of 1.96, 2.33 or 2.58 for 95%, 98% or 99% confidence level (Hassan, 1991).

The formular used in this study is formular used for determining sample size in studies where a single dichotomous stratification parameter is employed (eg rural- urban, large-small, high or low). The formular is given below as adopted from Hassan (1991).

Ns = (Z/E)2 (p) (1 – p).

Where Ns = the required Sample size.

|  |  |  |
| --- | --- | --- |
| Z | = | the standard score corresponding to a given confidence level. |
| E | = | the proportion of sampling error in a given situation, and |
| p | = | the estimated proportion or incidence of cases in the population. |

In this formular, p (1 – p) is maximized when p = 0.5. This ensures a sample size large enough to satisfy the precision and confidence constraints. However, if a researcher has sufficient evidence to believe that p differs from p = 0.5, substitution of his best estimate can result in a significant reduction in the required sample size (Hassan, 1991). For this study, p = 0.5 and confidence level of 95% with a tolerable amount of error not greater than 0.1 was used. Thus, the calculated sample size was 96.

#### COLLECTION OF SAMPLES

Between January 2003 and July 2003, 106 fresh faecal samples were collected from 106 patients with acute diarrhoea in various hospitals in Abuja, Nigeria. The faecal samples were all collected into a clean, dry, leak proof container in

accordance to standard routine procedure. A structurally designed questionnaire was used for obtaining information concerning each patient (Appendix A1). Faecal specimens were stored at 4°C initially at the hospitals, transported by cold box, and stored at the same temperature in the Microbiology laboratory of National Hospital, Abuja. All samples were analyzed on the same day of collection.

* 1. **ISOLATION OF *Escherichia coli* O157:H7**

Two techniques were used for the isolation of E. coli O157:H7 from the faecal samples according to the method of Chapman and Siddons (1996). These include: direct culture, and enrichment culture followed by immunomagnetic separation (EC - IMS).

* + 1. **Direct Culture of *E. coli* O157:H7**

Materials

* + - * Sorbitol MacConkey agar (SMAC) (Oxoid CM 813)
      * Cefixime – Tellurite sorbitol MacConkey agar (CT-SMAC)
      * E. coli O157:H7 latex Agglutination Test kit (Oxoid DR 620m)

Method

Approximately 10µl volumes of faecal samples were innoculated directly on to SMAC (March & Ratnam, 1986) and CT-SMAC (Zadik et. al., 1993). The plates were incubated at 370C for 18 - 24 hours. Non-sorbitol fermenting, (NSF), colonies that appear as colourless colonies on the two plates were tested for E. coli O157:H7 using the E. coli O157: H7 latex agglutination test kit. After this, the NSF colonies

were identified using conventional biochemical tests - kligers iron agar (KIA), simmon citrate agar, urease agar, methyl red test, and peptone water for indole and motility tests. All colonies identified as E. coli were stored on nutrient agar slants for polymerase chain reaction (PCR) used for detection of virulence genes of Enterohaemorhagic Escherichia coli.

#### Enrichment culture followed by Immunomagnetic Separation technique

In addition to direct culture, stool samples were enriched in buffered peptone water, supplemented with vancomycin, cefsulodine and cefixime. After enrichment, immunomagnetic separation was done followed by culture on sorbitol macConkey agar and CT - SMAC. The principle and procedure is as described below.

Immunomagnetic Separation Technique

Principle: Dynabeads, anti-E. coli O157 is designed for rapid, selective concentration of E. coli O157 directly from a pre-enriched sample using the buffered peptone water supplemented with vancomycin, cefixime and cefsulodin (BPW- VCC) or the bead retriever. During the incubation process, the antibodies coated onto the beads will specifically bind the target bacteria. Washing of the beads is achieved by repeated washing with the washing buffer (PBS-tween) and passage through the magnetic particle concentrator (MPC-S). At the final stage, the bead-bacteria complexes were resuspeded in PBS-tween for further processing to detect and/or isolate the target organism.

Materials:

* Buffered Peptone water with VCC supplement (BPW-VCC)
* Vortex Mixer
* Magnetic beads coated with an antibody against E. coli 0157 (Dynabeads anti-E. coli O157)
* 1.5ml micro centrifuge tube
* Rotating Mixer
* Magnetic separator Rack (MPC - S, Dynal)
* Phosphate Buffered saline (PBS) with Tween 0.05% v/v (PBST)

Method

About 0.5g of faeces was innoculated into 5 ml of BPW - VCC. The tube was vortex mixed and broth incubated at 37oC for 6 hours. Thereafter, 1 ml of broth was added to 20 µl of magnetic beads in a 1.5 ml micro centrifuge tube. The suspension was vortex mixed to suspend beads in broth culture. The tubes were then placed in a rotating mixer for 30 mins at room temperature. After that, tubes were placed in a magnetic separator rack and the magnets put in place for 5 mins. The culture supernate was removed by aspiration with a Pasteur pipette. The magnetic plate was then removed from the rack. The beads were washed by resuspension in 1 ml of PBS, PH 7.2 with Tween - 20 0.05% v/v (PBST) and the magnetic slide were replaced for 2 mins. The immediate step above was subsequently repeated. Then, the supernate was removed and resuspended in about 50µl of PBS. Beads were then innoculated onto SMAC and CT-SMAC, incubated at 37°C overnight, and examined for non-sorbitol fermenting colonies as above. Identification of NSF colonies was by

standard series of biochemical tests as listed above. All isolates confirmed as E. coli

by biochemical tests were stored for polymerase chain reaction (PCR).

#### POLYMERASE CHAIN REACTION (PCR) FOR THE DIAGNOSIS OF DIARRHOEAGENIC *ESCHERICHIA COLI*

* + 1. **Introduction**

An alternative approach for the detection of EHEC serotypes is the use of PCR to detect virulence factors of EHEC such as shiga toxins (stx), E. coli attaching and effacing (eae) and enterohaemolysin (Ehly) genes (Desmachelier et. al., 1998).

All primary isolates of E. coli from faecal samples by both direct culture and IMS were stored for Polymerase Chain Reaction (PCR). This aspect was aimed at finding out whether any of the non-sorbitol fermenting E. coli isolates from our environment were shiga-toxin-producing (STEC) otherwise known as verocytotoxin- producing (VTEC) or Enterohaemorrhagic E. coli (EHEC).

Due to non-availabilty of equipment and materials for this aspect of the study in Nigeria, contacts were made with the National Reference Laboratory for Escherichia coli and Shigella, Foodborne and Diarrhoeal Diseases Branch, CDC, Georgia, USA through Dr Nancy A. Strockbine, who accepted to have the isolates tested.

The PCR was done using lightcycler (Cebula et. al., 1995; Reischl et. al., 2002; Reischl et al., 2003).

#### Primers and Probes

Primers and probes were obtained from Roche Diagnostics, Mannheim, Germany.

Working concentration for primers used was 25 M and for probes: 5 M. Example of how to calculate this is shown in Appendix B9. The water provided by the LightCycler kits was used to prepare the working concentrations. After preparation, they were stored at 4°C until ready to use. The primer and probe stock concentrations were allocated and stored at -20oC.

Quality control (QC) for primers and probes:

A set of strains that represent different variants (different Tm: s) of E coli virulence factors formed the QC test panel. A large volume of DNA was extracted from the QC strains and the same template was used to test each new lot of primers and probes.

Sequences for primers (from 5’ to 3’):

|  |  |  |
| --- | --- | --- |
| ***stx1* and *stx2:*** | STEC-1 | gARa CRA AAT AAT TTA TAT gTg |
| ***eae:*** | STEC-2  eaeAF | TgA TgA TgR CAA TTC AgT AT  gAC CCg gCA CAA gCA TAA gC |
| **E*-hly***: | eaeAR  hlyAF | CCA CCT gCA gCA ACA AgA gg  gCA TCA TCA AgC gTA CgT TCC |
|  | hlyAR | AAT gAg CCA AgC Tgg TTA AgC T |

a IUB code for A or G (wobble base)

Sequences for probes (from 5’ to 3’):

***stx1:***

STEC I-HP-1 TTT ACg TTT TCg gCA AAT ACA gAg ggg AT-[FAMa]

STEC I-HP-2 [Red 640b]-TCg TAC AAC ACT ggA TgA TCT CAg Tgg g-Phc

For ipah-Stx1-multiplex:

STECI-HP-2 [Red 705b]-TCg TAC AAC ACT ggA TgA TCT CAg Tgg g-Phc

## stx2:

STEC II-HP-1 TCA ggC ACT gTC TgA AAC TgC TCC TgT gTA-[FAMa] STEC II-HP-2 [Red 705d]-ACC ATg ACg CCg ggA gAC gTg gAC CT-Phc

## eae:

eaeA-HP-1 ACA gTT CTg AAA gCg AAA TgA TgA Agg C-[FAMa] eaeA-HP-2 [Red 640b]- CCT ggT CAg CAg ATC ATT TTg CCA CT-Phc

## E-hly:

hlyA-HP-1 gCA Tgg CTC TTg ATg AAT TgC TgA gA-[FAMa]

hlyA-HP-2 [Red 705d]-CAA Cgg gAA ggA gAg gAT ATA AgT CAg-Phc a [FAM], flourescein

b [Red 640], LightCycler-Red 640-N-hydroxy-succinimide ester;

c Ph, 3’-phosphate

d [Red 705], LightCycler-Red 705-phosphoramidite

#### Extraction of DNA Template

Whole cell template:

A small amount (about half the growth of a 2mm diameter colony) of bacterial growth was suspended into 300µl of distilled water. This was boiled for 10mins and

then centrifuged at 4,500 rpm for 2mins. 2µl/reaction was used. To prepare enrichment broth, 500µl of broth culture was boiled for 10mins and centrifuged at 4,500 rpm for 2mins. 2µl/reaction was also used.

Purified DNA:

PureGene kit (Gentra Systems, Minneapolis, MN; Cat No. D-5000A): About half of loop (10 l loop) of overnight growth from agar plate was suspended in 600

l PureGene Lysis Buffer.The test proceeded, thereafter, following manufacturer’s instructions. 2 l / reaction was used.

DNeasy® Tissue Kit (Qiagen Inc., Valencia, CA; Cat. No. 69506): Half of loop (10 l loop) of overnight growth from agar plate was again suspended in 180 l DNeasy ATL Buffer. The test proceeded following manufacturer’s instructions. 2 l / reaction was used.

MagnaPure automatic DNA extractor (Roche Diagnostics; Cat. No. 2 236 931): About 1” sweep (1 l loop) from the confluent first streaking area of overnight bacterial growth was made and suspended in 230 µl of 0.01 M PBS (pH 7.2). This was boiled for 10 min. 200 µl was used as a sample input volume in MagnaPure. The test proceeded by following manufacturer’s instructions. 2 µl / reaction was used.

#### Mastermixes for one sample

(All reagents were kept in ice or in a cooling block)

HP mastermix = LightCycler - DNA Master Hybridization Probes, 10x

The water and MgCl2 provided by the kit was used to adjust the final reaction volume and MgCl2 concentration, respectively.

***stx1 & stx2*** (Shiga toxin genes 1 and 2 in STEC): dH2O 11.4 l

HP mastermix 2.0 l

MgCl2 (25mM) 1.6 l

STEC-1 primer (25 M) 0.4 l

STEC-2 primer (25 M) 0.4 l

STEC I-HP-1 (5 M) 0.8 l

STEC I-HP-2-LC640 (5 M) 0.8 l

STEC II-HP-1 (5 M) 0.8 l

STEC II-HP-2-LC705 (5 M) 0.8 l

= 19.0 l

***eae &* E*-hly*** (intimin and enterohemolysin genes in STEC and EPEC, and STEC, respectively):

dH2O 11.0 l

HP mastermix 2.0 l

MgCl2 (25 mM) 1.6 l

eaeAF primer (25 M) 0.4 l

eaeAR primer (25 M) 0.4 l

eaeA-HP-1 (5 M) 0.8 l

eaeA-HP-2-LC640 (5 M) 0.8 l

hlyAF primer (25 M) 0.2 l

hlyAR primer (25 M) 0.2 l

hlyA-HP-1 (5 M) 0.8 l

lyA-HP-2-LC705 (5 M) 0.8 l

= 19.0 l

#### Controls

Positive controls:

* + - * EDL 933 (STEC O157:H7; ATCC 43895): positive for stx1, stx2, eae,

E-hly and uidA.

* + - * S1191 (STEC O139:H1; CDC); positive for stx2e
      * 3157-02 (STEC O91: NM; CDC) positive for stx1, stx2-variant and E- hly

Negative controls: negative DNA control (non-pathogenic E. coli strain) and no DNA (water) control

#### Procedure

To get started, the computer and the light cycler were turned on. With the clicking of the right icons, the system runs its own self-test. After the self-test is completed and passed, the programming screen will become active. A colour compensation file is created following the instructions in the insert of light cycler colour compensation set.

After this, conditions in the temperature target segment of the first segment of the protocol - “Denaturation” is defined: 95°C 30 s with temperature transition rate 20°C / s

The second segment of the protocol was “PCR”. Cycling conditions in the temperature segment was defined as follows: 40 cycles of denaturation 95°C 0 s; annealing 50°C 20 s; extension 72°C 30 s; temperature transition rate 20°C / s in all steps; acquisition mode “Single” at the annealing step; analysis mode “Quantification”.

After this, condition for the third segment, “Melting Curves” was defined. These conditions are Rapid denaturation 95°C 0 s with temperatue transition rate 20°C / s; 10 s hold at 40°C with temperature transition raye of 0.2°C / s; Acquisition mode “Continous” during the slow denaturation step; Analysis Mode “Melting Curves”.

Finally, the conditions of the fourth segment “Cooling” were defined. This include 40°C 2mins with temperature transition rate 20°C / s.

After setting the conditions the required number of light cycler capillaries were placed in precooled light cycler centrifuge adapters with forceps. 18.0µl of mastermix per reaction was used. 2µl of DNA template was added. The cap was placed on the capillary with the forceps and pressed gently using the end of the forceps until the capillary is properly sealed. The sealed capillaries were then transfered in the light cycler sample carousel. The samples were then spun down in the light cycler carousel. The samples were then spun down in the light cycler carousel centrifuge at 3,000 rpm 15 s. After this, the capillaries were inspected for air bubbles or pipetting errors. The carousel was then placed in the light cycler and the samples were run, after which the results were analysed by following the instructions in the light cyclerTM operators manual version 3.5.

#### DETECTION OF OTHER SEROTYPES OF ENTEROHAEMORRHAGIC

***E.COLI* (EHEC)**

Other serotypes of Enterohaemorrhagic E.coli (EHEC) do not have a single distinguishing marker such as non-fermentation of sorbitol in 24 hours like E.coli OI57:H7. Thus, other toxin-positive strains can be detected using vero cells in a tissue culture cytotoxin assay (Baron et. al., 1994).

Faecal extracts were prepared from all samples analysed and stored for verocytotoxicity assay.

#### Preparation of Faecal Extracts

Materials needed

* + - * Modified Tryptone soybroth (mTSB) (Oxoid CM 989)
      * Bijou bottles

METHOD

Modified Tryptone soybroth (mTSB) was prepared according to manufacturer’s instruction and dispensed into sterile bijou bottles in 5 ml amounts. An aliquot of stool specimen (100 µl of liquid stool or 3 - 4 mm diameter of solid material) was inoculated into 5 mls of mTSB. Samples were incubated for 20 - 24 hours at 37oC without agitation. After this, centrifugation was done at 1,400 g for 10 mins. The supernatant was removed into sterile bijou bottles and stored at - 20 o C until ready for use (Pai et. al., 1984).

