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

BY

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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.....
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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.

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CALLISTA CHINYERE ASAMOLE-OSUOCHA

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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

1.1. BACKGROUND OF THE STUDY

1.1.1 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).

1.1.2 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:

i. Infectious**:** Viral damage to mucosal epithelium. Agents include rotavirus, Norwalk virus, and enteric adenovirus.

ii. Infectious: Enterotoxin mediated. Bacterial agents such as *Vibrio Cholerae*, *Escherichia coli*, *Bacillus cereus*, and *Clostridium perfringes* cause this.

iii. 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 ($MgSO_4$ 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.

1.1.3 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):

i. By producing a toxin, that affects fluid secretion, cell function or neurologic function (secretory, bacterial).

ii. By growing within or close to intestinal mucosal cells and destroying them, thus disrupting function (secretory, viral).

iii. By invading the mucosal epithelium causing cellular destruction and occasionally invading the bloodstream and going on to systemic disease (exudative, bacterial and parastic).

iv. 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/mm^3 (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:

i. 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.*

ii. 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:

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

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

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

In the case of infection, key bacterial properties are:

i. Ability to adhere to the mucosal epithelial cells and replicate.

ii. The ability to elaborate enterotoxins

iii. 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 V*ibrio 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).

1.2 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.

1.3 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.

1.4 AIMS AND OBJECTIVES

This study is aimed at:

i. Determininig the occurrence of *E. coli* O157:H7 in diarrhoeal illnesses in the FCT, Abuja.

ii. Assessing their involvement in haemorrhagic colitis and the haemolytic uraemic syndrome within the territory.

iii. Determining the prevalence of other Enterohaemorrhagic *E. coli* infections in diarrhoeal cases in the FCT.

iv. Determining the involvement or otherwise of other common bacterial pathogens in those diarrhoeal cases.

v. 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

2.1 *SALMONELLA*

2.1.1 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).

2.1.2 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.

i. *S. enterica* sub sp. *enterica* designated sub-species I

ii. *S. enterica* sub sp. *salamae* designated sub-species II

iii. *S. enterica* sub sp. *arizonae* designated sub-species IIIa

iv. *S. enterica* sub sp. *diarizonae* designated sub-species IIIb

v. *S. enterica* sub sp. *huntane* designated sub-species IV

vi. *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).

2.1.3 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, C_2, 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).

2.1.4 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 ($<10^3$) 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).

2.1.5 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.

2.1.6 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 H_2S 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 H_2S positive reaction, indicating that glucose is fermented with gas and H_2S 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).

2.1.7 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).

2.1.8 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%.

2.2 *SHIGELLA*

2.2.1 Description of the Genus

*Shigella*s 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).

2.2.2 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).

2.2.3 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).

2.2.4 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).

2.2.5 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).

2.2.6 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 H_2S. 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).

2.2.7 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).

2.2.8 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).

2.3 *VIBRIO*

2.3.1 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).

2.3.2 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).

2.3.3 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 (10^8 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 O*139 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 O*1 antisera or in O2 – 138 antisera. Thus, it was named O139 (Farmer III *et. al.*, 2003). Unlike *V. cholerae O*1, 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*).

2.3.4 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*).

2.3.5 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 36^{o}C. 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:

i. Agglutination of *V.cholerae* O1 antigens present in high numbers (often 10^6 -10^8 organisms/stool) in rice water stools from patients with cholera (Jesudaon, Thangaveli, and Lalitha, 1984).

ii. 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).

2.3.6 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).

2.3.7 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).

2.4 *ESCHERICHIA*

2.4.1 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).

2.4.2 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).

2.4.3 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*).*

2.4.4 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).

2.4.5 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).

2.4.6 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).

2.5 *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.

2.5.1 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).

2.5.2 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).

2.5.3 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 O*157: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): -

i. *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%.
ii. *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).
iii. *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).

2.5.4 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: -

i. They involve only children, who shed the organism longer than do adults.

ii. 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.

2.5.5 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: -

i. How to trace the infection back to its sources and

ii. 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 (Samadpour*et. 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).

2.5.6 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):

- Intimin – a protein necessary for their attachment to the intestinal wall.
- Hemolysin – a protein that affects the growth of other bacteria and that may also hemolyse human cells.
- 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.

2.5.7 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).

2.5.8 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).

2.5.9 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).

2.5.10 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/mm^3
- 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 e*t. 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).

2.5.11 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).

2.5.12 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).

2.5.13 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).

2.5.14 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

3.1 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).

3.2 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.

3.3 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).

3.4 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).

3.5 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
- σ, The standard deviation of the population parameter of the trait of interest.

Depending on α, 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).

$N_s = (Z/E)^2 (p) (1 - p).$

Where N_s = 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 $\pm$0.1 was used. Thus, the calculated sample size was 96.

3.6 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.

3.7 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).

3.7.1 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 37^0C 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*.

3.7.2 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 37^{o}C 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).

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

3.8.1 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).

3.8.2 Primers and Probes

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

Working concentration for primers used was 25 μM and for probes: 5 μM. 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 -20°C.

Quality control (QC) for primers and probes:

A set of strains that represent different variants (different T_m: 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′):

***stx*$_1$ and *stx*$_2$:**	STEC-1	gAR[a] CRA AAT AAT TTA TAT gTg
	STEC-2	TgA TgA TgR CAA TTC AgT AT
eae:	eaeAF	gAC CCg gCA CAA gCA TAA gC
	eaeAR	CCA CCT gCA gCA ACA AgA gg
E-*hly*:	hlyAF	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′):

stx$_1$:

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

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

For *ipah-Stx$_1$*-multiplex:

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

stx$_2$:

STEC II-HP-1 TCA ggC ACT gTC TgA AAC TgC TCC TgT gTA-[FAM[a]]

STEC II-HP-2 [Red 705[d]]-ACC ATg ACg CCg ggA gAC gTg gAC CT-Ph[c]

eae:

eaeA-HP-1 ACA gTT CTg AAA gCg AAA TgA TgA Agg C-[FAM[a]]

eaeA-HP-2 [Red 640[b]]- CCT ggT CAg CAg ATC ATT TTg CCA CT-Ph[c]

E*-hly:*

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

hlyA-HP-2 [Red 705[d]]-CAA Cgg gAA ggA gAg gAT ATA AgT CAg-Ph[c]

[a] [FAM], flourescein

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

[c] Ph, 3′-phosphate

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

3.8.3 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 µl loop) of overnight growth from agar plate was suspended in 600 µl PureGene Lysis Buffer.The test proceeded, thereafter, following manufacturer's instructions. 2 µl / reaction was used.

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

MagnaPure automatic DNA extractor (Roche Diagnostics; Cat. No. 2 236 931): About 1" sweep (1 µl 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.

3.8.4 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 $MgCl_2$ provided by the kit was used to adjust the final reaction volume and $MgCl_2$ concentration, respectively.

stx$_1$ & stx$_2$ (Shiga toxin genes 1 and 2 in STEC):

dH_2O	11.4 μl
HP mastermix	2.0 μl
$MgCl_2$ (25mM)	1.6 μl
STEC-1 primer (25 μM)	0.4 μl
STEC-2 primer (25 μM)	0.4 μl
STEC I-HP-1 (5 μM)	0.8 μl
STEC I-HP-2^{-LC640} (5 μM)	0.8 μl
STEC II-HP-1 (5 μM)	0.8 μl
STEC II-HP-2^{-LC705} (5 μM)	0.8 μl

= 19.0 μl

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

dH_2O	11.0 μl
HP mastermix	2.0 μl
$MgCl_2$ (25 mM)	1.6 μl
eaeAF primer (25 μM)	0.4 μl
eaeAR primer (25 μM)	0.4 μl
eaeA-HP-1 (5 μM)	0.8 μl
eaeA-HP-2^{-LC640} (5 μM)	0.8 μl
hlyAF primer (25 μM)	0.2 μl
hlyAR primer (25 μM)	0.2 μl
hlyA-HP-1 (5 μM)	0.8 μl
lyA-HP-2^{-LC705} (5 μM)	0.8 μl

= 19.0 μl

3.8.5 Controls

Positive controls:

- EDL 933 (STEC O157:H7; ATCC 43895): positive for s*tx*$_1$, *stx*$_2$, *eae,* E-*hly* and *uidA.*
- S1191 (STEC O139:H1; CDC); positive for *stx*$_{2e}$
- 3157-02 (STEC O91: NM; CDC) positive for *stx*$_1$, *stx*$_2$-variant and E-*hly*

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

3.8.6 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 cycler™ operators manual version 3.5.

3.9 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.

3.9.1 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 37^{o}C 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).

3.9.2 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 CO_2 incubator to maintain a proper balance of 5% CO_2 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).

3.10 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 37°C 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 37^{o}C.

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.

3.11 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.

3.12 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 37^{o}C 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 37^{o}C 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 as *V.cholerae* O1 serovar Hikojima whereas those that agglutinate Antisera O139 "Bengal" were designated as *V. cholerae* O139 "Bengal".

3.13 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 10^5 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.

3.14 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.

3.15 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 37^{o}C for 5-8 hrs, and Thiosulphate Citrate bile-salt sucrose agar (TCBS) - were also innoculated.
- All plates were incubated at 37^{o}C 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 –20^{o}C 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 37^{o}C 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 37^{o}C 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 37^{o}C 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 37^{o}C.
- 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.

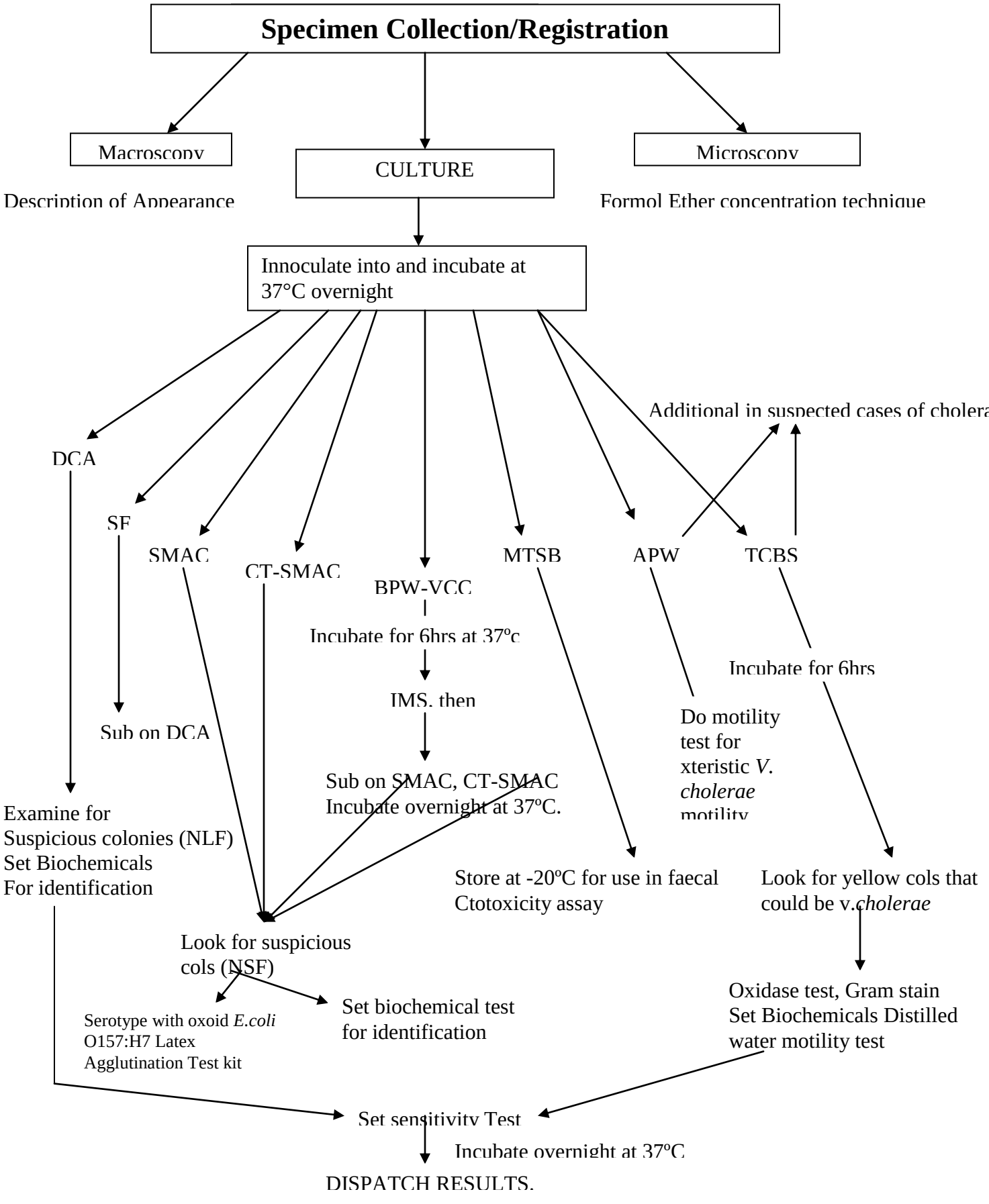

Figure 1: Algorithm of Procedures for the Study

CHAPTER FOUR
RESULTS

4.1 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%).

4.2 *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 ($P<0.05$).

4.3 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, *Stx*1 and *Stx*2 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).

4.4 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 $^{1}/_{12}$yrs 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.

4.5 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.

4.6 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 S*almonella 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

$X^2 = 306.755$, 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 ISOLATES	PERCENTAGES (%) Per Total No. of NSF's Isolated	Per Total No. of 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 A1	2	4.1	1.9
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		

$X^2 = 40.408$, 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 No	Age	Sex	Residence	App. Of Samples	Parasites Seen	Other Pathogens Isolated	ISOLATES OBTAINED FROM SMAC	CT-SMAC	IMS-SMAC	IMS-CT-SMAC	REMARKS
50	28yrs	F	Nyanya	Brownish watery, No Blood Nomucus	-	-	+	-	+	Not cultured on this	Isolate showed poor growth and no growth at all on CT-SMAC. It was this isolate that showed the need to inoculate SMAC after IMS.
54	47yrs	F	Wuse	Brownish, mucoid, Bloody.	-	-	+	-	+	-	This isolate seemed to require heavy innoculum to obtain a positive MR Result.
66	45yrs	M	Gwagwa	Brownish, loose, mucoid, No Blood.	-	-	+	+	+	-	Isolates from SMAC, CT-SMAC & IMS-CT-SMAC were exactly the same. IMS-SMAC yielded mixed growth of NSF of ? Motility (RVD Patient)
68	$1^{2}/_{12}$yrs	M	Old Karu	Brownish, watery, No blood, No mucus	-	-	+	-	-	-	Sample yielded just one colony on SMAC. On subculture showed poor growth on CT-SMAC. Isolate is non-motile (NM, NSF, LF *E. coli*).
75	$^{4.5}/_{12}$yrs	F	Area 11, Garki.	Brownish, loose, mucoid with blood stains	-	-	-	-	-	+	Identified as *Proteus vulgaris*. Only IMS-CT-SMAC yielded 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	$^{4}/_{12}$yr	M	Mararaba	Watery, No blood, No mucus	-	-	+	+	+	+	All four plates yielded NSF *E. coli* that gave the same biochemical result. NSF, NM, NLF *E. coli.*
87	44yrs	M	Kubwa	Dark brownish	Ova of	-	+	-	+	-	Case of chronic renal failure