#### Verocytotoxicity Assay

Preliminary stage

Faecal extracts stored at -20°C were removed from storage. Ultra centrifugation was done at 9,500 g for 5mins. Extracts were then filtered using 0.45µm membrane filters. The stool filtrates were stored at 4°C until ready to assay (Pai et al., 1984).

Repassage (Trypsinization) of tissue culture cells Requirements

The following items were used for the trypsinizatin of tissue culture cells: Stocks of the required continous cell line – African Green Monkey Kidney Cells (Vero cells); Hanks Minimum Essential Medium (HMEM); 10% Newborn Calf Serum; Hepes Buffer (1M Solution); Antibiotic Supplements - Penicillin/Streptomycin, Gentamicin, Fungizone; 24-well plastic dishes (Tissue Culture grade); Trypsin (0.25%, sterile and warmed to 37°C); Sterile Pippettes (10ml, 5ml and 1ml); Phosphate Buffered Saline (PBS); Tissue Culture flasks.

Method

The medium was decanted from flasks containing confluent growth of Vero cells, which were washed with warm PBS before being decanted. The procedure was repeated after which 0.25%-prewarmed trypsin was added to cover the cells. The trypsin was left on the cell sheet for 1min, and then poured off. The flask was kept at 37°Cfor 5 - 10 mins. The cells were examined under the microscope during this time to ensure that the cells were not peeling off too soon. Note was taken of when the sheets have holes and were beginning to peel from the surface of the glass. A small amount of freshly warmed medium containing 10% new born calf serum, hepes buffer and antibiotic supplements was added and the container gently shaken to loosen both the cellsas well as to stop the action of trypsin.

The antibiotic supplements were added to the medium at specified concentrations and for specific purposes as follows: Penicillin at a concentration of 100 iu/ml against Gram-positive oganisms; Streptomycin at a concentration of 100µg

/ml against gram-positive and gram-negative organism; Gentamicin at a concentration of 0.05mg/ml of medium against gram-positive, gram-negative organisms and mycoplasma; Fungizone at a concentration of 0.0025mg/ml against yeast and moulds.

A cell count was done to determine the number of cells per millilitre of harvested suspensions. The cells were then diluted at the ratio of 1: 4 to get the required concentration with fresh growth medium containing 10% NB calf serum and antibiotics.

5ml of the cells were dispensed into 25cm sterile tissue culture flask and incubated at 37°C for 48 hours untill a monolayer is formed.

0.5ml of the cells were dispensed into 24- well plastic dishes for cytotoxicity

assay.

These plastic dishes cannot be sealed and so require a CO2 incubator to maintain a proper balance of 5% CO2 and humidity to prevent a change in pH. Because this was not available, Hepes buffer at the rate of 15-20 mM/ml was added to the growth medium at the same time with serum and antibiotics before being dispensed into the plastic dishes. This stabilized the pH and allowed incubation in an ordinary incubator at 37°C. The 24-well plastic dishes were incubated for 48hrs to allow the cells to grow and become confluent.

All procedures were carried out in a Laminar Flow Hood (Class II Biosafety Cabinet).

Procedure for cytotoxicity assay

Verocytotoxicity assay was done according to the method of Konowalchuk et. al. (1977). 0.05ml aliquot of stool filtrate was added to each well of a 24-well plastic dish containing confluent growth of vero cells without removing growth medium. The plates were incubated for 24hrs at 37C. Vero cell monolayers were examined for cytopathic effect (CPE). Morphological effects were recorded as (+), (++) and (+++), ratings corresponding to roughly  25%, 50% and> 75% of cells affected. For controls, there were: (a) Vero cells that received nothing to show the normal architecture of the cells.

(b) Vero cells that received 0.05mls of sterile PBS instead of the filtrates as negative control.

All stool filtrates were used undiluted. All readings were done after 24hrs only. All procedures were carried out in a Laminar Flow Hood (Class II Biosafety cabinet).

* 1. **ISOLATION OF *SALMONELLA / SHIGELLA* SPECIES**

Routine method was used for the isolation Salmonella and Shigella species (Cheeseborough, 2000). A loopful of faecal sample was innoculated onto a plate of DCA and several loopfuls into 3 - 4 ml of SF in a test tube. Plates and test tubes were incubated aerobically at 37oC over- night. Suspicious colonies seen as colorless non-lactose fermenting (NLF) colonies on DCA were identified using conventional biochemical tests as done for non-sorbitol fermenters. In addition, suspicious colonies were innoculated onto nutrient agar plates (for serology) and macconkey

agar plates (to check for purity). A loopful of growth on SF broth was innoculated onto DCA. All plates and biochemical tests were again incubated overnight at 37oC.

The following day biochemical tests were read and isolates identified. Salmonella/Shigella isolates were serotyped for confirmation using the following antisera – Omni O (serum anti-Salmonella polyvalent A – 60); Serums anti- Salmonella polyvalents: T, A, B, C, Vi; Serum anti-Shigella dysenteriae polyvalent A1 and A2; and Serum anti-Shigella flexneri polyvalent. All were from Sanofi Diagnostics. Suspicious colonies on DCA plates subcultured from SF were treated like other NLF’s as described above.

All confirmed isolates of Salmonella and Shigella were tested for susceptibility to various antimicrobials.

* 1. **CHARACTERISATION OF *SHIGELLA FLEXNERI* ISOLATES**

Four (4) Shigella flexneri isolates from the study were sent to the Centre for Disease Control (CDC) in Atlanta, Georgia USA for confirmation and characterization courtesy of Cheryl A. Bopp, Chief of the Epidemic Investigations and Surveillance Laboratory, Foodborne and Diarrhoeal Diseases Laboratory Section.

Method

First, isolates were confirmed as Shigella flexneri using Denka Poly B antisera. Followed by serotyping using specific antisera. Antimicrobial Susceptibility Tests were then done using Disc diffusion method. Antimicrobials tested were chloramphenicol, trimethoprim-sulphamethoxazole, tetracycline, ampicillin, sulfisoxazole and streptomycin.

* 1. **ISOLATION OF *Vibrio Cholerae* WHEN CHOLERA WAS SUSPECTED**

Isolation of V. cholerae was also done using routine methods (Cheeseborough, 2000). Several loopfuls of specimen was innoculated into alkaline peptone water. This was incubated at 37oC for 5 - 8hrs at the end of which a drop of alkaline peptone water culture was placed on a slide and examined for typical V.cholerae darting motility. Several loopfuls of the peptone water culture were then subcultured on TCBS and incubated aerobically at 37oC overnight.

Suspicious colonies of sucrose fermenting V.cholerae that appeared as yellow 2

-3 mm diameter shiny colonies on TCBS were looked out for. When seen the following tests were performed: oxidase tests (V.cholerae are oxidase positive); gram stain (V. cholerae are gram-negative, coma-shaped rods); biochemical tests as listed above and distilled water motility tests to differentiate between Vibrio and Aeromonas (all Vibrio species are immobilized in distilled water but remain motile in peptone water. Aeromonas species remain motile in both distilled water and peptone water). Serology was performed to identify the specific V.cholerae serotype involved using Vibrio cholera anti-sera from Denka seiken. These include V. cholerae O1 – Polyvalent; V. cholerae O1 – Inaba type; V. cholerae O1 – Ogawa type; and V. cholerae O139 “Bengal”. All confirmed isolates were tested for susceptibility to various antimicrobials.

Serotyping of V. cholerae

Isolates, which agglutinate with Inaba type serum, were designated as V.Cholerae O1 serovar Inaba.Those that agglutinate with Ogawa type serum, were called V.cholerae O1 serovar Ogawa. Isolates, which agglutinate with Inaba and

Ogawa sera, were designated asV.cholerae O1 serovar Hikojima whereas those that agglutinate Antisera O139 “Bengal” were designated as V. cholerae O139 “Bengal”.

#### Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing was done on all confirmed isolates of Salmonella, Shigella and Vibrio cholera using the modified Kirby-Bauer disc diffusion method as described by Cheeseborough, (2000) using the following discs - tetracycline (30µg); chloramphenicol (30µg); trimethoprim-sulphamethoxazole (25µg); amoxycillin-clavulanic acid (30µg); ciprofloxacin (5µg). In addition, some of the S. flexnerii isolates were tested against ampicillin (10µg), and nalidixic acid (30µg). Mueller Hinton agar and Mueller Hinton broth were used for the sensitivity testing. 0.5M McFarland standard corresponding to a density of 105 organisms/ml of broth was used to standardize the innoculum size. Interpretation of zone diameters was done using the NCCLS interpretative standards. The organisms were reported as susceptible, intermediate, or resistant to the antimicrobial agents tested. For quality control, E coli ATCC 25922 was included in each batch of tests.

All results were dispatched to the clinician for further patients’ management.

#### EXAMINATION OF FAECAL SPECIMENS FOR PARASITES/YEASTS (FUNGI)

All faecal samples submitted for culture were also examined microscopically using x10 and x40 objectives. The aim is to rule out enteric parasites that could also cause diarrhoeal disease. Yeast cells were also identified using this method. The

technique used is the formol-ether sedimentation technique as described by Cheesborough, 2000.

Formol-ether sedimentation technique

Principle: Faeces was emulsified in formol-saline; the suspension was strained to remove large faecal particles. Formalin fixes the eggs, larvae and cysts to make them no longer infectious as well as preserve their morphology. Faecal debris is separated in a layer between the ether and the formol water freeing the sedimented parasitic elements from some of the extracts. Faecal fat is dissolved in the ether.

Method

1g of faecal sample was emulsified in 4 ml of formol saline contained in a test - tube. 3 ml of formol saline was added and the samples mixed thoroughly by vigorous shaking. The emulsified faeces was sieved into another test-tube. 3 ml of ether was then added and the suspension was thoroughly mixed for 1 minute. The suspension was centrifuged at 1,500 rpm for 2 minutes. Faecal debris was loosened from the side of the tube with a stick, after which, the tube was inverted to discard the ether, faecal debris and formol saline leaving the sediment at the bottom. The tube was tapped at the bottom to resuspend and mix the sediment. A drop of iodine was added into all tubes. The sediment was then transferred onto a slide, covered with coverslip and examined microscopically using the 10 x and 40 x objectives. Cysts or eggs seen were identified.

#### PROCEDURE FOR EXAMINATION OF ALL THE FAECAL SAMPLES

DAY 1

* + - Descriptions of appearance - The appearance of the entire specimen were described as in colour; whether formed, semi formed, or watery; presence of blood and/or mucus; and presence of adult worms or part of adult worms.
    - Microscopy – All samples were examined for ova/cysts of parasites using the Formol-ether sedimentation technique.
    - Culture of the specimen – All samples received was innoculated onto the following media: Deoxycholate Citrate Agar (DCA), Selenite F broth (SF), Sorbitol Mac-Conkey agar (SMAC), Cefixime-Tellurite sorbitol MacConkey Agar (CT-SMAC), Buffered Peptone Water with Vancomycin, Cefsulodin, and Cefixime (BPW-VCC), modified Tryptone soy broth (mTSB). For suspected cases of cholera, in addition to the above alkaline peptone water (APW) - incubated at 37oC for 5-8 hrs, and Thiosulphate Citrate bile-salt sucrose agar (TCBS) - were also innoculated.
    - All plates were incubated at 37oC for 18 – 24hrs.

The purposes for the media innoculated above were:

* + - For Routine Enteric Pathogens i.e Salmonella/Shigella species: DCA and Selenite F as an enrichment broth.
    - For E.coli O157:H7: Direct culture on SMAC and CT-SMAC; Enrichment in BPW-VCC for six hours followed by immunomagnetic separation (IMS) then subculture of beads into SMAC and CT-SMAC.
    - For Detection of other serotypes of Enterohaemorrhagic E.coli: Enrichment in modified tryptone soybroth. Faecal extracts of the culture on mTSB was made

for use in verocytotoxicity assay for these serotypes. The extracts were stored at –20oC until ready for use.

DAY 2

Examination and reporting of the cultures

* + - DCA – Here, non-lactose fermenters shown as colourless colonies that may be either Salmonella/Shigella species were looked for. When seen, they were innoculated into urea and incubated at 37oC for 4 hours. This was to rule out Proteus. If positive, it was disregarded but if negative, other biochemical tests were set up. These include kliglers iron agar (KIA), simmon citrate agar, peptone water (for motility and Indole Test), MR broth (for MRVP Test), nutrient agar (for serology), and macconkey agar (To ensure purity). All were incubated at 37oC for 18 – 24 hrs.
    - SF: Subculture was made from this fluid medium onto DCA.
    - SMAC, CT – SMAC, IMS-SMAC and IMS – CT – SMAC: Here, non-sorbitol fermenters that appeared as colourless colonies that could be E.coli O157:H7 were looked for. When seen, they were serotyped using Oxoid E.coli O157:H7 latex agglutination test kit. Biochemical tests were done on all NSF’s. These tests include innoculation into kliglers iron agar (KIA), simmon citrate agar, urea agar, peptone water (for motility and indole test), and MR broth (for MR VP tests). All were incubated at 37oC for 18 – 24 hours.
    - mTSB – Faecal extracts were made from this and stored in the freezer for use in verocytotoxicity assay.
    - Additional media innoculated for the suspected cases of cholera
    - Alkaline Peptone water: Motility test was done from this medium to check for the characteristic darting motility of Vibrio cholerae.
    - TCBS: Yellow colonies that could be V.cholerae were looked for. When present, the following tests were done: oxidase tests, Vibrios are oxidase positive; Gram Stain, Gram-negative coma-shaped rods were looked for on the gram stain. Other biochemical tests done include innoculation onto KIA, citrate, urea, peptone water, MR broth, and nutrient agar. All were incubated overnight at 37oC.
    - Distilled water motility tests were also done to distinguish Aeromonas from

Vibrios

DAY 3

All Biochemical tests were read. Results were recorded and isolates fully identified.

Antimicrobial sensitivity tests were done on all relevant isolates using the Modified Kirby –Bauer disc diffusion method (Cheesborough, 2000). Interpretation of zone diameters was done using the National Committee for Clinical Laboratory standards (NCCLS) interpretative chart (Bopp et. al., 1999).

DAY 4

Antimicrobial susceptibility tests were read. Results were written out and sent to clinicians for further patient management. Note: All samples were collected from sick patients. So, Clinicians were notified of all results for further patient management.

Description of Appearance Formol Ether concentration technique

CULTURE

Microscopy

Macroscopy

**Specimen Collection/Registration**

Innoculate into and incubate at 37°C overnight

Additional in suspected cases of choler

DCA

SF

SMAC

CT-SMAC

BPW-VCC

MTSB

APW

TCBS

Sub on DCA

Examine for

Suspicious colonies (NLF)

Incubate for 6hrs at 37ºc

IMS, then

Sub on SMAC, CT-SMAC

Incubate overnight at 37ºC.

Incubate for 6hrs Do motility

test for xteristic *V*. *cholerae* motility

Set Biochemicals For identification



Look for suspicious cols (NSF)

Store at -20ºC for use in faecal Ctotoxicity assay

Look for yellow cols that could be v.*cholerae*

Oxidase test, Gram stain

Serotype with oxoid *E.coli* O157:H7 Latex Agglutination Test kit

Set biochemical test for identification

Set Biochemicals Distilled water motility test

Set sensitivity Test

Incubate overnight at 37ºC DISPATCH RESULTS.