				watery, No blood, No mucus	*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 blood, No Mucus.	-	-	+	+	+	+ RVD Patient	Yielded 2 serotypes of NSF *E. coli*: (a) showed 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 smooth slightly raised moist NSF colonies with entire edges.
101	62yrs	M	Maitama	Brownish, Semi-formed, No blood, Nomucus	-	-	+	-	+	-	NSF's isolated on all 4 plates. 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.
102	$1^{6}/_{12}$yrs	M	Adisa Estate, Gudu District	Brownish,watery mucoid, No blood seen	-	-	-	+	-	-	Isolated only on CT-SMAC. 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 FROM THE STOOL CULTURE.
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	$2\ ^{5}/_{12}$yrs	M	Wuse II, Urban	Cerelac, small qty of rice	Nil	Yes
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	$^{9}/_{12}$yrs	M	BPE, Abuja, Urban	NA	NA	NA
043	$1^{8}/_{12}$ 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	$4^{5}/_{12}$ yr	F	Area11, Garki, Urban	Infant formular purchased at kaduna	Kaduna	Yes (older Brother)
081	$^{6}/_{12}$ yr	M	NA	NA	Nil	No
083	3yrs	F	Gausau (Zamfara State) Urban	NA	Nil	No
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	$2^{6}/_{12}$yr	M	Wuse II, Urban	NA	NA	NA
105	$1^{8}/_{12}$ 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

$X^2 = 12.500$, 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 MICROORGANISM	NUMBER ISOLATED	**PERCENTAGES (%)** Per Total No. of Pathogens Isolated	Total No of Samples analysed (% pos)
Vibrio cholera O1 serovar Ogawa	Bacteria	2	8.3	1.9
Salmonella typhi	"	2	8.3	1.9
Salmonella paratyphi B	"	1	4.2	0.95
Salmonella paratyphi C	"	1	4.2	0.95
Shigella specie (not identified to specie)	"	1	4.2	0.95
Shigella flexnerii	"	5	20.8	4.7
Shigella dysenteriae A1	"	2	8.3	1.9
Cyst of *Entamoeba histolytica*	Parasite	1	4.2	0.95
Egg of Hookworm	"	1	4.2	0.95
Egg of *Schistosoma mansonii*	"	1	4.2	0.95
Yeast cells (*Candida* sp).	Fungi	7	29.3	6.6
Total		24		22.6

KEY

n = 106

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

Sample No.	Age	Sex	Residence	App. of Sample	Parasite seen	Other Pathogens Isolated	ISOLATES OBTAINED FROM					
							SMAC	CT-SMAC	IMS-SMAC	IMS-CT-SMAC	DCA	SF-DCA
62	1½yrs	M	Lugbe	Brownish Watery, No blood NoMucus	-	-	+	+	+	-	-	-
79	42yrs	M	(Caucasian) Maitama	Brownish Watery, No blood NoMucus	-	-	+	-	-	-	+	+
90	44yrs	M	NA	Brownish Loose Mucoid ?Blood Stains	-	-	-	-	+	+	-	-
96	17yrs	F	Jabi	Brownish Mucoid Blood stains	-	-	+	-	-	-	-	-
103	2yrs	F	Asokoro	Yellowish Watery No Blood 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 Trimethoprim-Sulfamethoxazole Ampicillin Sulfosoxazole Streptomycin	Only one colony Type
K0816	62	+	Chloramphenicol Trimethoprim-Sulfamethoxazole Ampicillin Sulfosoxazole Streptomycin	Tested both smooth and irregular colony type
K0817	90	+	Chloramphenicol Trimethoprim-Sulfamethoxazole Ampicillin Sulfosoxazole Streptomycin	Tested both smooth and irregular colony type
K0818	96	+	Chloramphenicol Trimethoprim-Sulfamethoxazole Ampicillin Sulfisoxazole Streptomycin	Tested both smooth and irregular colony type

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

Symptoms	Cases with NSF *E. coli* Isolated			Cases with *Shigella* sp Isolated			Cases with *Salmonella* sp Isolated			Cases with *V. cholerae* Isolated			Cases with *Candida* sp Identified			Cases with Enteric parasitesseen			Caseswithnopathologic agentdetected		
	Yes	No	Un-Known	Yes	No	Un-Known	Yes	No	Un-Known	Yes	No	Un-Known	Yes	No	Un-Known	Yes	No	Un-Known	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
Abdominal Cramps	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
Watery Diarrhea	9(90%)	1	-	7(85.5%)	1	-	4(100%	-	-	2(100%)	-	-	6(86%)	1	-	2(67%)	1	-	58(78%)	16	-
Bloody Diarrhea	4(40%)	6	-	6(75%)	2	-	1(25%)	3	-	-	2	-	0(0%)	7	-	0(0%)	3	-	13(18%)	61	-
Presence of mucus	4(40%)	6	-	4(50%)	4	-	1(25%)	3	-	2(100%)	-	-	4(57%)	3	-	1(33%)	2	-	25(34%)	49	-
Vomiting	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	10			8			4			2			7			3			74		
Male/Female Ratios	7:3			4:4			4:0			1:1			3:4			2:1			29:45		
Age Distribution	$^{4}/_{12}$ yrs – 62yrs			1½yrs – 51yrs			$^{6}/_{12}$ yr – 25yrs			30 – 35yrs			$^{9}/_{12}$yrs – 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

ORGANISM	Percentage Susceptibility Pattern (%) Tetracycline			Chloramphenicol			Amoxy/Clav			Ciprofloxacin			Trime/sulpha			Ampicillin			Nalidixic Acid		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Shigella flexneri	0	0	100	20	40	40	40	20	40	100	0	0	40	0	60	75	25	0	40	40	20
Shigella dysenteriae A1	0	0	100	0	0	100	0	50	50	100	0	0	0	0	100	100	0	0	-	-	-
Shigella specie	0	0	100	0	0	100	100	0	0	100	0	0	-	-	-	-	-	-	-	-	-
Salmonella typhi	0	0	100	100	0	0	50	0	50	100	0	0	100	0	0	-	-	-	-	-	-
Salmonella paratyphi B	100	0	0	0	0	100	0	100	0	100	0	0	0	0	100	-	-	-	-	-	-
Salmonella paratyphi C	100	0	0	100	0	0	100	0	0	100	0	0	0	0	100	-	-	-	-	-	-
Vibrio cholerae O1 serovar Ogawa	100	0	0	50	50	0	100	0	0	100	0	0	0	0	100	-	-	-	-	-	-

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.

5.1 *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.

5.2 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 (*stl 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.

5.3 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).

5.4 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 [ϕ: 7,8] and Y [ϕ: 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.

5.5 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.

REFERENCES

Abu-Bobie, J., Frankel, G., Bain, C., Gonca Ives, A. G., Trabulsi, L. R., Douce, S. G., Knutton, S. and Dougan, G. (1998). Detection of α, β, γ and δ, four intimin derivatives expressed by attaching and effacing Microbial pathogens. *Infection and Immunity* 36: 662 - 668.

Ackman, D., Birkhefad, G., Root, T. (1995). Swimming - associated hemorrhagic colitis due to *Escherichia coli* O157:H7 infection. *In*: Abstracts of the 35th Interscience conference on Antimicrobial Agents and Chemotherapy, San Francisco, California, Washington, DC, September 17 - 20. *American Society of Microbiology* 1955:299.

Acheson, D. W. K., and Keusch, J. T. (1995). *Shigella* and enteroinvasive *Escherichia coli*. *In*: M. J. Blaser, J. I. Ravdin, H. B. Greenberg, and R. C. Guerrant (ed). Infections of the gastrointestinal tract. New York, NY, Raven press, pp 763-784.

Ahmed, R., Bopp, C., Borczyk, A. and Kasatiya, S. (1987). Phage-typing scheme for *Escherichia coli* O157:H7. *Journal of Infectious Diseases* 155: 806 - 809.

Albert, M. J. (1994). *Vibrio cholerae* O139 Bengal. *Journal of Clinical Microbiology* 32:2345 - 2349.

Albert, M. J., Faruque, S. M., Faruque, A. S., Bettelheim, K. A., Neogi, P. K., Bhuiyan, N. A. and Kaper, J. B. (1996). Controlled study of cytolethal distending toxin producing *Escherichia coli* Infections in Bangladeshi children. *Journal of Clinical Microbiology* 34:717 - 719

Allan-Jones, E., Mindel, A. and Sargeaunt, P. (1986). *Entamoeba histolytica as* a commensal intestinal parasite in homosexual men. *New England Journal of Medicine* 315: 353-356.

Akashi, S., Joh, K., Tsuji, A. (1994). A severe outbreak of hemorrhagic colitis and hemolytic uremic syndrome associated with *Escherichia coli* O157:H7 in Japan. *European Journal of Paediatrics* 153: 650 - 655.

Amaro, C., and Biosca, E. G. (1996). *Vibrio vulnificus* biotype 2, Pathogenic for eel is also an Opportunistic Pathogen for humans. *Applied Environmental Microbiology* 62:1454 – 1457.

Amirlak, I. and Amirlak, B. (2006). Haemolytic uraemic syndrome: An overview (Review Article). *Nephrology* 11 (3): 213 - 218.

Armstrong, G. L., Hollingsworth, J. and Glenn - Morris J. Jnr. (1996). Emerging food borne pathogens: *Echerichia coli* O157:H7 as a model of entry of a New Pathogen into the food supply of the developed world. *Epidemiological Review* 18 (1) 29 - 51.

Aspan, A., Lohikari, L., Eriksson, E. and Gurnarsson, A. (2002). Evaluation of the transmission of verotoxin positive *E. coli* O157 from animals to man in Sweden by Molecular Epidemiology. Culled from the Internet A:\EVALUA~2.HTM.

Aucott, J.N. and Ravdin, J.L. (1993). Amoebiasis and non-pathogenic intestinal protozoa. *Infectious Diseases Clinics of North America* 7:467-485.

Aysev, A.D., Guriz, H. and Endem, H. (2001). Drug resistance of *Salmonella* strains from community infections in Ankara, Turkey, 1993-1999. *Scandinavian Journal of Infectious Diseases* 33:420-422.

Beery, J.T. and Doyle, M.P. (1985). Variable colonization of chicken cecae by *Escherichia coli* associated with haemorrhagic colitis. *Applied Environmental Microbiology* 49:310 - 315.

Bell, B.P., Goldoft, M. and Griffin, P.M. (1994). A multistate outbreak of *Escherichia coli* O157: H7-associated bloody diarrhoea and haemolytic uraemic syndrome from hamburgers: the Washington experience. *Journal of American Medical Association* 272:1349-1353.

Bellomo, A.R., Perlman, D.C. and Kaminsky, D.L. (1992). *Pneumocystis* colitis in a patient with the Acquired Immunodeficiency Syndrome. *American Journal of Gastroenterology* 87:759-761.

Belongia, E. A., Macdonald, K.L. and Parham, G.L. (1991). An outbreak of *Escherichia coli* O157:H7 colitis associated with consumption of precooked meat patties. *Journal of Infectious Diseases* 164: 338 - 343.

Belongia, E.A., Osterholm, M.T., Soler, J.T., Ammend, D.A., Braun, J.E. and Macdonald, K.L. (1993). Transmission of *Escherichia coli* O157:H7 infections in Minnesota child day care facilities. *Journal of American Medical Association* 269: 883 - 888.

Bern, C., Martins, J., de Zoysa, I. and Glass, R.I. (1992). The magnitude of the global problem of diarrhoeal disease: a ten-year update. *Bulletin of the World Health Organisation* 70 (6): 705 - 714.

Besser, R.E., Lett, S.M., Weber, J.T. (1993). An outbreak of diarrhoea and the haemolytic uraemic syndrome from *Escherichia coli* O157:H7 in fresh-pressed apple cider. *Journal of American Medical Association* 269:2217-2220.

Besser J, Beebe J and Swaminathan B. (2003). Investigation of foodborne and waterborne disease outbreak. *In*: P. R. Murray, E. J. Baron, J. A.Jorgensen, M. A. Pfaller, R. H. Yolken (ed). Manual Clinical Microbiology (8 edn). ASM press, vol 1, pp 162 - 313.

Bettelheim, K. A. (1995). The genus *Escherichia*. *In*: Balows, A., Truper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H. (ed.). The Prokaryotes. (2edn) Berlin Germany: Springer-Verlag K G, pp 2696-2736.

Beutin. L., Gleier, K. and Zimmermann, S, (2001). Comparative evaluation of different diagnostic methods for the detection of Shiga-Toxin producing *Escherichia coli* (STEC) in human clinical stool specimens. www.research.teaagasc.ie/vteceurope/beutin.html.

Beutin, L., Gelar, D. and Steinruck, H. (1993). Prevalence and some properties of Vero toxin (shiga-like toxin) - producing *Escherichia coli* in seven different species of healthy domestic animals. *Journal of Clinical Microbiology* 31:2483-2488.

Beutin, L., Montenegro, M. A., Orskov, I., Orskov, F., Proada, J., Zimmerman, S. and Stephen, R. (1989). Close association of verocytotoxin (Shiga-like toxin) production with enterohemolysin production in strains of *Escherichia coli*. *Journal of Clinical Microbiology* 27: 2559- 2564.

Beutin, L., Zimmerman, S. and Gleies, K. (1998). Human infections with shiga toxin-producing *Escherichia coli* other than serogroup O157 in Germany. *Emerging Infectious Diseases* 4:2.

Bisharat, N., Agman, V., Finkelstein, R., Raz, R., Ben-Dror, G., Lerner, L., Soboh, S., Colodor, R., Cameron, D. N., Wykstra, D. L., Swerdlow, D. L., Farmer III, J. J. and the Israeli *Vibrio* study group (1999). Clinical, Epidemiological, and

Microbiological features of *Vibrio vulnificus* biogroup 3 causing outbreaks of wound infection and bacteraemia in Israel. *Lancet* 354: 1421 – 1424.

Bitzan, M., Moebius, E., Ludwig, K., Muller-Wiefel, D.E., Hesseman, J. and Karch, H. (1991). High incidence of serum antibodies to *Escherichia coli* 0157 lipopolysacharide in children with haemolytic-uraemic syndrome. *Journal of Pediatrics* 119:380-385.

Blake, P. A., Merson, M. H., Weaver, R. E., Hollis, D. G. and Heushin, P. C. (1979). Disease caused by a Marine *Vibrio*: Clinical characteristics and Epidemiology. *New England Journal of Medicine* 300:1 - 5.

Bohn, H. and Karch, H. (1992). DNA fingerprinting of *Escherichia coli* O157:H7 strains by pulsed-field get electrophoresis. *Journal of Clinical Microbiology* 30:2169-2172.