#### Figure 1: Algorithm of Procedures for the Study

**CHAPTER FOUR RESULTS**

#### Age and sex Distribution of Diarrhoeal Patients

A total number of one hundred and six (106) faecal samples were analysed. Sixty- two of these were from children between the ages of one month to fifteen years while forty-four were from adults. They were forty-nine males and fifty-seven females.

Table 2 shows the age distribution of diarrhoeal patients included in the study. It shows that age is statistically significantly associated with diarrhoeal disease in Abuja (P< 0.05). This statistical analysis and subsequent ones were done using SPSS V10 (Computer software). The highest incidence of diarrhoeal diseases occurs between the ages of zero to five years (54.7%).

* 1. ***Escherichia coli* O157:H7**

E. coli O157 was not isolated from any of the 106 faecal samples analysed.

However, 49 non-sorbitol fermenting organisms were isolated. The non-sorbitol fermenting bacteria isolated from diarrhoeal cases in the Federal Capital Territory, Abuja are shown in Table 3. Non-sorbitol fermenting (NSF) Escherichia coli (i.e. NSF E. coli other than E. coli O157:H7) constituted 22.4% of all the NSF’s isolated and 10.4% of the total number of samples analysed. Other non-sorbitol fermenters include Vibrio cholerae (1.9% of the total number of samples analysed), Klebsiella pneumoniae (0.9%), Pseudomonas spp (8.5%), Shigella flexneri (4.7%), Shigella dysenteriae A1 (1.9%), and Shigella species (0.9%) (The isolate was not typable but gave all the biochemical reactions of Shigella species but did not react with the three anti-Shigella antiserum

available i.e. anti-Shigella dysenteriae A1, A2 and anti-Shigella flexneri). Considering the fact that antiserum was not available for other Shigella species like Shigella boydii and Shigella sonneii, the isolate was just reported as Shigella species. The rest of the non- sorbitol fermenters isolated include Morganella morganii (1.9%), Proteus mirabilis (1.9%), Proteus vulgaris (2.8%), Providencia retgerii (0.9%) and Enterobacter aerogenes (0.9%). There were nine non-sorbitol fermenters (8%) isolated that were not identified due to oversight at the early stages of the study. However, isolation of non- sorbitol fermenters in sorbitol macconkey agar was more significantly associated with E. coli and Pseudomonas specie, statistically, than other bacterial species (P<0.05).

* 1. **Non-sorbitol fermenting *E. coli* other than *E. coli* O157:H7**

The eleven NSF E. coli isolates are analysed as shown in Table 4. They were isolated from 10 samples, one of which yielded two clearly different serotypes of NSF E. coli. Nine of the samples appeared completely watery or loose. Only one sample from a chronic case of diarrhoea appeared semi-formed. These organisms were isolated from male and female, children and adults. The place of residence of patients from whom these organisms were isolated seems to be fairly spread out within the Federal Capital Territory ie there is no clustering of these cases geographically within the territory. One of the samples, however, was from a retroviral disease (RVD) patient resident in Makurdi on referral to National Hospital, Abuja.

Eight of the eleven isolates were motile, six of which exhibited a sluggish kind of motility. One of the isolates with normal motility gave a false postive reaction (auto- agglutination). It reacted positively with both test and control latex of E. coli O157:H7 serological kit. Three of the isolates were non-motile (Sample nos 068, 084, 087). Two

of the three non-motiles (084, 087) were equally non-lactose fermenting. Thus, they are non-sorbitol fermenting (NSF), non-lactose fermenting (NLF) and non-motile (NM) E. coli (NSF, NLF, NM E. coli). The remaining non-motile one was lactose fermenting (NSF, LF, NM, E. coli). However, all the eight motile ones were lactose fermenting.

None of the ten samples yielded any other bacterial pathogen. Only one of the ten

(087) yielded egg of Schistosoma mansonii. This was from a patient with chronic renal failure who later died from complications of the renal failure.

Generally, sorbitol macconkey agar (SMAC) supported the growth of these NSF E. coli’s better than cefixime-tellurite macconkey agar. Inclusion of the Immunomagnetic separation procedure did not improve the rate of isolation.

To determine whether any of these eleven NSF E. coli’s is shiga-toxin or vero- cytotoxin producing, they were tested against a panel of virulence markers by Polymerase chain reaction (PCR). Results of this are shown on Table 5. None was positive for the shiga toxins, Stx1 and Stx2 and enterohaemolysin (Ehly) genes. Two were positive for the E. coli attaching and effacing (eae) gene. One of the two that were positive for eae gene serotyped as Escherichia coli O Rough: Non-Motile. The other serotyped as Escherichia albertii (formerly called Shigella boydii 13).

#### Other serotypes of Enterohaemorrhagic *E. coli* (EHEC)

Cytotoxic activities of the stool filtrates were examined in all the 106 faecal samples used for the study. Free faecal toxin was demonstrated in 16 (15.09%) of the 106 samples examined (Table 6). Microscopically, the cytopathic effect (CPE) on the cells started by the cells rounding up. Eventually, they got detached from the cell sheet within the next 24hrs (Plate Ib). Plate Ia shows the appearance of the normal vero

monolayer. The undiluted stool filtrates of all the samples except one induced the above effect in about 25% of the monolayer within 24hrs. The exception, sample no 058, from the five year old daughter of a diplomat induced the CPE in 50% 0f the monolayer within 24hrs. It was not observed whether the CPE advanced with time.

Out of the 16 samples that gave cytotoxic activity, only one yielded a common enteric pathogen – Shigella dysenteriae A1 from stool culture (Table 6). No other usual pathogens were isolated from the remaining 15 samples. However, three of these 16 samples yielded non-sorbitol fermenting E. coli. In addition to the NSF E. coli, one of the three yielded Schistosoma mansonii from the stool microscopy. The remaining twelve

(12) did not give any significant baceterial yield. However, yeast cells were seen in the microscopy of one of them.

The characteristics of the sixteen patients are shown on Tables 7, 8, and 9. Table

7 details the age, sex, residence, food history, travel history and the presence or absence of diarrhoea in family members of the sixteen patients. The youngest patient was 1/12yrs and the oldest 44yrs. Age predominance was observed (Table 8). 12 (75%) out of the 16 patients were children between the ages of zero to 3years. This is statistically significant (P< 0.05). Two (12.5%) were children aged between 4years and 5yrs. The remaining 2 (12.5%) were from adults, 27yrs and 44yrs respectively. No sex predominance was observed; there was no clustering of cases geographically or in time within the Federal Capital Territory. No specific food item could be implicated as the source of infection. Food history was not obtained from majority of the cases. However, the few children that had their feeding pattern listed in the questionnaire either were on breast milk or unnamed Infant formular. There was no history of recent travel abroad for

all the sixteen cases. Two of the children had a member of their family also down with diarrhoea at the same time.

Out of the 16 patients, the male female ratio is 9:7; age distribution was 0.08yr – 44yrs with the mean being ± 6yrs. The clinical features of the diarrhoeal Patients are as shown on Table 9. 25% had nausea, 37.5% had abdominal cramps, 81.25%, watery diarrhoea, 18.75% bloody diarrhoea, 50% has mucus in their stool samples, 31.25% vomiting and 62.5% had fever.

#### Enteric Pathogens Isolated from the study

There were 14 bacterial pathogens isolated, 3 parasites and 7 Candida species (Table 10). Shigella species have the highest frequency of eight out of the 24 pathogens isolated giving an isolation rate of 33.3% of the pathogens isolated and 7.7% of the total number of samples analysed. Among these, Shigella flexneri has the highest percentage positive (20.8% of the pathogens isolated and 4.7% of the samples analyzed). Others are Shigella dysenteriae A1, 8.3% and 1.9% respectively; unidentified Shigella species, 4.2% and 0.95% respectively. Other bacterial pathogens isolated include: - Vibrio cholerae O1 serovar Ogawa, Salmonella typhi, Salmonella paratyphi B and Salmonella paratyphi C. Both V. cholerae and Salmonella typhi have isolation rates of 8.3% per total number of pathogens isolated and 1.9% of the total number of samples analyzed while both Salmonella paratyphi B and C have 4.2% and 0.95% respectively. The V. cholerae isolates were from sporadic cases of cholera. There was no outbreak during the study period.

The Candida species have an isolation rate of 29.2% of the total number of pathogens isolated and 6.6% of the total number of samples analysed. The three

parasites – Entamoeba histolytica, Schistosoma mansonii and hookworm have the same rates of isolation of 4.2% of the pathogens isolated and 0.95% of the samples analyzed. In all, the 24 pathogens isolated were 22.6% of the samples analyzed.

Table 11 shows the analysis of the Shigella flexnerii isolates from diarrhoeal cases in the FCT, Abuja. Out of the five isolates, only two from No. 079 and 103 were isolated from Deoxycholate Citrate Agar (DCA) and Selenite F (SF) broth-media routinely used for stool culture. The three others, which would, ordinarily, have been missed, were isolated from the sorbitol macconkey agar of various compositions. However, one common pattern of four out of the five isolates was growth on sorbitol macconkey agar. The only one that did not grow on this (No 090) grew after immunomagnetic separation and culture on IMS-SMAC and IMS-CT-SMAC.

Thus, Sorbitol MacConkey Agar was found to support the growth of Shigella flexnerii better than the Deoxycholate Citrate Agar (DCA) routinely used for stool culture. This raised the need to have a more detailed study done on these isolates to find out if there could be anything genetically distinct in them that could account for this inability. The isolates were, therefore, sent to CDC for characterization. Results of this study are shown on Tables 12 and 13.

Table 12 shows serology results of the isolates. All four isolates were confirmed as Shigella flexneri. Two are Shigella flexnerii 2a(II: 3,4) while the other two are Shigella flexnerii serotype 1b(I: 6). The antigenic formular is in parenthesis describing the specific antigens. One of the isolates that serotyped 2a(II: 3,4) grew on both SMAC and DCA. The rest grew on SMAC only. From the results, there is nothing antigenically distinct in any of them from established serotypes in literature.

Table 13 shows antimicrobial susceptibility test result of the isolates. All four isolates showed 100% resistance to the six antibiotics used – chloramphenicol, trimethoprim-sulphamethoxazole, tetracycline, ampicillin, sulfisoxazole and streptomycin.

Table 14 details the summary of the clinical features of all the patients included in the study according to the isolates obtained. 30% of patients with NSF E. coli isolated from their stool samples presented with nausea, 40% abdominal cramps, 90% had watery diarrhoea, 40% had bloody diarrhoea, 40% showed presence of mucus in their sample, 30% presented with vomiting and 40% with fever. These patients had male to female ratio of 7:3; age distribution was 4/12yrs - 62yrs with the mean being ±26.9 years.

Patients that had Shigella species isolated from their samples presented with the following clinical features: - nausea (20%), abdominal cramps (50%), watery diarrhoea (85.5%), bloody diarrhoea (75%), presence of mucus (50%), vomitting (35%) and fever (87.5%). The male to female ratio was 4:4. The age distribution was 1.5yrs - 51 yrs with the mean being ± 20.5 yrs.

For those with Salmonellae infections the following presentations were observed: - nausea (25%), abdominal cramps (25%), watery diarrhoea (100%), bloody diarrhoea (25%), presence of mucus (25%), vomitting (75%), fever (100%). The male to female ratio was 4:0; age distribution 0.5-25 years while the mean was ± 9.1 years.

Patients with cholera had the following presentation: - nausea (50%), abdominal cramps (50%), watery diarrhoea (100%), bloody diarrhoea (0%), presence of mucus (100%), vomitting (100%), fever (50%). The male to female ratio was 1:1, age distribution 30-35 years while the mean was ± 32.5 years.

57% of patients with Candida infection had nausea. The rest of the clinical features are as follows: - abdominal cramps (29%), watery diarrhoea (86%), bloody diarrhoea (0%), presence of mucus (57%), vomitting (43%), fever (57%). The male to female ratio was 3:4; age distribution 0.7-58 years while the mean was ± 19.2 years.

For patients with enteric parasites, the clinical features observed were nausea (0%), abdominal cramps (33%), watery diarrhoea (67%), bloody diarrhoea (0%), presence of mucus (33%), vomitting (0%), fever (33%). The male to female ratio was 2:1, age distribution 30-44 years while the mean was ± 37.3 years.

The clinical features of the rest of the patients with no enteric pathogens detected from their stool samples were also analysed. The following figures were obtained; - nausea (26%), abdominal cramps (47%), watery diarrhoea (78%), bloody diarrhoea (18%), presence of mucus (34%), vomitting (31%), fever (38%). The male to female ratio was 29:45; age distribution four days-66 years while the mean was ± 14.2 years.

#### Antimicrobial Susceptibility Patterns

The percentage antimicrobial susceptibility patterns of all the entire bacterial pathogens isolated from the study are shown on Table 15. 100% of the Shigella species isolated from the study were resistant to tetracycline while 100% of them were sensitive to ciprofloxacin. Out of the Shigella flexneri isolates, only 20% were sensitive to chloramphenicol, 40% were intermediate while 40% were resistant. In the case of Shigella dysenteriae A1 and the Shigella species isolates, 100% of them were resistant to chloramphenicol. For amoxycillin-clavulanic acid, Shigella flexneri isolates gave 40% susceptible, 20% intermediate and 40% resistant; Shigella dysenteriae A1 gave a pattern of 0% sensitive, 50% intermediate and 50% resistant while the Shigella species

gave 100% susceptibility to Amoxycillin-clavulanic acid. 40% of the Shigella flexneri isolates was sensitive to Trimethoprim-sulphamethoxazole while 60% were resistant. 100% of Shigella dysenteriae were resistant to the drug. However, the Shigella species were not tested against Trimethoprim-sulphamethoxazole. Out of the Shigella flexneri isolates from the study 75% were susceptible to Ampicillin, 25% showed intermediate susceptibility while 100% of the Shigella dysenteriae A1 were sensitive. Ampicillin also was not tested against the Shigella species isolated. The susceptibility pattern of the Shigella flexneri isolates to Nalidixic acid is 40% susceptible, 40% intermediate and 20% resistant. Shigella dysenteriae A1 and the Shigella species isolates were not tested against Nalidixic acid.

Analysis of the susceptibility pattern of the Salmonella isolates showed that 100% of the Salmonella typhi isolates were resistant to tetracycline, while 100% of Salmonella paratyphi B and 100% Salmonella paratyphi C were sensitive to the same drug. The pattern for chloramphenicol is as follows: 100% susceptible for Salmonella typhi and Salmonella paratyphi C while Salmonella paratyphi B was 100% resistant. For amoxycillin-clavulanic acid, Salmonella typhi was 50% susceptible and 50% resistant, 100% of the Salmonella paratyphi B was of intermediate category while 100% of Salmonella paratyphi C was sensitive. All the Salmonella species were 100% susceptible to ciprofloxacin. Then, for trimethoprim-sulphamethoxazole, 100% of Salmonella typhi was susceptible while 100% of Salmonella paratyphi B and 100% of Salmonella paratyphi C were resistant. The Salmonella isolates were not tested against ampicillin and nalidixic acid.

The V. cholerae isolates were 100% susceptibility to tetracycline, amoxycillin- clavulanic acid, ciprofloxacin, and 100% resistant to trimethoprim-sulphamethoxazole.

For chloramphenicol, 50% were sensitive while 50% were of intermediate category. The

V. cholerae isolates were not tested against ampicillin and nalidixic acid.

#### Table 2: Age Distribution of Diarrhoeal Patients in the Federal Capital Territory, Abuja, 2003

|  |  |  |
| --- | --- | --- |
| AGE GROUP | NUMBER | PERCENT (%) |
| 0 – 5yrs | 58 | 54.7 |
| 5 – 10yrs | 3 | 2.8 |
| 10 – 15yrs | 1 | 0.9 |
| 15 – 20yrs | 3 | 2.8 |
| 20 – 25yrs | 2 | 1.9 |
| 25 – 30yrs | 9 | 8.5 |
| 30 – 35yrs | 7 | 6.6 |
| 35 – 40yrs | 8 | 7.5 |
| 40 – 45yrs | 6 | 5.7 |
| 45 – 50yrs | 2 | 1.9 |
| 50 – 55yrs | 4 | 3.8 |
| 55yrs and above | 3 | 2.8 |
| Total | 106 | 100.0 |

X2 = 306.755, P value = 0.000, df = 11

#### Table 3: Non-Sorbitol Fermenting Organisms Isolated from Diarrhoeal cases in the Federal Capital Territory, Abuja, 2003

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| SPECIES | NUMBER | OF | PERCENTAGES | (%) |  |
|  | ISOLATES |  | Per Total No. | of Per Total No. | of |
|  |  |  | NSF’s Isolated | Samples |  |
|  |  |  |  | Analysed(n) |  |
| NSF E.coli (Not O157) | 11 |  | 22.4 | 10.4 |  |
| Vibrio cholerae | 2 |  | 4.1 | 1.9 |  |
| Klebsiella pneumoniae | 1 |  | 2.0 | 0.9 |  |
| Pseudomonas specie | 9 |  | 18.4 | 8.5 |  |
| Shigella flexneri | 5 |  | 10.2 | 4.7 |  |
| Shigella dysenteriae | 2 |  | 4.1 | 1.9 |  |
| A1  Shigella specie | 1 |  | 2.0 | 0.9 |  |
| Morganella morgani | 2 |  | 4.1 | 1.9 |  |
| Proteus mirabilis | 2 |  | 4.1 | 1.9 |  |
| Proteus vulgaris | 3 |  | 6.1 | 2.8 |  |
| Providencia retgerri | 1 |  | 2.0 | 0.9 |  |
| Enterobacter  aerogenes | 1 |  | 2.0 | 0.9 |  |
| NSF’s (not identified) | 9 |  | 18.4 | 8.5 |  |
| TOTAL | 49 |  |  |  |  |

X2 = 40.408, P value = 0.000, df = 12

#### PLATE I & II

**Table 4: Analysis of Non-Sorbitol Fermenting *E. coli* from Diarrhoeal Cases in the Federal Capital Territory Abuja, 2003**

Sample

Age Sex Residence App. Of

Parasites

Other

ISOLATES OBTAINED FROM

No Samples

Brownish watery, No

Seen

Pathogens Isolated

SMAC CT- SMAC

IMS- SMAC

IMS-CT- SMAC

REMARKS

Isolate showed poor growth and no growth at all on CT-

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 50 | 28yrs | F | Nyanya | Blood Nomucus | | | | - - + | | | | - | + | Not cultured on this | SMAC. It was this isolate that showed the need to inoculate |
|  |  |  |  |  | | | |  | | | |  |  |  | SMAC after IMS. |
| 54 | 47yrs | F | Wuse | Brownish, | | | | - - + | | | | - | + | - | This isolate seemed to require |
|  |  |  |  | mucoid, Bloody. | | | |  | | | |  |  |  | heavy innoculum to obtain a |
|  |  |  |  |  | | | |  | | | |  |  |  | positive MR Result. |
| 66 | 45yrs | M | Gwagwa | Brownish, loose, | | | | - - + | | | | + | + | - | Isolates from SMAC, CT-SMAC |
|  |  |  |  | mucoid, No | | | |  | | | |  |  |  | & IMS-CT-SMAC were exactly |
|  |  |  |  | Blood. | | | |  | | | |  |  |  | the same. IMS-SMAC yielded |
|  |  |  |  |  | | | |  | | | |  |  |  | mixed growth of NSF of ? |
|  |  |  |  |  | | | |  | | | |  |  |  | Motility (RVD Patient) |
| 68 | 12/12yrs | M | Old Karu |  | Brownish, |  | | - - + - - - Sample yielded just one | | | | | | | |
|  |  |  |  |  | watery, | No | | colony on SMAC. On | | | | | | | |
|  |  |  |  |  | blood, | No | | subculture showed poor | | | | | | | |
|  |  |  |  |  | mucus |  | | growth on CT-SMAC. Isolate is | | | | | | | |
|  |  |  |  |  |  |  | | non-motile (NM, NSF, LF E. | | | | | | | |
|  |  |  |  |  |  |  | | coli). | | | | | | | |
| 75 | 4.5/12yrs | F | Area | 11, | Brownish, | loose, | | - - - - - + Identified as Proteus vulgaris. | | | | | | | |
|  |  |  | Garki. | mucoid | | | with |  | | | |  |  |  | Only IMS-CT-SMAC yielded |
|  |  |  |  | blood stains | | |  |  | | | |  |  |  | NSF E. coli. Since the plate |
|  |  |  |  |  | | |  |  | | | |  |  |  | showed mixed growth of |
|  |  |  |  |  | | |  |  | | | |  |  |  | NSF’s, it is possible that |
|  |  |  |  |  | | |  |  | | | |  |  |  | growth of NSF E. coli may |
|  |  |  |  |  | | |  |  | | | |  |  |  | have been masked in the |
|  |  |  |  |  | | |  |  | | | |  |  |  | other plates. |
| 84 | 4/12yr | M | Mararaba | Watery, | | | No | - - + | | | | + | + | + | All four plates yielded NSF E. |
|  |  |  |  | blood, | | | No |  | | | |  |  |  | coli that gave the same |
|  |  |  |  | mucus | | |  |  | | | |  |  |  | biochemical result. NSF, NM, |
|  |  |  |  |  | | |  |  | | | |  |  |  | NLF E. coli. |
| 87 | 44yrs | M | Kubwa | Dark brownish | | | | Ova | of | - | + | - | + | - | Case of chronic renal failure |

|  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | | | | watery, blood,  mucus | No No | Schistosoma mansonii |  |  |  |  | (CRF) biochemically similar to the immediate one above,  NSF, NM, NLF E. coli. R. I. P. |
| 98 | 40yrs | M | Markurdi | Yellowish, watery, | No | - | - + | + | + | + | Yielded 2 serotypes of NSF E. coli: (a) showed |
| 101 | 62yrs | M | Maitama | blood, No Mucus.  Brownish, Semi- | | autoagglutination ie positive rxn on both test and control latex of E. coli O157:H7 serological kit. This isolate also showed gas production  +. (b) The second isolate was completely negative on both test and control latex. It showed gas production ++. Both isolates were observed on SMAC plate. Only (b) was seen on CT-SMAC. Growth wasgenerally poorer on CT- SMAC than smac. The 2 isolates showed larger colonies on SMAC – 2-3mm  RVD Patient smooth slightly raised moist  NSF colonies with entire edges.  - - + - + - NSF’s isolated on all 4 plates. | | | | | |
| 102 | 16/12yrs | M | Adisa | formed, No blood, Nomucus  Brownish,watery | | Isolates on SMAC yielded NSF  E. coli. Isolates on CT-SMAC & IMS-SMAC yielded Morganella morganii. Bio on NSF from IMS-CT-SMAC was not found. CONCLUSION – isolate have poor growth on CT-SMAC.  - - - + - - Isolated only on CT-SMAC. | | | | | |
|  |  |  | Estate, Gudu  District | mucoid, No blood seen | | Showed no motility. | | | | | |
| **Key** | |  |  |  | |  | | | | | |
| + =  - = | Growth No Growth |  |  |  | |  | | | | | |

#### Table 5: Screening of Non-Sorbitol Fermenting *E. coli* Isolates from Diarrhoeal cases in Federal Capital Territory, Abuja, 2003, for Virulence Markers using PCR

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Sample No | Stx 1 gene | Stx 2 gene | Eae  gene | Ehly  gene | Serotype | | |
| Is 50 | - | - | - | - | - | | |
| Is 54 | - | - | - | - | - | | |
| Is 66 | - | - | - | - | - | | |
| Is 68 | - | - | - | - | - | | |
| Is 75 | - | - | - | - | - | | |
| Is 84 | - | - | + | - | Escherichia albertii (formerly  Shigella boydii 13) | | |
| Is 87 | - | - | - | - | - | | |
| Is 98a | - | - | - | - | - | | |
| Is 98b | - | - | - | - | - | | |
| Is 101 | - | - | - | - | - | | |
| Is 102 | - | - | + | - | Escherichia | coli | OROUGH: |
|  |  |  |  |  | NONMOTILE. |  |  |

**KEY:**

+ - Positive

- - Negative

PCR – Polymerase Chain Reaction.

CDC – Center for Disease Control, Atlanta Georgia USA.

eae - E. coli attaching and effacing.

Stx 1 - Shiga Toxin 1

Stx 2 – shiga toxin 2

Ehly - Enterohemolysin

#### Table 6: Cytotoxic Activity of Stool Filtrates on Vero Cells and Bacterial Yield from Diarrhoeal Cases in FCT, 2003

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| SAMPLE NO | ACTIVITY OF STOOL FILTRATES ON VERO MONOLAYER. | ORGANISMS ISOLATED STOOL CULTURE. | FROM | THE |
| 010 | + | \_ |  |  |
| 025 | + | \_ |  |  |
| 026 | + | \_ |  |  |
| 028 | + | \_ |  |  |
| 030 | + | \_ |  |  |
| 043 | + | \_ |  |  |
| 053 | + | \_ |  |  |
| 058 | ++ | \_ |  |  |
| 075 | + | NSF E. coli |  |  |
| 081 | + | \_ |  |  |
| 083 | + | Shigella dysenteriae A1 |  |  |
| 084 | + | NSF, NLF, NM E. coli |  |  |
| 087 | + | NSF, NLF, NM E. coli |  |  |
| 094 | + | \_ |  |  |
| 097 | + | \_ |  |  |
| 105 | + | \_ |  |  |

**Key**

+ - Rating of morphological effect corresponding to roughly <25% of cells affected

++ - Rating of Morphological effect corresponding to roughly 50% of cells affected

#### Table 7: Analysis of Samples with Cytotoxic Activity from Stool Filtrates of Diarrhoeal Cases in the Federal Capital Territory Abuja, 2003

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Sample No | Age | Sex | Residence | Food History | Travel History | Diarrhoeal | illness |
|  |  |  |  |  |  | infamilymembers | |
| 010 | 2 5/12yrs | M | Wuse II, Urban | Cerelac, small | Nil | Yes | |
|  |  |  |  | qty of rice |  |  | |
| 025 | 6/12 yr | F | Asokoro, Urban | Breast milkonly | Benin | No | |
| 026 | 27yrs | M | Suleja, Urban | NA | NA | NA | |
| 028 | 6/12 yr | F | Karmo, Periurban | NA | NA | NA | |
| 030 | 9/12yrs | M | BPE, Abuja, Urban | NA | NA | NA | |
| 043 | 18/12 yrs | F | Area1, Garki, Urban | Frisocrem, Tea | Kano | NA | |
| 053 | 11/12 yr | M | Wuse, Urban | NA | NA | NA | |
| 058 | 5yrs | F | Maitama, Urban | NA | Gurara falls | No | |
| 075 | 45/12 yr | F | Area11, Garki, Urban | Infant formular | Kaduna | Yes (older Brother) | |
|  |  |  |  | purchased at |  |  | |
|  |  |  |  | kaduna |  |  | |
| 081 | 6/12 yr | M | NA | NA | Nil | No | |
| 083 | 3yrs | F | Gausau (Zamfara State) | NA | Nil | No | |
|  |  |  | Urban |  |  |  | |
| 084 | 4/12 yr | M | Maraba, Periurban | NA | Nil | No | |
| 087 | 44yrs | M | Kubwa, Urban | NA | NA | NA | |
| 094 | 1/12 yr | M | Maitama, Urban | Breast Milk | Nil | No | |
| 097 | 26/12yr | M | Wuse II, Urban | NA | NA | NA | |
| 105 | 18/12 YRS | F | NA | NA | NA | NA | |

**Key**

NA – Information not available

#### Table 8: Age Groups of Diarrhoeal Patients with Cytotoxic Activity from Stool Filtrates in Abuja, 2003

|  |  |  |
| --- | --- | --- |
| AGE GROUP | FREQUENCY | PERCENT (%) |
| 0 – 3yrs | 12 | 75.0 |
| 4 – 5yrs | 2 | 12.5 |
| Above 5yrs | 2 | 12.5 |
| Total | 16 | 100.0 |

X2 = 12.500, P value = 0.002, df = 2

#### Table 9: Clinical Features of Diarrhoeal Patients in the Federal Capital Territory Abuja, 2003 whose Stool Filtrates gave Cytotoxic Activity on Vero Cell

|  |  |  |  |
| --- | --- | --- | --- |
| SYMPTOMS | Yes (%) | No | No Response |
| Nausea | 4 (25%) | 11 | 1 |
| Adominal Cramps | 6(37.5%) | 8 | 2 |
| Watery Diarrhoea | 13 (81.25%) | 3 | - |
| Bloody Diarrhoea | 3 (18.75%) | 13 | - |
| Presence of mucus | 8 (50%) | 8 | - |
| Vomiting | 5(31%) | 9 | 2 |
| Fever | 10 (62.5%) | 3 | 3 |
| Total no. of Patients | 16 |  |  |
| Male/Female Ratio | 9: 7 |  |  |
| Age Distribution | 0.08yr – 45yrs |  |  |
| Mean | 6yrs |  |  |

**Table 10: Pathogens Isolated from Diarrhoeal Cases in the Federal Capital Territory Abuja, 2003**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| SPECIES | TYPE | OF | NUMBER | **PERCENTAGES** | **(%)** |  |
|  | MICROORGANISM |  | ISOLATED | Per Total No. of | Total No | of |

Pathogens Isolated

Samples analysed (% pos)

Vibrio cholera O1 Bacteria 2 8.3 1.9

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| serovar Ogawa  Salmonella typhi | " | 2 | 8.3 | 1.9 |
| Salmonella paratyphi | " | 1 | 4.2 | 0.95 |
| B  Salmonella paratyphi | " | 1 | 4.2 | 0.95 |
| C  Shigella specie (not | " | 1 | 4.2 | 0.95 |
| identified to specie)  Shigella flexnerii | " | 5 | 20.8 | 4.7 |
| Shigella dysenteriae | " | 2 | 8.3 | 1.9 |
| A1  Cyst of Entamoeba | Parasite | 1 | 4.2 | 0.95 |
| histolytica |  |  |  |  |
| Egg of Hookworm | " | 1 | 4.2 | 0.95 |
| Egg of Schistosoma | " | 1 | 4.2 | 0.95 |
| mansonii  Yeast cells (Candida | Fungi | 7 | 29.3 | 6.6 |
| sp). |  |  |  |  |
| Total |  | 24 |  | 22.6 |

**KEY**

n = 106

162

**Table 11: Analysis of *Shigella flexnerii* Isolated from Diarrhoeal Cases in the Federal Capital Territory, Abuja, 2003**

Sample

Age Sex Residence App. of

Parasite

Other

ISOLATES OBTAINED FROM

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| No. |  |  | Sample seen | Pathogens |  | | | |
|  |  |  |  | Isolated | SMAC | CT- IMS- | IMS- | DCA SF- |
|  |  |  |  |  |  | SMAC SMAC | CT- | DCA |
|  |  |  |  |  |  |  | SMAC |  |
|  |  |  | Brownish |  |  |  |  |  |
|  |  |  | Watery, |  |  |  |  |  |
|  |  |  | No blood |  |  |  |  |  |
| 62 | 1½yrs M | Lugbe | NoMucus - | - | + | + + | - | - - |
|  |  |  | Brownish |  |  |  |  |  |
|  |  |  | Watery, |  |  |  |  |  |
|  |  | (Caucasian) | No blood |  |  |  |  |  |
| 79 | 42yrs M | Maitama | NoMucus - | - | + | - - | - | + + |
|  |  |  | Brownish |  |  |  |  |  |
|  |  |  | Loose |  |  |  |  |  |
|  |  |  | Mucoid |  |  |  |  |  |
|  |  |  | ?Blood |  |  |  |  |  |
| 90 | 44yrs M | NA | Stains - | - | - | - + | + | - - |
|  |  |  | Brownish |  |  |  |  |  |
|  |  |  | Mucoid |  |  |  |  |  |
|  |  |  | Blood |  |  |  |  |  |
| 96 | 17yrs F | Jabi | stains - | - | + | - - | - | - - |
|  |  |  | Yellowish |  |  |  |  |  |
|  |  |  | Watery |  |  |  |  |  |
|  |  |  | No Blood |  |  |  |  |  |
| 103 | 2yrs F | Asokoro | NoMucus - | - | + | + - | - | + + |

**Key**

+ = Growth

- = No Growth NA = Not Available

#### Table 12: Characterization of *Shigella flexnerii* Isolated from Diarrhoeal Cases in the Federal Capital Territory, Abuja 2003

|  |  |  |  |
| --- | --- | --- | --- |
| CDC EISL # | Sample No | Denka Seiken Poly B | S. flexneri serotype |
| K0815 | 103 | + | 2a(II: 3,4) |
| 04-3440 |  |  |  |
| K0816 | 62 | + | 1b(I: 6) |
| 04-3441 |  |  |  |
| K0817 | 90 | + | 2a(II: 3,4) |
| 04-3442 |  |  |  |
| K0818 | 96 | + | 1b(I: 6) |
| 04-3443 |  |  |  |

**Key**

+ = Positive

CDC = Centre for Disease Control, Atlanta, Georgia USA.