Bokete, T.N., O'Callahan, C.M. and Clausen, C.R. (1993). Shiga-like toxin-producing *Escherichia coli* in Seattle children: a prospective study. *Gastroenterology* 105: 1724 - 1731.

Bopp, C. A., Brenner, F. W., Wells, J. G. and Strockbine N. A. (1999). *Escherichia*, *Shigella* and *Salmonella*. *In*: P. R. Murray, E. J. Baron, N. A. Pfaller, F. C. Tenover and R. H. Yolken, (eds). Manual of Clinical Microbiology (7 edn) pp 459 - 474

Bopp, C.A., Brenner, F.W., Fields P. I., Wells J. G. and Strockbine N. A. (2003). *Escherichia*, *Shigella* and *Salmonella. In*: P. R. Murray, E. J. Baron, J. A. Jorgensen, M. A. Pfaller and R. H. Yolken (eds). *Manual of Clinical Microbiology* (8 edn) pp 654 - 671.

Borczyk, A.A., Karmali, M.A. and Lior, A. (1986). Bovine reservior for verotoxin-producing *E.coli* O157:H7. (Lett). *Lancet* 2:1043.

Boyce, T.G., Swerdlow, D.L. and Griffin, P.M (1995). *Escherichia coli* O157:H7 and the Haemolytic Uraemic Syndrome. *New England Journal of Medicine* 333 (6): 364 - 368.

Brenner, F. W. and McWhorter-Muslin, A. C. (1998). Identification and serotyping of *Salmonella*. Centers for Disease Control and Prevention, Atlanta, Ga.

Brenner, F. W., Villar, R.S., Angulo, F. J., Tauxe, R. and Swaminathan, B. (2,000). *Salmonella* Nomenclature*. Journal of Clinical Microbiology* 38: 2465 - 2467.

Brenner, D. J., Faming, G. R., Skerman, P. J, and Falkow, S. (1972). Polynucleotide sequence divergence among strains of *Escherichia coli* and closely related organisms. *Journal of Bacteriology* 109: 933 - 965.

Brooks, J. T., Sowers, E. G., Wells, J. G., Greene, K. D., Griffin, P. M. and Hoekstra, R. M. (2005). Non-O157 Shiga-toxin-producing *Escherichia coli* infections in the United States, 1983-2002. *Journal of Infectious Diseases* 192: 1422-1429.

Cahoon, F.E., Thomson, J.S. (1987). Frequency of *E.coli* O157:H7 isolation from stool specimens. *Canadian Journal of Microbiology* 33:914-915.

Caprioli, A., Falbo, V., Ruggeri, F. M., Baldassari, C., Bisichina, R., Ippolito, G., Romoli, E. and Donelli, G. (1987). Cytotoxic necrotizing factor production by hemolytic strains of *Escherichia coli* causing extra intestinal infections. *Journal of Clinical Microbiology* 25:146 - 149.

Caprioli, A. and Tozzi, A. E. (1998). Epidemiology of shiga toxin - producing *Escherichia coli* Infections in continental Europe. *In*: J. B. Kaper and A. D. O'Brien (eds.). *Escherichia coli* O157:H7 and other shiga toxin - producing *E.coli* strain. ASM, Washington DC. pp 38-48.

Carter, A. O., Borczyk, A. A. and Carlson, J.A. (1987). A severe outbreak of *Escherichia coli* O157:H7-associated haemorrhagic colitis in a Nursing home. *New England Journal of Medicine* 317: 1496 - 1500.

Cebula, T. A., Payne, W. L. and Feng, P. (1995). Simultaneous identification of strains of *Escherichia coli* serotype O157:H7 and their shiga-like toxin type by mismatch amplification assay-multiplex PCR. *Journal of Clinical Microbiology* 33: 248-250.

Centers for Disease control (1994). *Escherichia coli* O157:H7 outbreak linked to commercially distributed dry-cured salami-Washington and California. *Mortality and Morbidity Weekly Report* 44: 157 - 160.

Centers for Disease Control and Prevention (1997). *Salmonella* Surveillance, 1996. Centers for Disease Control and Prevention, Atlanta, G S.

Centers for Disease Control and Prevention (1997). Multidrug-Resistant *Salmonella* serotype Typhimurium - United States, 1996. *Morbidity and Mortality Weekly Report* 46: 308 - 310.

Centers for Disease Control and Prevention. (1999). Laboratory Methods for the diagnosis of Epidemic Dysentery and Cholera. WHO/ CDS/ CSR/ EDC/ 99.8. pp 108.

Centers for Disease Control and Prevention (2000). NARMS 2000 Animal Report. Centers for disease control and prevention, Atlanta, Ga.

Centers for Disease Control and Prevention (2001). Preliminary food net data on the incidence of foodborne illnesses - selected sites, United States, 2000. *Morbidity and Mortality Weekly.* 50: 241 - 246.

Centers for Disease Control Prevention (2001). *Salmonella* Surveillance, 1996. Centres for Disease Control and Prevention, Atlanta. Ga.

Chapman, P. A., Daly, C.M. (1993). Evaluation of non-radioactive trivalent DNA probe (LTh, ST1a, ST1b) for detecting enterotoxigenic *Escherichia coli*. *Journal of Clinical Pathology* 46: 309-312.

Chapman, P.A., Jewes, L., Siddons, C.A., Norman, P. and George, S.L. (1990). Verocytotoxin-producing *Escherichia coli* Infection in Sheffield 1985 - 1989. *PHLs Microbiological Digest* 7: 163 - 166.

Chapman, P.A. and Siddons, C.A. (1994). A comparison of strains of *Escherichia coli* O157 from human and cattle in Sheffield United Kingdom (Lett). J*ournal of Infectious Diseases* 170: 251 - 253.

Chapman, P.A. and Siddons, C.A. (1996). A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin - producing *E.coli* 0157 from cases of bloody diarrhoea, non-bloody diarrhoea and asymptomatic contacts. *Journal of Medical Microbiology* 44: 267 - 271.

Chapman, P.A., Siddons, C.A. and Harkin, M.A. (1996). Sheep as a potential source of verocytotoxin - producing *E.coli* O157". (Lett). *Veterinary Record* 138:23-24.

Chapman, P.A., Siddons, C.A., Zadik, P.M. and Lewes, C. (1991). An improved selective medium for the isolation of *E.coli* O157. *Journal of Medical Microbiology* 35:107-110.

Chapman, P.A., Wright, D.J. and Norman, P. (1989). Verotoxin - producing *Escherichia coli* in Sheffield: Cattle as a possible source. *Epidemiology and Infection 102*: 439 - 445.

Chapman, P.A., Siddons, C.A., Wright, D.J., Norman, P., Fox, J. and Crick, E. (1993). Cattle as a possible source of verocytotoxin-producing *Escherichia coli* infections in man. *Epidemiology and Infection* 111: 439 - 447.

Chapman, P.A., Wright, D.J., Siddons, C.A. (1994). A comparison of Immunomagnetic separation and direct culture for the isolation of verocytotoxin-producing *Escherichia coli* O157 from bovine faeces. *Journal of Medical Microbiology* 40:424-427.

Chapman, P.A., Wright, D.J. and Wiggins, R. (1993). Untreated Milk as a source of verotoxigenic *Escherichia coli* O157 (Lett). *Veterinary Record* 133: 171 - 172.

Chart, H., Smith, H.R., Scotland, S.M., Rowe, B. Milford, D.V. and Taylor, C.M. (1991). Serological identification of *Escherichia coli* O157:H7 infection in haemolytic uraemic syndrome. *Lancet* 337: 138 - 140.