EISL # = Epidemic Investigations and Surveillance Laboratory No.

#### Table 13: Antibacterial Susceptibility Test Results of *Shigella flexneri* Isolates From Diarrhoeal Cases in Federal Capital Territory, Abuja, 2003

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| CDC EISL# | Sample No | Denka Seiken Poly B | Resistance | Comment |  |
| K0815 | 103 | + | Chloramphenicol | Only one colony |  |
|  |  |  | Trimethoprim- | Type |  |
|  |  |  | Sulfamethoxazole |  |  |
|  |  |  | Ampicillin |  |  |
|  |  |  | Sulfosoxazole |  |  |
|  |  |  | Streptomycin |  |  |
| K0816 | 62 | + | Chloramphenicol | Tested both smooth | and |
|  |  |  | Trimethoprim- | irregular colony type |  |
|  |  |  | Sulfamethoxazole |  |  |
|  |  |  | Ampicillin |  |  |
|  |  |  | Sulfosoxazole |  |  |
|  |  |  | Streptomycin |  |  |
| K0817 | 90 | + | Chloramphenicol | Tested both smooth | and |
|  |  |  | Trimethoprim- | irregular colony type |  |
|  |  |  | Sulfamethoxazole |  |  |
|  |  |  | Ampicillin |  |  |
|  |  |  | Sulfosoxazole |  |  |
|  |  |  | Streptomycin |  |  |
| K0818 | 96 | + | Chloramphenicol | Tested both smooth | and |
|  |  |  | Trimethoprim- | irregular colony type |  |
|  |  |  | Sulfamethoxazole |  |  |
|  |  |  | Ampicillin |  |  |
|  |  |  | Sulfisoxazole |  |  |
|  |  |  | Streptomycin |  |  |
| **Key** |  |  |  |  |  |

+ = Positive.

CDC = Centre for Disease Control Atlanta, GA, USA.

EISL# = Epidemic Investigations and Surveillance Laboratory No.

**Table 14: Summary of Clinical Features of Studied Diarrhoeal Patients in the Federal Capital Territory, Abuja 2003**

Cases with NSF E. coli

Isolated

Cases with Shigella sp Isolated

Cases with

Salmonella sp

Cases with V. cholerae Isolated

Cases with Candida

sp Identified

Cases with Enteric parasitesseen

Caseswithnopathologic agentdetected

Sympto Isolated

ms Yes No Un- Kno wn

Yes N

o

Un- Kno wn

Yes N

o

Un- Kno wn

Yes N

o

Un- Kno wn

Yes N

o

Un- Kno wn

Yes N

o

Un- Kno wn

Yes No Un- Known

Nausea 3(30%) 7 - 2(20%) 5 1 1(25%) 2 1 1(50%) 1 - 4(57%) 1 2 0(0%) 3 - 19(26%) 39 16

Abdomi nal Cramps Watery Diarrhea Bloody Diarrhea Presenc e of

mucus Vomitin g

4(40%) 6 - 4(50%) 3 1 1(25%) 2 1 1(50%) 1 - 2(29%) 3 2 1(33%) 2 - 35(47%) 23 16

9(90%) 1 - 7(85.5%) 1 - 4(100% - - 2(100%) - - 6(86%) 1 - 2(67%) 1 - 58(78%) 16 -

4(40%) 6 - 6(75%) 2 - 1(25%) 3 - - 2 - 0(0%) 7 - 0(0%) 3 - 13(18%) 61 -

4(40%) 6 - 4(50%) 4 - 1(25%) 3 - 2(100%) - - 4(57%) 3 - 1(33%) 2 - 25(34%) 49 -

3(30%) 7 - 2(25%) 5 1 3(75%) - 1 2(100%) - - 3(43%) 2 2 0(0%) 3 - 23(31%) 35 16

Fever 4(40%) 6 - 7(87.5%) - 1 4(100%

)

- - 1(50%) 1 - 4(57%) 1 2 1(33%) 2 - 28(38%) 30 16

Total No. of Patients Male/Fe male Ratios Age Distribut ion

10 8 4 2 7 3 74

7:3 4:4 4:0 1:1 3:4 2:1 29:45

4/12 yrs – 62yrs 1½yrs – 51yrs 6/12 yr – 25yrs 30 – 35yrs 9/12yrs – 58yrs 30yrs – 44yrs 4days – 66yrs

Mean ±26.9yrs ±20.5yrs ±9.1yrs ±32.5yrs ±19.3yrs ±37.3yrs ±14.2yrs

#### Table 15: Percentage Susceptibility Patterns of Enteric Pathogens Isolated from Diarrhoeal Cases in the Federal Capital Territory Abuja 2003

Percentage Susceptibility Pattern (%)

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ORGANISM | Tetracycline S I R | | | Chloramphenicol S I R | | | Amoxy/Clav S I | | R | Ciprofloxacin S I R | | | Trime/sulpha S I R | | | Ampicillin S I R | | | Nalidixic Acid S I R | | |
| Shigella | 0 | 0 | 100 | 20 | 40 | 40 | 40 | 20 | 40 | 100 | 0 | 0 | 40 | 0 | 60 | 75 | 2 | 0 | 40 | 40 | 20 |
| flexneri |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  | 5 |  |  |  |  |
| Shigella | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 50 | 50 | 100 | 0 | 0 | 0 | 0 | 100 | 100 | 0 | 0 | - | - | - |
| dysenteriae |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| A1 |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Shigella | 0 | 0 | 100 | 0 | 0 | 100 | 100 | 0 | 0 | 100 | 0 | 0 | - | - | - | - | - | - | - | - | - |
| specie |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Salmonella | 0 | 0 | 100 | 100 | 0 | 0 | 50 | 0 | 50 | 100 | 0 | 0 | 100 | 0 | 0 | - | - | - | - | - | - |
| typhi |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Salmonella | 100 | 0 | 0 | 0 | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 0 | 0 | 0 | 100 | - | - | - | - | - | - |
| paratyphi B |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Salmonella | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 100 | - | - | - | - | - | - |
| paratyphi C |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Vibrio | 100 | 0 | 0 | 50 | 50 | 0 | 100 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 100 | - | - | - | - | - | - |
| cholerae |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| O1 serovar |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| Ogawa |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |

**Key**

S = Susceptible

I = Intermediate R = Resistant Amoxy/Clav = Amoxycillin - Clavulanic Acid. Trime/Sulpha = Trimethoprim – Sulphamethoxazole.

#### CHAPTER FIVE DISCUSSION

This study showed that the highest frequency of diarrhoeal diseases in the Federal Capital Territory, Abuja occurs within the age group of zero to five years. This is in line with the internationally accepted fact that children suffer more from diarrhoeal diseases than adults. A fact that has been attributed to lower immunity seen with children than with adults, which makes them more susceptible to infections with enteric pathogens.

* 1. ***Escherichia coli* O157:H7**

E. coli O157:H7 was not isolated from this study despite the use of internationally accepted optimum procedure for its isolation. This is in contrast to studies in some parts of the world, mostly the developed countries tailored towards detecting sporadic occurrences of E. coli O157:H7 in diarrhoeal diseases (Bokete et. al., 1983; Ratnam and March, 1986). Most reported outbreaks of haemorrhagic colitis and haemolytic uraemic syndrome due to E. coli O157 has been in the developed countries with only a few reports coming from Africa (Issacson et. al., 1993; Germain et. al., 1998). Consumption of undercooked hamburgers has mostly been implicated in these outbreaks with just a few exceptions. Numerous studies in several of these countries have shown this organism to be present in the gastrointestinal tract of varying percentages of cattles (Armstrong et. al., 1996) from where they could lead to contamination of their meat products. There is a paucity of literature in Nigeria to show the presence or absence of this organism in the bowel flora of healthy cattles in the country. Possibility exists, however, that these organisms may not be part of the bowel flora of cattle in our

environment. Traditionally, in most parts of Nigeria meat and meat products are thoroughly cooked before consumption. Therefore, even in the event of contamination, the heat from the cooking could reduce the bacterial load so drastically or denature the organisms in such a way as to render them incapable of causing human infection. Thus, the inability to detect any sporadic case of diarrhoeal disease due to E. coli O157:H7 from this study can be attributed to these facts. In addition, sorbitol macconkey agar does not detect all E.coli O157: H7, which are non-sorbitol fermenting (Smith et. al., 1987) so, failure to detect E. coli O157: H7 by this technique does not mean that there is no E. coli O157: H7.

A study by Voravuthikunchai et. al., (2005) in Thailand found the presence of

humoral immune response to O157 lipopolysaccharide in healthy blood donors and patients with disease unrelated to diarrhoea. They concluded that possible exposure to cross-reacting antigens in these subjects could be the reason for finding this response in their sera, and a possible protection aginst colonization and disease associated with O157:H7 strains (Voravuthikunchai et. al., 2005). Similarly, serum obtained from Mexican adults and children aged less than one year not only responded to O157 LPS, but also to two other cross-reacting LPSs: O7 and O116 (Navarro et. al., 2003). They concluded that colonization by any of these strains could have a protective immune response against infection by O157 strains, which could explain why strains belonging to this serotype are seldom isolated from the Mexican population, especially when a similar immune response to these LPSs was also found in breastmilk samples obtained by the authors (Navarro et. al., 2003).

The presence of anti-LPS antibodies in serum could, also, reflect the presence of a similar response in the intestine. In this case, the presence of specific IgA antibodies

against cross-reacting non-O157 bacterial antigens could inhibit the pathogenic capacity of O157:H7 strains through the temporal loss of critical virulence factors used by these strains to cause disease in humans, as shown by Mellmann et. al. (2005).

The results outlined here from studies in other less-developed areas of the world are possibilities that could also explain the non-isolation of E. coli O157:H7 in this study. However, further studies are required to confirm this.

* 1. **Non-sorbitol fermenting *E.coli* other than *E. coli* O157:H7**

The phenotypic characteristic of most E. coli serotypes used in this study is their ability to ferment sorbitol within a 24-hr period as similarly reported by Armstrong et. al., 1996. Krishnan et. al., 1987; Padhye & Doyle, 1992 has it that E. coli O157:H7 is the only E. coli among clinical isolates which does not ferment sorbitol within 24hrs and which is glucuronidase-negative. This study has shown that in our environment among clinical isolates, there are E. coli strains other than E. coli O157 that do not ferment sorbitol within a 24hr period (Table 3). No other stool pathogen was isolated from all samples yielding NSF E. coli except one. The isolation of NSF E. coli other than O157 is in line with the findings of Chapman and Siddons (1996). The current studies show that NSF E. coli other than O157 could be isolated from clinical samples thus contradicting the earlier works. However, Chapman and Siddons (1996) did not state clearly whether these NSF E. coli strains should be regarded as normal gastrointestinal flora or not.

Apart from NSF E.coli, eleven other non-sorbitol fermenters were isolated from direct culture on SMAC and after IMS technique (Table 3). These isolates are similar to those obtained by Chapman and Siddons (1996). Among these, Non-sorbitol fermenters are some pathogens. They are Vibrio cholerae, Shigella dysenteriae A1 and Shigella

flexneri. All these are known Non-sorbitol fermenters. The Vibrio cholerae were isolated from two sporadic cases of cholera. There was no outbreak during the study period.

From the number of non-sorbitol fermenters isolated from SMAC in this study, it is obvious that despite its usefulness as a screening method for E. coli O157: H7 its specificity is quite low. This in line with the findings of Smith et. al, (1987) who found that the sensitivity of sorbitol macconkey agar method when compared with DNA probes was 73% while the specificity was only 39%. March and Ratnam (1986) found E.coli O157 to have a sensitivity of 100% and a specificity of 85% on Sorbitol Macconkey (SMAC).

Similarly, the main problem found with the IMS technique in this study has also been reported by other workers (Chapman and Siddons, 1996). This problem is the number of sorbitol non-fermenting microorganisms other than E. coli O157 that adhered non-specifically to the magnetic beads. In their study, ten different types, of such organisms were found with the two most common groups being E. coli strains of other serogroup (32.4%) and Proteus spp. (19.6%). In this study, 12 different types of these organisms were found with the two most common groups being E. coli strains of other serogroup (10.4%) and Pseudomonas specie (8.5%) (Table 3). These organisms are also a problem with direct culture technique as seen in this study and others reported in literature (Chapman et. al., 1991).

There seems to be an even distribution of patients with NSF E. coli within the FCT, as the persons from whom these organisms were isolated have their residences fairly spread out within the Federal Capital Territory, Abuja (Table 4). In other words, there is no clustering of these cases geographically within the FCT. One of the samples, however, was from a Retroviral Disease (RVD) patient resident in Markurdi on referral to

National Hospital, Abuja. This shows that some of these NSF E. coli whatever their role is in disease pathogenesis may well extend beyond the borders of the FCT in this country.

No sex or age predominance was seen among patients from whom NSF E. coli was isolated. Sorbitol MacConkey agar (SMAC) supported the growth of these NSF E. coli’s better than Cefixime-Tellurite Sorbitol MacConkey agar (CT-SMAC). Inclusion of the immunomagnetic step (IMS) procedure did not improve the rate of isolation. One of the recognized phenotypic characteristics of E. coli O157:H7 is its ability to resist cefixime and tellurite while most other normal stool flora are sensitive to them. This property led to the incorporation of these two agents into SMAC to improve its selectivity for E. coli O157:H7. Thus, the finding here simply shows that these NSF E. coli strains are most probably sensitive to Cefixime and Tellurite. In addition, the IMS procedure is specifically meant to improve the isolation of E. coli 0157:H7 because the magnetic beads are coated with an antibody against E. coli O157. Therefore, inclusion of the IMS procedure should not really improve the rate of isolation of other NSF E. coli strains.

One significant feature of the NSF E.coli that gave a false-positive reaction with E.coli O157 serological test kit is that it came from a patient with Retroviral Disease (RVD). From this patient alone, two serotypes of NSF E.coli were isolated. Out of 106 faecal samples analyzed, five were from RVD patients. Out of these five, two samples yielded NSF E.coli, 1 yielded Entamoeba histolytica, one from a one-year-old baby gave yeast cells (Candida species) from microscopy and the last one yielded no isolate at all. The two samples that yielded NSF E.coli yielded three out of the eleven NSF E.coli obtained from the study. With this data, there is need therefore, to consider the involvement of these NSF E.coli in RVD diarrhoea along with the consideration of these organisms in the pathogenesis of diarrhoeal disease as a whole. This is essential, even

though, no literature is available to me about the involvement of Enterohaemorrhagic E.coli in RVD diarrhoea because bacteria are known to be the second most common group of organisms to cause colitis in patients with AIDS (Riley & Riley, 2003). Although, in this study no bacterial pathogen was isolated from RVD patients included in the study; bacterial colitis in patients with AIDS, versus bacterial colitis in normal hosts, is associated with more severe diarrhoea, higher rates of septicemia, and more frequent recurrences (especially when Salmonella species are involved) (Nelson, et. al., 1992). Reports in literature have it that as many as 35% of AIDS patients with severe diarrhoea have either Salmonella or Campylobacter species as contributing pathogens (Smith et. al., 1988). This has not been found to be so in this study, at least with Salmonella species even though the number studied is too few for this statement to be conclusive. Campylobacter on the other hand was not included in this study as it is not routinely sought for in diarrhoeal cases within our environment.

Entamoeba histolytica is a ubiquitous organism that can be isolated from up to 10% of the world’s populations most of whom are asymptomatic (Aucott et. al., 1993). Entamoeba histolytica may be found in the colon in up to 40% of patients with AIDS, but only a minority of patients will have significant disease (Allan – Jones et. al., 1986). Cramping, abdominal pain, tenesmus and bloody stools mark acute colitis from amoebiasis. Common infectious organisms (Atypical Parasites) known to cause disease in AIDS patients but not usually thought to be gastrointestinal pathogens, including P. carinii and Toxoplasma gondii, have been reported to cause colonic disease (Bellomo et. al., 1992; Pauwels et. al., 1992).

Candida albicans on the other hand was described in an HIV-infected individual who presented with a three-month history of watery diarrhoea, weight loss, and abdominal pain (Jayagopal et. al., 1992). Endoscopies showed multiple ulcers throughout the colon. Biopsy was considered consistent with cytomegalovirus (CMV), and ganciclovir was initiated. After 2 days of treatment, the patient died as a result of cardiac arrest. The autopsy showed no evidence of CMV infection but did show extensive C. albicans in association with widely disseminated disease.

To find out whether any of the 11 NSF E. coli could be STEC or VTEC, the isolates were sent to CDC where they were screened for shiga toxin/verocytotoxin (st/vt) genes. None was positive for these genes. However, two were positive for the E. coli attaching and effacing (eae) gene (Table 5). The presence of eae gene alone has not been shown to be of clinical significance. Results, therefore, show that none of the sorbitol-negative

E. coli isolated from this study is VTEC/STEC. This is not surprising because most non- O157 STEC strains are known to ferment sorbitol (Bopp et.al., 2003). There is need, therefore, for further studies ained at identfying the specific serotype responsible for the CPE observed.

* 1. **Other serotypes of Enterohaemorrhagic *Escherichia coli***

In literature, haemorrhagic colitis has been defined as an acute episode of diarrhoea associated with blood in the stool and from which Salmonella, Shigella or Campylobacter was not isolated (Smith et. al., 1987). This condition has been associated with vero-toxin-producing Escherichia coli also known as shiga-toxin-producing E. coli (STEC) and Enterohaemorrhagic E. coli (EHEC). Detection of free faecal toxin in stool filtrates has been established in literature as one of the methods for the laboratory

diagnosis of the aetiology of gastroenteritis due to this group of organisms (Karmali et. al., 1983; Pai et. al., 1984; Polland et., al., 1990).

Despite considerable advances in recent years in the development of diagnostic methods such as enzyme immunoassays that detects shiga-like toxins in stool samples and DNA-based methods, many EHEC infections are still undiagnosed. Accurate diagnosis requires isolation of the pathogen since only then can the aetiology and the extent to which the patient is contagious be determined (Karch, 1996). Because, there is no selective isolation medium for non-O157 STEC, testing for shiga toxin in the stool is the best option for the laboratory to detect these organisms (Bopp et. al., 2003).

In this study, cytotoxic activities were examined in all 106 faecal samples using the vero cell cytotoxicity assay method. Free faecal toxin was detected in 16 (15.09%) out of the 106 faecal samples analysed (Table 6) indicating the possible involvement of VTEC other than E. coli O157:H7 in diarrhoeal diseases in the FCT, Abuja.

The lack of isolation of O157:H7 strains in less-developed areas of the world, as shown ealier, does not mean however, that STEC strains are not part of the burden of diarrhoeal disease in children from these areas (Journal of Health, Population and Nutrition, 2005). The findings from this study have given more credence to this statement. E. coli belonging to serotypes other than O157:H7 have been associated with both outbreaks and sporadic diseases in animals and humans in various parts of the world (Scheutz, Beutin, and Smith, 2000; Brooks et. al., 2002).

One of the 16 samples also yielded Shigella dysenteriae A1 from stool culture. It is

known that certain sublines of Hela and Vero cells are highly sensitive to shiga toxins (O’Brien & Holmes, 1987). Therefore, the finding that stool filtrate of a sample that yielded Shigella dysenteriae A1 gave cytotoxic activity on vero cells is not completely out

of place. However, this finding is also indicating the possibility of a mixed infection of

Shigella dysenteriae A1 and non-O157 VTEC.

The incidence of 15.09% makes non-O157 VTEC infection more common than any other enteric pathogen isolated during the study. This is similar to the finding in a study of stool specimens submitted to a children’s Hospital in Seattle where non-O157 VTEC were found to be more common than Yersinia or Shigella species (Bokete et. al, 1993). In a study from Germany carried out in hospitalized Children with diarrhoea, EHEC infections were found to be the second most common bacterial cause of diarrhoeal diseases (Karch, 1996). In this study, it can be said to have been found the leading cause wih its incidence of 15.09% being higher than those of other enteric pathogens isolated.

In the last decade, VTEC have emerged as important pathogens of the gastrointestinal tract of individual of all ages but with an increased incidence and severity in young children and the elderly (Griffin & Tauxe, 1991) as also seen in this study. The incidence found was much higher in children between age one month to three years (75%) as against 12.5% for children between ages 5-10 years and 12.5% for adults. No specific food item could be implicated as being the source of infection (Table 7). The intestinal tract of domestic animals, particularly, cows have been identified as the major reservoirs of E. coli in many parts of the world (Mariani- Kurkjidiani et. al., 1993). EHEC serotypes have also been isolated from a wide range of foodstuffs (CDC, 1994). There is, therefore, need for research into these areas to try to identify the reservoirs of these organisms, if any, in our environment.

Two of the children had a member of their family also down with diarrhoea at about the same time. It is not known whether this contributed to the diarrhoea in

anyway. However, in considering sources of infection, person-to-person transmission should not be overlooked. In the study done by Pai et. al., (1984), no probable source of infection could be identified in one of the 20 cases and person-to-person transmission was suspected. In an outbreak of E. coli O157:H7 infection that occurred in Ottawa in November 1982, person-to-person spread was suspected in 3 of 31 cases (LCDC, 1983).

Karch, (1996), noted that the extensive movement of people and foodstuffs within and between European countries makes EHEC infections a cross-border problem. This possible source of infection should also not be overlooked in the country as highlighted, in this study, by the fact that the strongest cytopathic effect observed is from the stool filtrate of a 5year old daughter of a diplomat resident in FCT, Abuja. Other study in literature also indicates that VTEC should be considered a cause of travelers’ diarrhoea (Smith et. al., 1987).

EHEC infections cause a variety of clinical conditions ranging from near asymptomatic carriage of the organisms, with or without mild abdominal symptoms to severe haemorrhagic colitis and haemolytic uraemic syndrome (Karch, 1996). In addition, in the year 2002, Griffin stated that E. coli O157 and other STEC cause illness that can present as mild non-bloody diarrhoea, severe bloody diarrhoea (haemolytic colitis) and HUS. In 1987, Smith et. al., found 75% of cases of VTEC isolated having abdominal pain, 38% vomiting and 25% had fever. In a clinical series, bloody stool were observed in over 75% of patients, half the patient had vomiting but only one third developed fever (Ostroff et. al., 1989). Comparing these reports with findings here (Table 9), this study recorded a lower incidence of bloody diarrhoea and a higher incidence of fever. The patients, however, exhibited the varied clinical symptoms associated with EHEC infections. Nevertheless, it is important to note that non - O157

shiga toxin-producing E. coli are a heterogenous group, the members of which have varying associations with human disease (Tarr and Neill, 2001).

#### Enteric Pathogens Isolated from the study

Only 22.6% of samples analysed yielded enteric pathogens routinely sought for (Table 10). This goes to show that the greater percentage of enteric infections within the territory remains undiagnosed possibly because they may be caused by pathogens other than the ones routinely looked for. This indicates the need for a surveillance study aimed at identifying additional pathogens that may be significantly associated with diarrhoea in the Federal Capital Territory, Abuja.

The focus of this study was to search for E.coli O157 and other enterohaemorrhagic E. coli. It was on this basis that the inclusion criteria were established. It was, therefore, not surprising when Shigella species gave the highest rate of isolation among other bacterial pathogens isolated from the study considering the fact that the criteria is the common presentation associated more with Shigella diarrhoea than with diarrhoea caused by most other bacteria. Out of the 106 samples studied, eight Shigella species (Table 10) were isolated while none of E. coli O157:H7 was isolated. This is in contrast with findings in the USA where E. coli O157 was found to be more frequently isolated from diarrhoeal cases than Shigella species (MacDonald et. al., 1988).

The Shigella flexneri isolates showed a remarkable feature of enhanced isolation rate from SMAC as opposed to the DCA and SF combinations (Table 11). Four of the five isolates showed a common pattern of growth on SMAC as opposed to only two that grew on the routine culture media. The only one that did not grow on this media grew

only after IMS followed by culture on IMS-SMAC. Their presence on sorbitol macconkey agar may have been masked by overgrowth of sorbitol fermenters. It can be difficult to detect a small number of sorbitol non-fermenters on plates with a large number of sorbitol fermenters (Smith et. al., 1987). It is recommended that in situations of heavy growth of sorbitol fermenters where discrete colonies could not be isolated, a repeat streaking on another culture plate for dicrete colonies is suggested as the non-sorbitol fermenters present may have been masked by the overgrowth of sorbitol fermenters. From the above, one could see that in routine cultures, these isolates would have been missed and the results would have gone out as “No Salmonella/Shigella species isolated”.

Considering the fact that Sorbitol MacConkey agar supported the growth of Shigella flexnerii better than the routinely used Deoxycholate Citrate Agar and Selenite F combinations (Table 11), the isolates were sent to CDC for more detailed study. The aim of this is to find out whether there are any distinct genetic differences that could account for this phenomenon. Serology result on Table 12 shows that two are Shigella flexnerii serotype 2a(II: 3,4) while the other two are Shigella flexnerii serotype 1b(I: 6).

Shigella is divided ino 4 subgroups, A (S. dysenteriae), B (S. flexnerii), C (S. boydii) and D (S. sonnei). Subgroup A has 15 serotypes; subgroup C has 19 serotypes while subgroup D is made up of a single serotype. Subgroup B, Shigella flexnerii has 8 serotypes. Serotypes 1 to 5 are subdivided into 11 subserotypes – 1a [I: 4]; 1b [I: 4,6]; 2a [II: 3,4]; 2b [II: 7,8]; 3a[III:(3,4), 6,7,8]; 3b [III: (3,4), 6]; 4a[IV: 3,4]: 4b[IV: 6];

4c[IV: 7,8]; 5a[V: (3,4)]; 5b[V: 7,8]. Serotype 6 is subdivided into three different

bioserotypes - 6[VI: 4] bioserotype Boyd 88; 6[VI: 4], bioserotype Manchester; 6[VI: 4],

bioserotype Newcastle. The remaining two are serotypes X [: 7,8] and Y [: 3,4] (Bopp

et al, 2003).

The four isolates fit into established serotypes in literature. There is, therefore, nothing antigenically distinct in any one of them to account for the phenomenon observed. Initially, I had proposed the inclusion of SMAC in routine cultures for Shigella species because of the enhanced support of the growth of these isolates by SMAC. From personal communication with Cheryl Bopp, 2005, this would not be particularly useful for Shigella as a group because some ferment sorbitol, while some do not. On the other hand, MacConkey is useful because all strains are lactose-negative (Cheryl Bopp, CDC, Personal Communication, 2005). Therefore, the best is to emphasise the inclusion of MacConkey agar in addition to DCA in routine stool culture. This is, actually, the best isolation procedure for Shigella in current literature – ‘It is recommended that for the optimal isolation for Shigellae, two different selective media should be used: a general purpose plating medium of low selectivity (eg MAC) and a more selective medium {eg Xylose, Lysine, Desoxycholate agar (XLD); DCA and Hektoen Enteric Agar (HE)} (Bopp et. al., 2003).

Table 13 shows antimicrobial susceptibility test results from CDC. All four isolates showed 100% resistance to the six antimicrobials used. This is alarming. It confirms and emphasizes the need for rational use of antibiotics in the FCT.

The main clinical features of diarrhoeal patients in the FCT vary depending on the causative agent (Table 14). The findings here are in line with established facts about enteric infections. A study among HIV-patients stated that presenting symptoms provide a clue to the most likely bacteria pathogen (Nelson et. al., 1992). In this study, abdominal cramps were seen most with patients whose stool samples yielded Shigella

species (50%) and V. cholera (50%) while it presented least among patients with Salmonella infections. Nausea was seen with the various categories of patients except cases that yielded enteric parasites. However, the percentage frequency of occurrence of nausea among these patients was lowest when compared against other clinical presentations. Watery diarrhoea was seen in very high level of frequency in all categories of patients. This is in order considering the fact that the inclusion into the study was based on the presence of watery diarrhoea. Bloody diarrhoea was found mainly among patients with NSF E. coli, Shigella species and Salmonella species isolated from their samples. There were no cases of bloody diarrhoea among patients with V. cholerae, Candida species and enteric parasites isolated from them. 18% of those with no pathologic agents detected had bloody diarrhoea. Among those who yielded specific organisms, 75% of cases with Shigella species had bloody diarrhoea followed by those who yielded NSF E. coli (40%), the lowest is those with Salmonella species that have 25% showing bloody diarrhoea. This is in line with a study that stated that Salmonella is unlikely to present with blood in the stool whereas 15%-25% of patients with Campylobacter and Shigella have bloody stool (Nelson et al, 1992). However, the number of patients with Shigella that presented with blood in this study (75%) is much higher than what they found in their study. Presence of mucus was also seen in all categories of all patients. The highest was those with V. cholerae (100%), followed by Candida species (57%), Shigella species (50%) then NSF E. coli (40%), enteric parasites (33%). The least was Salmonella species (25%).