Cheeseborough, M. (2000). District Laboratory Practise in Tropical Countries, 4edn. Cambridge: Cambridge University Press, Part2 pp 434.

Chen, S., Yee, A. and Weller, J. (1997). Molecular typing of Verotoxigenic *Escherichia coli* using amplified fragment length polymorphism (AFLP) analysis, abstract. V169/V1. In: VTEC 97: Third International Symposuim and workshop on Shiga-Toxin (Verocytotoxin) -producing *Escherichia coli* Infections. pp 105

China, B., Goffaux, F., Pirson, V. and Mainil, J. (2001). Multiple polymerase chain reactions to Detect and characterize enterohaemorrhagic *Escherichia coli*. Culled from the Internet A:\MULTIP~1.HTM.

Chiou, C. S., Hsu, S. Y., Chiu, S. I, Wang, T. K. and Chao, C. S. (2000). *Vibrio parahaemolyticus* serovar 03:K6 as cause of unusually high incidence of foodborne disease outbreaks in Taiwan from 1996 to 1999. *Journal of Clinical Microbiology* 38: 4621 - 4625.

Cimolai, N., Carter, J.E., Morrison, B.J. and Anderson T.D. (1990). Risk factors for the progression of *Escherichia coli* O157:H7 enteritis to haemolytic-uraemic syndrome. *Journal of Paediatrics* 116: 589 - 592.

Clarke, R.C., Mc Ewen, S.A. and Gannon, V.P. (1989). Isolation of verocytotoxin-producing *Escherichia coli* from Milk filters in South - Western Ontario. *Epidemiology and Infection* 102: 253 - 260.

Clarkson, W.K., Bonacini, M. and Peterson, I. (1991). Colitis due to *Histoplasma capsulatum* in the Acquired Immunodeficiency Syndrome. *American Journal of Gastroenterology* 86: 913-916.

Cody, S., Glynn, M. and Farmer, J. l (1999). An outbreak of *Escherichia coli*. O157:H7 infection from Unpasteurized Commercial Apple Juice. *Annals of Internal Medicine* 130: 202.

Committee on Infection Diseases, American Academy of Pediatrics: 2,000 Red Book. EIK Grove Village, I L, American Academy of Pediatrics, 2002.

Consensus Conference Statement. (1994). *Escherichia coli* O157:H7 infections - an Emerging National Health Crisis. *Gastroenterology* 108: 1923 – 1934.

Cordovez, A., Brado, V. and Maggi, L. (1992). Enterheomorrhagic *Escherichia coli* associated with haemolytic-uraemic syndrome in Chilean children. *Journal of Clinical Microbiology* 30: 2153 - 2157.

Corvallis, C.D. (1993). Yet another outbreak of haemorrhagic colitis. Published by: Center for Disease Prevention and Epidemiology, Oregon Health Division, Department of Human Resources Portland OR. *Summary*: 4231 - 4232.

Crawford, J. A. (1999). The Gastrointestinal Tract. *In*: R. S. Cotran, V. Kumar and T. Collins (ed). Pathologic Basis of disease (6 edn). WB Saunders Company. pp 805-807.

Cray, W.C.J. and Moon, H. W. (1996). Experimental infection of calves and adult cattle with *Escherichia coli* O157:H7. *Applied Environmental Microbiology* 61: 1586 - 1590.

Cruchaga, S., Echeita, A., Aladuena, A., Garcia-Pena, J., Fias, N. and Usera, M.A. (2001). Antimicrobial resistance in *Salmonellae* from humans, food and animals in Spain in 1998. *Journal of Antimicrobial Chemotherapy* 47: 315-321.

Dalton, C. B., Mintz, E. D., Wells, J. G., Bopp, C. A. and Tauxe, R. V. (1999). Outbreaks of enterotoxigenic *Escherichia coli* Infection in American adults: a clinical and epidemiological profile. *Epidemiology and Infection* 123: 9 - 16.

Daniels, N. A., Evans, M. C. and Griffin, P. M. (2000). Non-Cholera *Vibrios* .P. 137 – 147. *In*: W. M. Scheld, W. A. Craig and J. M. Hughes (ed). Emerging infections (4edn) Washington, DC: ASM Press pp 137-147.

Desmachelier, P. M., Bilge, S. S., Fegan, N., Mills, L, Vary, J. C. (Jnr) and Tarr, P. I. (1998). A PCR specific for *Escherichia coli* 0157 based on the *rfb* locus encoding 0157 Lipopolysacharide. *Journal of Clinical Microbiology* 36(6): 1801 - 1804.

Dev, V.J., Main, M. and Goward, J. (1991). Waterborne outbreak of *Escherichia coli* O157. (Lett). *Lancet*: 341 - 961.

Donnenberg, M. S., Tacket, C. O., James, S. P., Losonsky, G., Nataro, J. P., Wassermen, S. S., Kaper, J. B. and Levine, M. M. (1993). Role of the *eae* gene in experimental enteropathogenic *Escherichia coli* Infection. *Journal of Clinical Investigation* 92: 1412 - 1417.

Donnenberg, M. S., Tzipori, S. J., Mickee, M. L., O'Brein A. D., Acroy, J. and Kaper J. B. (1993). The role of the *eae* gene of enterohemorrhagic *E.coli* in intimate attachement in vitro and in a porcine model. *Journal of Clinical Investigation* 92: 1418 - 1424.

Donnenberg, M. S., Tacket, C. O., Losonky, G., Frankel, S., Nataro, J. P., Dougan, G. and Levine, M. M. (1998). Effect of prior experimental human enteropathogenic *Escherichia coli* Infection on illness following homologous and haterologous rechallenge. *Infection and Immunity* 66: 52 - 58.

Doorduyn, Y., de Jager, C. M., van der Zwaluw, W. K., Friesma, I. H. M., Heuvelink, A. E., de Boer, E., Wannet, W. J. B. and van Duynhoven, Y. T. H. P. (2006). Shiga toxin-producing Escherichia coli (STEC) O157 outbreak, The Netherlands, September- October 2005. *Archives: Eurosurveillance monthly releases* 11: 7 - 8

Doyle, M. P. and Schoenii, J.L. (1987). Isolation of *Escherichia coli* O157:H7 from retail fresh meats and poultry. *Applied Environmental microbiology* 53: 2394 - 2396.

Duguid, J. P. and North, R. A. E. (1991). Egg and *Salmonella* food–poising: An evaluation. *Journal of Medical Microbiology* 34: 65 - 67.

Elloit, S. J., Wainwright L. A., McDonald, T. K., Jarvis, K. S., Deng, Y. K., Lai, L. C., McNamara B. P., Donnenberg, M S and Kaper, J. B. (1998). The complete

sequence of the locus of enterocyte effacement (LEE) from Enteropathogenic *Escherichia coli* E 2348/69. *Molecular Microbiology* 28: 1 - 4.

Ewing, W. H. (1986). Edwards and Ewings Identification of Enterobacteriacese. (4ed). New York, N.Y. Elsevier Science publishing co. Inc.

Farina, C., Goglio, A., Conedera. G., Minelli, F. and Caprioli, A. (1996). Antimicrobial susceptibility of *Escherichia coli* O157 and other enterohemorrhagic *Escherichia coli* Isolated in Italy. *European Journal Clinical Microbiology Infectious Diseases* 15: 351 - 353.

Farmer III, J. J. and Hickman - Brenner, F. W. (1992). Vibrio and Photobacterium. *In*: A. Ballows, H. G. Truper, M. Dworkin, W. Harder and K. H. Schleifer (ed.). The Prokaryotes (2 edn). Berlin Germany: Springer-Verlay, pp 2952-3011

Farmer III, J. J., Janda, J. M., BirkHead, K. (2003). Vibrio. *In*: P. R. Murray, E. J. Baron, J. A. Jorgensen, M. A. Pfaller and R. H. Yolken (ed). Manual of Clinical Microbiology (8edn) Vol 1. Washingtoh DC: ASM Press. pp 706-719.

Feng, P. (1995). *Escherichia coli* serotype O157:H7; Novel vehicle of infection and emergence of phenotypic variant. *Emerging Infectious Diseases* 1:47:52.

Fey, P. D., Wickert, R. S., Rupp, M. E., Safranek, T. J. and Hinrichs, S. H. (2000). Prevalence of Non-O157: H7 Shiga Toxin-Producing *Escherichia coli* in Diarrhoeal stool samples fro Nebraska. *Emerging Infectious Diseases* 6(5): 530-533.

Frankel, G., Phillips, A.D., Rosenshine, I., Dougan, G., Kaper, J. B. and Knutton, S. (1998). Enteropathogenic and Enterohaemorrhagic *Escherichia coli*: More subversive elements. *Molecular Microbiology* 30: 911 - 921.

Furowicz, A. J. and Orskov, P. (1972). Two New *Escherichia coli* O antigens, O150 and O157 and one new K antigen K 92, in strains isolated from veterinary diseases. *Acta Pathologica Microbiologica Scandinavica* [B], 80: 441 - 444.

Germain, Y., Cunin, P. and Tedjouka, E. (1998). Enterohaemorrhagic *Escherichia coli*. in Ngoila (Cameron) during an outbreak of bloody diarrhoea. *Lancet* 352: 625.

Gilligan, P. H., Janda, J. M., Karmali, M. A. and Miller, J. M. (1992) Cumitech 12 A, Laboratory Diagnosis of Bacterial Diarrhoea. Nolte, F. S.(coordinating ed) ASM press, Washington DC.

Grandsen, W.R., Damm, M. A., Anderson, J. D., Carter J.E. and Lior, H. (1986). Further evidence associating haemolytic uraemic syndrome with infection by verotoxin-producing *Escherichia coli* O157:H7. *Journal of Infectious Diseases* 154: 522-524.

Griffin, P. M., Mead, P. S. and Sivapalasingam, S. (2002). *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli. In*: M. J. Blaser, J. I. Ravdin, H. B. Greenberg and R.L. Guerrant (ed.). Infections of the Gastrointestinal tract (2 edn). New York N.Y. Lippincott Williams and Wilkins. pp 627-642.

Griffin, P. M. (1995). *Escherichia coli* O157:H7 and other Enterohaemorrhagic *Escherichia coli*. *In*: M. J. Blaser, P .D. Smith, J. I. Ravdin, H. B. Greenberg, R. L. Guerrant (eds). Infections of the gastrointestinal tract. New York, NY: Raven press. 739 - 761.

Griffin, P. M. (1991). Symposium on new and emerging pathogens *Escherichia coli* O157:H7 – beyond the burger. *In*: Abstract of the 35th inter-science conference on antimicrobial Agents and chemotherapy. San Francisco, California. September 17 – 20. Washington DC: American Society of Microbiology pp 355.

Griffin, P. M., Olmstead, L.C. and Petras, R. E. (1990). *Escherichia coli* O157:H7 - associated colitis, a clinical and histological study of 11 cases. *Gastroenterology* 99: 142 - 149.

Griffin, P.M., Ostroff, S.M. and Tauxe, R. V. (1988). Illnesses associated with *Escherichia coli* O157:H7 infections: a broad clinical spectrum. *Annals of Internal Medicine* 109: 705 - 712.

Griffin, P.M. and Tauxe, R. V. (1991). The epidemiology of infections caused by *Escherichia coli* O157:H7, other Enterohaemorrhagic *E. coli* and the associated haemolytic uraemic syndrome. *Epidemiological Review* 13: 60 - 98.

Guerrant, R. L., Van Gilder, T., Stainer, T. S., Thielman, N. M, Slutsker, L., Tauxe, R. V., Henessy, T., Griffin, P. M., Dupont, H., Sack, R. B., Tarr, P., Neill, M., Nachamkin, I., Peller, L.B, Osterholm, M. T., Bennish, M. L., and Pickering, L. K. (2001). Practice Guidelines for the management of infectious Diarrhoea. *Clinical Infectious Disease.* 32: 331 - 351.

Hancock, D.N., Besser, T. E. and Rice, D.H. (1995). Non-bovine sources of *Escherichia coli* O157:H7. *In*: Abstract of the proceedings of the conference of Research workers in Animal Diseases Chicago, illinois. Nov. 13 - 14, (Abstract no 66).

Hassan, T. (1991). Inferential Statistics. *In*: Handbook of Research Methods in Medicine. Prof. Bankole (ed). Lagos NERDC Press pp 167-211.

Heneghan, S.J., Li, J. and Petrossian, E. (1993). Intestinal perforation from gastrointestinal histoplasmosis in Acquired Immunodeficiency Syndrome: Case Report and Review of the Literature. *Archives of Surgery* 128: 464-466.

Heuvelink, A.E., Van de kar, N. C. A. J., Meis, J.F.S.M., Monnens, L. .A H. and Melchers W.J.G. (1995). Characterization of Verocytotoxin-producing *Escherichia coli* O157 isolates from patients with haemolytic uraemic syndrome in Western Europe. *Epidemiology and Infection* 115: 1 - 14.

Hickman - Brenner, F. W., Stubbs, A. D. and Farmer III, J. J. (1991). Phage - typing of *Salmonella enteritidis* in the United States. *Journal of Clinical Microbiology* 29:2 817 - 2823.

Hockin, J. C. and Lior, A. (1986). Haemorrhagic colitis due to *Escherichia coli* O157:H7: A rare disease? *Canadian Medical Association Journal* 134: 25 - 26.

Hohmann, E. L. (2001). Nontyphoidal Salmonellosis. *Clinical Infectious Diseases* 32: 263 - 269.

Honda, T., Arits, M., Takeda, T., Yoh, M. and Miwaten, I T. (1985). Non - O1 *Vibrio chderae* produces two newly identified toxins related to *Vibrio parahemolyticus* haemolysin and *Escherichia coli* Heat - stable enterotoxin. *Lancet* 11: 163 - 164

Hook, E. W. (1990). *Salmonella* species (including Typhoid Fever). *In*: G. L. Mandel, R. S. Douglas Jnr., J. E. Bennett (eds). Principles and Practice of Infectious Diseases. Churchill Living Stone. pp 1700 - 1716.

Huq, M. I., Alam, A. K. M. J., Brenner, D. J., and Morris, G. R. (1980). Isolation of *Vibrio*-like group, EF - 6, from patients with diarrhoea. *Journal of Clinical Microbiology* 11: 621 - 624.

Isaacson, M., Carter, P.A. and Effler, P, (1993). Haemmorrhagic colitis epidemic in Africa (Lett). *Lancet*: 341 – 961.

Janda, J. M. (1998). *Vibrio*, *Aeromonas*, and *Pleisiomonas*. *In*: Collier, L. (ed.). Topley & Wilsons Microbiology and Microbial Infections (9edn). New York, NY: Oxford University Press, pp 1065-1089.

Jayagapol, S. and Cervia, J.S. (1992). Colitis due to *Candida albicans* in a patient with AIDS. *Clinical Infectious Disease* 15: 555.