Vomiting occurred in all categories of patients except those that yielded enteric parasites. The highest incidence of vomiting occurred with cholera cases (100%),

followed by Salmonella species (75%), Candida species (43%), NSF E. coli (30%),

Shigella species (25%) and cases with no pathological agents detected (23%).

Fever was observed in all patients group. Salmonella cases had 100% followed by cases with Shigella species (87.5%), Candida species (57%), V. cholera (50%), NSF E. coli (40%), and no pathologic agent detected (36%), while those with enteric parasites had the least (28%). The finding here is similar to those found in literature. One study, found fever common with Salmonella (80%), in contrast only 40% to 50% of Campylobacter and Shigella cases presented with fever (Nelson et. al., 1992). The figure in my study was much higher with Shigella species (87.5%) than in this study. The reason might be in the differences among the patient population studied.

In one study, cases with Verocytotoxin-producing Escherichia coli (VTEC) isolated from their stool samples had abdominal pain (75%), vomiting (38%) and fever (25%) (Smith et al, 1987). It can be seen that the patients in this study with NSF E. coli isolated from their stool samples have lower incidence of abdominal pain (40%) and higher incidence of fever (40%) than the study cited above. However, the incidence of vomiting in the two studies seems similar. These NSF E. coli have been found not to be shiga toxin/ verocytotoxin-producing from PCR studies of virulence markers done on them.

#### Antimicrobial Susceptibility Patterns

The mainstay of treatment of Shigella dysenteriae A1 infection and infection with most bacterial pathogens is appropriate antimicrobial therapy, which lessens the risk of serious complications and death (CDC, 1999). The percentage susceptibility patterns are as presented in Table 15. Antimicrobial agents currently recommended by WHO for

treatment of Shigella dysenteriae A1 and possibly, for the treatment of other Shigella species include ampicillin, trimethoprim-sulphamethoxazole, nalidixic acid, privimecillinam, ciprofloxacin, norfloxacin and enofloxacin (CDC, 1999). Looking closely at this list and the susceptibility pattern of Shigella isolates from this study (Table 15), one could also see that among the recommended drugs, ciprofloxacin is about the only one that the organisms are still sensitive to. This is a highly worrisome situation and calls for the authorities to look into the rational use of antibiotics in the Federal Capital Territory.

However, the finding here is in line with some reports in literature that states that in certain areas of African and Asia, S. dysenteriae A1 are resistant to all locally available antimicrobial agents including nalidixic acid but are still susceptible to the fluoroquinolones (Sack et. al., 1997, Bopp et. al., 2003). Because of the widespread antimicrobial resistance among Shigella strains, it is recommended that all isolates should undergo susceptibility testing (Bopp et. al., 2003).

The antimicrobial susceptibility pattern of Salmonella isolates from this study is

shown on Table 15. The finding here, too, is also in line with reports in literature where investigations have found an increasing prevalence of isolates resistant to at least one antimicrobial agent in the United States and other parts of the world such as Turkey, Spain, Poland, United Kingdom and Belgium (Lee et. al, 1994; MacDonald et. .al, 1987; Aysev et. al., 2001; Cruchaga et. al., 2001; Szych et. al., 2001; Threlfall et. al., 2001, van Loovern et. al., 2001). However, in contrast to reports from United Kingdom where Salmonella enterica serotype Typhi (Threlfall et. al., 2001) showed reduced susceptibility to ciprofloxacin, the Salmonella isolates from this study are still highly susceptibility to ciprofloxacin.

Successful treatment of cholera patients depends on rapid replacement of fluid and electrolyte losses. With proper treatment, mortality is less than 1% of reported cases (CDC, 1999). Antimicrobial agents are, still recommended by WHO for treating cholera as shown in the literature review. The antimicrobial susceptibility pattern of V.cholerae O1 serovar Ogawa isolates from the two sporadic cases of cholera included in this study is also shown on Table 15. Analysis of this report shows that these two isolates are still sensitive to WHO recommended drugs for the treatment of cholera except for trimethoprim/sulfamethoxazole. Hopefully, this might be representative of the true situation of the majority of V.cholerae organisms in the FCT environment. Confirmation of this would require analysis of antibiogram pattern of more V.cholerae isolates from the Territory. However, except for trimethoprim/sulfamethoxazole, the use of any of the WHO recommended drugs for the treatment of sporadic cases of cholera within the Federal Capital is recommended in the absence of antimicrobial susceptibility tests results or before the result of the antimicrobial susceptibility test is made available to the clinician.

Even though, this study did not yield any E. coli O57:H7, it does not completely rule out its involvement in diarrhoeal diseases in the Federal Capital Territory, Abuja, considering the sample size. There is need, therefore, for a population-based study in search of this organism before its involvement in diarrhoea diseases within this region could be conclusively ruled out.

The study has, however, shown high involvement of non-O157 STEC in diarrhoeal diseases in FCT. It was not possible to establish the specific serotype of STEC involved. This is, mainly, because the focus of the study in the isolation procedure was for the isolation of E. coli O157:H7, which is known to be a non-sorbitol fermenter. None of the

non-sorbitol fermenting E. coli isolated proved to be STEC. However, most non-O157 STEC strains are known to ferment sorbitol (Bopp et. al., 2003). This calls for further study in search of the specific serotype involved.

The study, also, showed the involvement of various common enteric pathogens in diarrhoeal diseases in the FCT, Abuja and their antimicrobial susceptibility patterns. Furthermore, it has shown the need for a surveillance study in the FCT and various sections of the country aimed at identifying additional pathogens that are significantly associated with diarrhoea and place them in context with traditional pathogens. The new knowledge on the aetilogy of diarrhoea in the surveillance patients, thus acquired, would help plan studies into various aspects of diarrhoeal diseases in the country.

#### FURTHER STUDIES

As a result of findings from this study, the following are areas of further work that need to be done.

* Population-based survey for E. coli O157:H7.
* Study designed to identify the specific serotype of Non-O157 STEC involved in diarrhoeal disease in the FCT.
* More elaborate research into the STEC group of organisms with a view to understand their epidemiology, modes of transmission, best approach for diagnosis, treatment and methods of control.
* Research into Cattles and foodstuffs in our environment with a view to identify the reservoir of these organisms.

#### SUMMARY OF RESULTS

The study demonstrates some important findings:

* + 1. The possible non-involvement of E.coli O157:H7 in studied diarrhoeal cases within the FCT.
    2. The presence of other non-sorbitol fermenting E.coli among clinical isolates of E.coli in our environment.
    3. The age group most afflicted with diarrhoeal diseases in the FCT is from one month to five years.
    4. Involvement of non-O157 STEC in diarrhoeal diseaes within the FCT.
    5. The high prevalence rate of non-O157 STEC infections in the FCT especially among children between ages 0 - 3 years.
    6. Implication of non-O157 STEC more than any other common enteric pathogen in infantile diarrhoea in the FCT, Abuja.
    7. None of the sorbitol-negative E. coli isolated from the study was STEC indicating the possibility that sorbitol-positive E. coli in the samples might be responsible for the ST/VT production.
    8. High Sensitivity of Sorbitol MacConkey agar for the isolation of Shigella flexnerii from faecal samples.
    9. Need to include MacConkey agar among the media used for routine cultures of enteric pathogens from diarrhoeal stools.
    10. The study also showed that gastrointestinal diseases were not clustered geographically within the territory.
    11. The need for Public Health Officials to look into diarrhoeal diseases in the Federal Capital Territory and methods of control.
    12. The high resistance level of Shigella isolates from FCT to first-line antimicrobial agents used for the treatment of Shigellosis.
    13. The antimicrobial sensitivity pattern of Salmonella isolates from within the FCT is in line with the current trend reported in literature from various parts of the world.
    14. Salmonella organisms isolated from within the FCT are still sensitive to most of the recommended drugs.
    15. The susceptibility of V. cholerae isolates from FCT to most of the first-line antimicrobial drugs used for the treatment of cholera.
    16. The need for the authorities to look into the rational use of antibiotics in the Federal Capital Territory, Abuja, Nigeria.

#### CONTRIBUTION TO KNOWLEDGE

Apart from the fact that this study has detailed the current antimicrobial susceptibility status of common enteric pathogens in the FCT, Abuja, it has the following novel findings, which constitute major contributions to knowledge:

1. That E. coli O157:H7 is not involved in diarrhoeal diseases in the FCT, Abuja, Nigeria as it is in the developed countries of the world.
2. The involvement of non-O157 STEC in diarrhoeal diseases in the Federal Capital Territory, Abuja Nigeria.
3. The high prevalence rate of non-O157 STEC associated diarrhoea especially among children between 0 – 3 years.
4. The implication of non-O157 STEC more than any other common enteric pathogen in infantile diarrhoea within the Federal Capital Territory, Abuja Nigeria.

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#### Appendix A1

**QUESTIONNAIRE**

#### TOPIC: - A study of Bacterial Agents associated with Diarrhoeal cases in the Federal Capital Territory, Abuja.

1. **Characteristics of patients:**

1. Name………………………………………..

2. Clinic/Hospital…………………………….. Hosp. No:………………

3. Sex………………………………………….

4. Age………………………………………….

1. Race or Ethnic Group………………………
2. Location of Residence………………………
3. Clinical Features Yes No

* Nausea
* Abdominal Cramps
* Watery Diarrhoea
* Bloody Diarrhoea
* Vomiting
* Fever (note exact temp)

1. Complications:

* Haemolytic Uraemic Syndrome (HUS)……………………..
* Thrombotic Thrombocytopaenic Pupurae (TTP)…………………

1. Date, Month, Year of Illness……………………………………………….

10. Educational Levels:………………………………………………………...

11. Occupation:………………………………………………………………

#### Duration of illness in patients:

* 1. Illness before presenting to the hospital……………………………

ii. Diarrhoea……………………………………………………………

1. Illness before bloody diarrhoea…………………………………….

iv. Bloody diarrhoea……………………………………………………

v. Hospitalisation……………………………………………………...

* + Without complications…………………………………………
  + With complications…………………………………………….

vi. Illness from onset to cure…………………………………………..

#### Others:

* 1. Possible food eaten before onset of infection and the time lapse between them……………………………………………………
  2. Food history for 7 days before the onset of symptoms…………

(i.e. Regularly consumed food, for children include breast milk or infant weaning formular).

* 1. Drugs taken since onset of symptoms…………………………..

(include before visit to the hospital).

#### Exposure Variables:

* 1. Diarrhoeal illness in family members……………………………
  2. Diarrhoeal illness in close contact (for children, day-care and schools are to be included)……………………………………….

#### Travel History:

i. Any recent travels…………………………………………………

ii. If yes:

a. Indicate where to………………………………………………

b. When…………………………………………………………..

#### ANY OTHER INFORMATION CONSIDERED RELEVANT TO THE STUDY.

**Appendix A2**

Microbiology/Parasitology Department, National Hospital,

Abuja. Date.....................................

............................................

.............................................

.............................................

.............................................

Sir/Madam,

**REQUEST FOR COLLABORATION**

I wish to inform you that I want to embark on a research project titled: - “A study of bacterial agents associated with diarrhoeal cases in the Federal Capital Territory, Abuja.” The major focus of the study is to search for Escherichia coli O157:H7 and other Enterohaemorrhagic E. coli in those diarrhoeal cases.

Escherichia coli O157:H7 is one of the many emerging infectious diseases around the world and a base-line data will be beneficial to our Health and Academic community. In order to achieve this, I request your collaboration by sending in stool samples of patients with diarrhoea and filling the accompanying questionnaire.

Thanks in anticipation of your willingness to cooperate.

#### OSUOCHA, CALLISTA C. (MRS).

**Appendix A3**

#### APPROVALS RECEIVED FOR THE STUDY.

**APPENDIX B1**

#### LIST OF CONTROL STRAINS USED IN THE STUDY

Control strains used in the study are: -

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| i. Enterococcus faecalis ATCC 29212. |  | | | | |
| 1. E.coli ATCC 25922 2. Shigella flexnerii obtained from clinical | materials | and | stored | for | this |
| purpose. |  |  |  |  |  |
| iv. Salmonella typhi obtained from clinical | materials | and | stored | for | this |
| purpose. |  |  |  |  |  |
| v. Proteus vulgaris obtained from clinical | materials | and | stored | for | this |
| purpose. |  |  |  |  |  |
| vi. Pseudomonas aeruginosa ATCC 27853. |  |  |  |  |  |

#### Appendix B2

**CONSIDERATIONS FOR QUALITY CONTROL OF MEDIA**

Each batch of medium prepared from the dehydrated medium were tested for the following characteristics:

1. Sterility
2. Ability to support the growth of target pathogens
3. Ability to produce appropriate biochemical reactions
4. pH

b and c above are known as Performance Testing.

#### Sterility:

One plate or tube from each autoclaved batch of medium was incubated overnight at 37°C and examined for contamination. Anyone that remained sterile is recorded to have passed quality control test (PQCT). In the course of the study, no batch failed the sterility test.

#### Ability to support growth of the target organism(s)

For the selective media used such as DCA, Shigella flexnerii was used to test the ability of the media to support growth of the target pathogen. Production of appropriate colour on the test medium is also noted.

#### Ability to produce appropriate biochemical reactions

* + For the selective media: One pathogen and one non-pathogen were used to test for the ability of the medium to differentiate target organism from competitors.
  + For biochemical medium: One organism that will produce a positive reaction and one organism that will produce a negative reaction were used.

#### PH Testing: -

Most culture media have a pH of near Neutral, 7.2, except alkaline peptone water. PH testing was done using a narrow range pH paper. For the fluid medium, a piece of the paper was dipped into a sample of the medium when it is at room temperature. The color of the paper was compared against the pH color chart. For the agar medium, the pH paper was laid on the surface of the solidified agar medium. The color was then compared against the pH color chart.

#### Appendix B3

**QUALITY CONTROL OF REAGENTS**

The date of opening of all reagent kits was clearly marked on the packet. The expiration dates were also noted. The manufacturers indicated this for all reagents.

Each reagent was tested to make sure that the expected reactions are obtained. This was done using the positive and negative controls when included in the packet of the reagent under investigation. All reagents were tested at an interval of two weeks to ensure that no deterioration has occurred.

Method Used for Quality Control of Antisera

Some of the antisera came with their controls. For others known positives were used as control strains.

A drop of antiserum was placed on one end of a slide and a drop of 0.85% saline was placed on the other end of the slide. The saline was used to test each antigen for roughness or autoagglutination. A dense suspension of the control isolate, preferably from a non-selective agar such as nutrient Agar, was prepared in normal saline. One drop of the antigen suspension or the positive control was added to the antisera and the saline. This was thoroughly mixed with an applicator stick or a wire loop and then rocked back and forth for about one minute. Agglutination reaction was then read. The saline control was always negative before reagent was accepted to have passed quality control test or before agglutination for the test was regarded to be valid.

#### Appendix B4

**PREPARATION AND QUALITY CONTROL OF VARIOUS BACTEROLOGICAL MEDIA USED IN THE STUDY.**

#### Sorbitol Macconkey Agar (SMAC)

The medium was prepared according to manufacturers instructions. Sterilization was by autoclaving at 121oC for 15 minutes. The medium was allowed to cool and poured into petridishes. E.coli ATCC 25922 produced excellent growth of pink colonies. However, no E.coli 0157:H7 control isolate was available for use. Petridishes containing this medium were labelled SMAC.

Cefixime -Tellurite Sorbitol MacConkey agar

Materials needed include:

* Sorbitol MacConkey Agar (Oxoid, CM 813).
* Cefixime Tellurite Selective supplement (Oxoid, SR 172E). The box contains 10 vials. Each vial is sufficient to supplement 500 ml of sorbitol MacConkey Agar (CM 813). The vial contents are Potassuim Tellurite (1.25mg) and Cefixime (0.025 mg).

Method: Aseptically, 2 ml of sterile distilled water was added to one vial and inverted gently to dissolve. The vial content was added to 500 ml of sterile sorbitol macconkey agar prepared according to manufacturer’s instruction and cooled to 50oC. They were

mixed well, poured into sterile petridishes, and allowed to set. The petridishes were then labeled CT - SMAC.

Quality control: E.coli ATCC 25922 was used to quality control this medium. The organism was inhibited in this medium and produced very very scanty growh with very very tiny colonies. No E.coli O157:H7 control isolate was available for use.

#### Desoxycholate citrate agar

Preparation was according to manufacturers instruction. For quality control of DCA, E. coli ATCC 25922 and Shigella flexnerii was used to confirm its selective and inhibitory growth characteristics. E.coli produced pink colonies while Shigella flexnerii produced colourless colonies.

#### Kliglers iron agar (KIA)

KIA is a carbohydrate containing screening media widely used for identification of enteric pathogens. It differentiates lactose fermenters from non-lactose fermenters and have a hydrogen sulfide indicator. H2S producing organisms will cause blackening of the medium in both KIA and TSI. KIA contains glucose and lactose. Organisms which ferment glucose cause the butt of the tube to become acid (Yellow); some also produce gas. Lactose fermenting organisms will produce an acid (Yellow) slant; lactose non- fermenting organism will have an alkaline (red) slant. Preparation of KIA was done according to manufacturers instructions dispensed in test-tubes so that the volume of medium is sufficient to give a deep butt and a long slant.

For quality control, E.coli ATCC 25922 and Salmonella typhi was used for confirmation of biochemical response characteristics. E.coli gave an acid slant and butt,

with the production of gas but no H2S, Salmonella typhi gave an acid butt, alkaline slant, production of a little gas and H2S seen as blackening of the medium.

#### Macconkey agar

Prepared according to manufacturers instruction. For quality control, E.coli ATCC 25922 and Shigella flexnerii was used. E.coli ATCC 25922 produced pink colonies while Shigella flexnerii produced colourless colonies.

#### Urea medium

Manufacturers instruction was followed for the preparation of this medium. The urea agar base was prepared as directed on the bottle. This was sterilized at 121oC for 15 minutes. It was allowed to cool to about 50oC before urea was added to the agar base. This was then mixed and distributed into sterile bijou bottles and slanted to produce a deep butt.

For quality control, E.coli ATCC 25922 and Proteus vulgaris was used. E.coli is urea negative while Proteus vulgaris is urea positive.

#### Simmon citrate agar

Prepared according to manufactures instruction and dispensed into bijou bottles. For QC – E. coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853 were used. E. coli produced no changes while P. aeruginosa produced a blue colour.

#### Nutrient agar

Prepared according to manufacturers instruction and poured into petridishes. This is a general-purpose medium. For QC – E. coli ATCC 25922 was used. The medium supported the growth of the organism.

#### MR Broth

Prepared according to manufacturers instruction and dispensed into bijou bottles. For QC, E. coli ATCC 25922 and P. aeruginosa ATCC 27853 were used. E. coli showed positive reaction with the production of a red colour while P. aeruginosa was negative giving no colour change.

#### Peptone water

Also prepared according to manufacturers instruction. For QC, E. coli ATCC 25922 was used. This showed the production of a pink colour with the addition of indole reagent after overnight incubation.

#### Thiosulphate citrate bile salts sucrose agar (TCBS)

This medium was prepared according to manufacturers instructions. No Vibrio cholerae isolates was available for quality control.

#### Mueller Hinton agar

Medium was prepared following manufacturer’s instructions. Agar was carefully poured into petridishes to a uniform depth of 4mm. This is because more or less agar will affect the susceptibility results. The PH was checked and maintained at 7.2.

The medium was quality controlled by testing it with a control strain of Enterococcus faecalis ATCC 29212 and co-trimoxazole disc. The zone of inhibition obtained was averagely 22mm. For quality control of each test run, E.coli ATCC 25922 was used.

#### Buffered peptone water with VCC supplement (BPW – VCC)

Materials needed

1. Buffered peptone water (Oxoid, CM 809).
2. VCC selective supplement (Oxoid, SR 190A). Each box contains 10 vials. Each vial is sufficient to supplement 225 ml of Buffered peptone water CM 509. The vial contents include vancomycin(1.8 mg), cefixime(0.01125 mg), and cefsulodin(2.25 mg). Method: Aseptically, 4 mls of sterile distilled water was added to one vial and gently mixed. The contents were aseptically added to 225 ml of sterile Buffered peptone water (CM 509) prepared according to manufacturers instructions and allowed to cool to 50o C. This was then dispensed in 5 ml amounts into sterile bijou bottles and labeled BPW-VCC.

#### Appendix B5

**PREPARATION OF HANKS MINIMUM ESSENTIAL MEDIUM (HMEMS)**

The following was used for the preparation of HMEMS.

* + Hanks Salt with Glutamine
  + Sodium Bicarbonate (NaHCO3)
  + 0.22 m millipore filter
  + Distilled water

9 liters of distilled water was measured out (i.e 90% of the final required volume). The water was left at room temperature. While gently stirring the water, 106.8grams of HMEM’S salt with Glutamine was added. This was stirred until dissolved without heating the water. To the solution, 3.5g of NaHCO3 was added and stirred until dissolved. The solution was thoroughly mixed. While stirring the pH was adjusted to 7.2 using 1N NaOH. Additional 1litre of water was added to bring the medium to a final volume of 10 litres. The medium was immediately sterilized using 0.22m millipore filter. After sterilization, the medium was first kept in an incubator at 37C for 48hrs. Change of colour indicates contamination. The type of colour depends on the contaminating organisms. If the medium remained clear after 48hrs, it was transferred to a dark place at room temperature for 7 days. This is essential because some of the micronutrients are ligt sensitive. They diminish with light. Storage in the dark also aids the growth of fungi indicating contamination by fungi/mold, which are slow-growing and grow better at room temperature. If at the end of this period, the medium remains clear, it has passed Quality Control Test and can be passed on for use.

#### Appendix B6

**PREPARATION OF ANTIBIOTIC CONCENTRATIONS USED.**

#### Preparation of A/B concentration

A/B concentration is a mixture of antibiotics (Penicillin/Streptomycin) with a diluent (sterile PBS, PH 7.4). The preparation is based on the volume required.

Requirements include Penicillin (106 iu) - 5vials, Streptomycin (5gms) – 2vials, Sterile Phosphate Buffered saline (PBS) pH 7.4, 500mls bottle, Syringe and Needle,

5mls or 10mls bijou bottles, and Sterile UV Room.

To prepare, 5mls of PBS was aspectically added to each vial of Penicillin and streptomycin and left for few minutes to dissolve. The contents of each vial was then transfered into a common sterile bottle (maybe 500mls). The vials were rinsed with another 5mls of PBS and transfered again to the sterile bottle. The entire content was well mixed and dispensed in quantities of 5mls into sterile bijou bottles and stored at -

20°C.

For cell culture, 1ml of this solution was used for each 500mls of medium to give a

final concentration of Penicillin 100iu/ml and Streptomycin 100g/ml.

#### Preparation of fungizone

The following were used Fungizone (100mg) – 1vial, Distilled water – 100mls, Sterile bottle.

Aseptically, 5mls of distilled water was added to a vial of fungizone. Contents were withdrawn into another sterile bottle. The vial was rinsed twice. The solution was made up to 100ml. 5mls of the solution was dispensed into bijou bottles and stored at

–20°c. It was used at a concentration of 2.5mg/litre i.e 2.5ml/ litre of Media.

#### Preparation of Gentamicin

Gentamicin comes in solution of 50mg/ml or 10mg/ml. If 50mg/ml solution – add 1ml/litre of medium.

If 10mg/ml solution – add 5ml/litre of medium..

This gives the required working concentration of 50mg/L Solution is stored at 4c.

#### Appendix B7 PREPARATION OF HEPES BUFFER

Molecular Weight (MW) of Hepes is 238.3. To prepare a 1 Molar solution, molecular weight (238.3g) was dissolved in 1000ml of distilled water.

OR 23.83g in 100ml of distilled water

ON the other hand, to use 10g of Hepes salt calculation was as follows: 238.3g - 1000ml

10g - 10/238.3 x 1000

=41.9 ≈ 42mls of distilled water.

Therefore, 10g of Hepes salt was dissolved in 42mls of distilled water to give IM Solution.

NOTE: 1 Molar solution means that 1ml of solution contains 1M.

The Hepes buffer was used at a concentration of 15-20mm/ml. Calculation was as follows:

1ml of medium to contain – 15mM.

* 500ml of medium would contain - 500 x 15 = 7500mM. BUT

1M=1000mM

If 1000mM is contained in 1ml of Hepes Buffer.

* 7500mM will be contained is 7500/1000 =7.5mls of solution.

Therefore, 7.5mls of Hepes solution prepared above is removed and added to 500ml of media.

This gives a final concentration of 15mm/ml of media.

#### Appendix B8

**CELL COUNTING PROCEDURE**

Before tissue cells could be dispensed into tissue culture flasks or plastic dishes, they were diluted to concentration, which wouldl enable them to grow. If the cells are too numerous or too few, a single-layer- thick sheet (monolayer) of cells will not form. To make this dilution, knowledge of the number of cells available is needed.

Requirements include: Tissue Cell Suspension, Sterile Hanks Growth medium, 0.5% Trypan blue solution, Test tubes, 12 x 72mm, Sterile Pippettes, 1.0ml Sterile, Funnel, Sterile Graduated Cyinder, Sterile Gauze square, 4” x 4’’, Improved Neubauer Counting Chamber, Test tube Rack and Microscope.

Aseptically, the stem of a sterile glass funnel was placed into the mouth of a graduated cylinder. About 4 pieces of sterile guaze was, also aseptically, placed over the funnel opening. The trypsinized cells were poured from the flask through the sterile gauze into the graduated cylinder. When the suspension looks very heavy, some Hanks growth medium was added until one can just see through the suspension. The funnel was then removed and the cylinder covered tightly with a sterile aluminium foil cap.

To count the cells procedure was as follows. A cover glass was first placed over the ruled area of a counting chamber. Using a sterile 1.0ml pipette, 0.5ml of well-suspended cells was aseptically, removed from the graduated cylinder and placed in a small test- tube. Then, 1.0ml of 0.5% Trypan Blue solution using a fresh pipette was added making it a 1:3 dilution of the cells. Dead cells will stain blue while live ones will be colourless.

Content was thoroughly mixed by gentle aspiration with a sterile pipette. Then, 0.5ml of the sample was removed and used to charge the chamber by placing the tip of the pipette to the edge of the coverslip without allowing the liquid to overflow.

The cells were allowed to settle for 2mins before placing the chamber onto the microscope stage.

Using the low power objective, the ruled area of the chamber was focused.

All the cells with clear-cut nuclei and surrounding cytoplasm that appeared in the white cell areas (four-corner squares) were enumerated. To find the average number of cells per square, the total number of cells in all four-corner squares was divided by four.

To compute how many cells there would have been had the material been counted undiluted, the average number of cells per square was multiplied by the dilution factor which is 3. This gives the total number of cells per 0.1mm³ (cubic millitres) of concentrate. To correct this value to a count per ml (millitre) of concentrate, it was multiplied by 10,000. The rationale is as follows: -

1ml= 1cc (cubic centimeter).

A cube represents 1cc with 1cm (or 10mm) on each edge. 1cc has 10 x 10 x 10mm =1,000 cubic mm.

The count of the average number of cells per square was based on the volume of that square i.e 0.1mm³. To bring this to a 1cm value, multiply by 10. To bring 1cm value to 1ml value, multiply by 1000 or combining the two, multiply the average number of cells per square by 10,000.

In brief, average number of cells per square x dilution factor x 10,000 = number of cells/ml of concentrate.

Therefore, the average number of cells per square x 10,000 (correction factor) x 3 (dilution factor) will give the cell count/ml i.e the average number of cells in each millitre (ml) of fluid in the graduated cylinder.

Cells were diluted to give about 150,000cells/ml to 300,000cells/ml. Concentration that would produce good confluent growth. To determine the dilution factor (i.e how much to dilute the concentrated cells in order to obtain 150,000 to 300,000 cells/ml, the cell count/ml determined above was divided by the number of cells/ml desired.

In this study,

Cells per ml of concentrate counted =800,000. Cells per ml desired =200,000

Therefore, dilution factor = 800,000/200,000 = 4.

This means that the concentrated cells would be diluted four times to obtain 200,000 cells/ml i.e one part of cell concentrate was diluted with three parts of diluent.

As a result of this, 1:4 dilution was done in the procedure for repassaging of the Vero cells.

#### Appendix B9

**Calculation of working concentrations for primers and probes**

An example how to calculate working concentrations for primers

STEC-1:

Length of the primer: 20 bases Concentration of the stock: 0.64 g/l

To prepare 200 l of 25 M working stock:

The molecular weight of the primer is calculated by using the average weight of a DNA base (330):

20 x 330 = 6600

The DNA concentration of the working stock has to be changed from M to g/l using the molecular weight of the primer:

1 M = 6600 g/l; 1M = 0.0066 g/l; 25 M = 25 x 0.0066 g/ = 0.165 g/l The amount of primer stock needed (Y) is calculated by using equation

vol1 x conc1 = vol2 x conc2:

200 l x 0.165 g/l = Y x 0.64 g/l Y = 51.56 l

The amount of water needed: 200 l - 51.56 l = 148.44 l

An example of how to calculate working concentrations for probes:

STECI-HP-1:

Concentration of the stock: 135.17 M To prepare 200 l of 5 M working stock:

The amount of primer stock needed (Y):

200 l x 5 M = Y x 137.17 M Y = 7.40 l

The amount of water needed:

200 l - 7.40 l = 192.60 l

**Note**: Large quantities of primers and probes are usually shipped in dry form. The concentrations in dry form are often expressed in nmols. For long term storage, a convenient stock concentration is 100 M. In order to prepare a 100 M stock, 1:10 the amount of primer / probe nmols was dissolved into distilled water.

For example: the amount of primer / probe in dry form: 53 nmol. Add 530 l of dH2O →

100 M stock.

#### Appendix B10 List of LightCycler equipment and supplies

LightCycler Instrument (Roche Diagnostics, Mannheim, Germany; Cat. No. 2 011 468) LightCycler - Centrifuge Adapters (Cat. No.1 909 312)

LightCycler - Sample Carousel (Cat. No. 1 909 282) LightCycler - Color Compensation Set (Cat. No. 2 158 850)

LightCycler - DNA Master Hybridization Probes (Cat. No. 2 158 825) LightCycler - Capillaries (Cat. No. 1 909 339)

Optional: LightCycler Carousel Centrifuge (Cat. No. 2 189 682)

#### Additional equipment and supplies

“Clean” hood / PCR chamber

Conventional block cycler or boiling water bath Table top centrifuge

Two sets of pipettors: “clean” set for setting up the mastermixes and another set for preparation of the DNA template

Filtered pipette tips

0.5 ml and 1.5 ml sterile eppendorf tubes Forceps