Jerse, A. E., Yu, J., Tall, B. D. and Kaper, J. B. (1990). A genetic locus of enteropathogenic *Escherichia coli* necessary for the production of attaching and effacing lesions on tissue culture cells. *Proceedings of the National Academy of Science* 87: 7839 - 7843.

Jesudason, M. V., Thangaveli, C. P. and Lalitha, M. K. (1984). Rapid screening of faecal samples for *V. cholerae* by agglutination technique. *Journal of Clinical Microbiology* 19: 712 - 713.

Johnson, W. M. and Lior, H. (1988). A new heat-glabile cytolethal distending toxin (CLDT) produced by *Escherichia coli* isolates from clinical material. *Microbial Pathology* 4: 103 - 113.

Journal of Health, Population and Nutrition. (2005). Importance of *Escherichia coli* strains producing verotoxins. Editorial *Journal of Health, Population and Nutrition* 23 (4): 303 - 304.

Karch, H. (1996). Control of Enterohaemorrhagic *Escherichia coli* Infection: The need for a Network involving Microbiology laboratories and Clinical and Public Health Institutions. _*European Journal of Clinical Microbiology and Infection* 15:276-280.

Karch, H., Russman, H. and Schmidt, H. (1995). Long-term shedding and clonal turnover of Enterohaemorrhagic *Escherichia coli* O157 in diarrhoeal diseases. *Journal of Clinical Microbiology* 33: 1602 - 1605.

Karch, H., Stockbine, N. A. and O' Brien, A. D. (1986). Growth of *Escherichia coli* in the presence of trimethoprim-sulfamethoxazole facilitates detection of shiga-like toxin-producing strains by colony blot assay. *FEMS Microbiological letters* 35: 141 - 145.

Karmali, M. A., Steele, B. T., Petric, M. and Lim C. (1983). Sporadic cases of Haemolytic Uraemic Syndrome associated with faecal cytotoxin and cytotoxin - producing *Escherichia coli* in stools. *Lancet* 1: 619 - 620.

Karmali, M. A., Petric, H., Lim, C., Flemming, P.C., Arbus, G. S. and Lior, H. (1985). The association between idiopathic haemolytic uraemic syndrome and infection by verotoxin - producing *Escherichia coli*. *Journal of Infectious Diseases* 15: 775 - 782.

Keene, W. E., Mc Anulty, J.M., Hosby, F.C., Williams, P., Herdberg K, Oxman, G.L., Barrett, T. J., Pfaller, M. A. and Fleming, D.W. (1994). A Swimming-associated outbreak of hemorrhagic colitis caused by *Escherichia coli* O157:H7 and *Shigella sonneii. New England Journal of Medicine* 331: 579 - 584.

Keene, W.E., Mc Anulty, J. M. and Williams, L.P. (1993). A two – restaurant outbreak of *Escherichia coli* O157:H7 enteritics associated with consumption of mayonnaisse. *In*: Abstracts of the 33rd Inter-science conference on Antimicrobial Agents and Chemotherapy. New Orleans, Louisiana. October 17 – 20, Washington DC. American Society of Microbiology pp 354.

Kleanthrous, H., Smith, H.R. and Scotland, S.M, (1990). Haemolytic uraemic syndrome in the British Isles, 1985 – 1988, association with verocytotoxin-producing *E. coli* Part 2, Microbiological aspects. *Archives of Diseases of Children* 65: 722 – 727.

Knutton, S., Baldwin, T., Williams, P. H. and McNeigh, A. S. (1989). Actin accumulation at sites of bacterial adhesion to tissue culture cells: basis of a new diagnostic test for enteropathogenic and enterohaemorrhagic *Escherichia Coli*. *Infection and Immunity* 57: 1290 - 1298.

Konowalchuk, J., Spiers, J. I., Stavric, S. (1977). Vero response to a cytotoxin of *Escherichia coli. Infections and Immunity* 18: 775 - 779.

Kotloff, K. L. (1999). Global burden of *Shigella* infections: Implications for various development and implementation control strategies. *Bulletin of World Health Organization 77* (8): 651 - 666.

Krishnan, C., Fitzgerald, V.A., Darkin, S.J and Behme, R. (1987). Laboratory investigation of outbreak of haemorrhagic colitis caused by *Escherichia coli* O157:H7. *Journal of Clinical Microbiology* 25: 1043-1047.

Kudva, I.J., Hatfield, P.S. and Hoode, C.J. (1996). *Escherichia coli* O157:H7 in Microbial flora of sheep. *Journal of Clinical Microbiology* 34: 431 - 433.

Laboratory Centre for Disease Control. (1983). Haemorrhagic colitis in a home for the aged – Ontario. *Canadian Disease Weekly Report* 9:29 - 32.

Le Sauxe, N., Spika, J.S. and Friesen, B. (1993). Groundbeef consumption in non-commercial settings is a factor for sporadic *Escherichia coli* O157:H7 infection in Canada. (Lett). *Journal of Infectious Diseases* 167: 500-502.

Lee, L. A., Puhr, N. D., Mahoney, E. K., Bean, N. A. and Tauxe, R. .V (1994). Increase in antimicrobial resistant *Salmonella* infections in the United States, 1989 – 1990. *Journal of Infectious Diseases* 120:128 - 134

Ling, J. M. and Cheng, A. F. (1993). Infectious Diarrhoea in Hong Kong. *Journal of Tropical Medicine and Hygiene* 96: 107 -112.

Lingwood, C. A. (1994). Verotoxin - binding to human renal sections. *Nephron* 66: 21 - 28.

Lior, H. (1994). Classification of *Escherichia coli*. *In*: C. J. Gyles (ed). *Escherichia coli* in domestic animals and humans. CAB International, Wallingford. pp 31 – 72.

MacDonald, K.L., Cohen, M.L., Hargrett-Bean, N.T. Wells, J.G., Puhr, N.D. Collin, S.F. and Blake, P.A. (1987). Changes in antimicrobial resistance of *Salmonella* isolated from humans in the United States. *Journal of American Medical Association* 258:1496-1499.

MacDonald, K. L., O' Leary, M. J., Cohen, M. L., Norris Wells, J. G., Noll, E., Kobayashi, J.M. and Blake, P. A. (1988). *Escherichia coli* O157:H7, an emerging gastrointestinal pathogen. Results of a one-year prospective population - based study. *Journal of American Medical Association* 259:3567 - 3570.

Maloney, W. J. and Guerrant, R. L. (1992). Epidemiology, Therapy and Prevention of Infection with *Salmonella* organisms. *Current Opinion in Infectious Disease* 5: 74 – 79.

March, S.B. and Ratnam, S. (1986). Sorbitol MacConkey medium for detection of *Escherichia coli* O157:H7 associated with hemorrhagic colitis. *Journal of Clinical Microbiology* 23: 869 -872.

March, S.B. and Ratnam, S. (1989). Latex agglutination test for detection of *Escherichia coli* serotype O157. *Journal of Clinical Microbiology* 27: 1675 - 1677.

Mariani-Kurkdjiani, P., Denamur, E. and Milton, A. (1993). Identification of a clone of *Escherichia coli* O103: H2 as a potential agent of haemolytic - uraemic syndrome in France. *Journal of Clinical Microbiology* 32: 530 J.

Martin, M. L., Shipman, L.D. and Wells, J. G. (1987). Isolation of *Escherichia coli* O157:H7 from dairy cattle associated with two cases of haemolytic uraemic syndrome. (Lett). *Lancet* 1:98.

Martin, 1.E., Tyler, S.D., Tyler, K.D, Khakhira, R. and Johnson, W. M, (1996). Evaluation of ribotyping as epidemiological tool for typing *Escherichia coli* serogroup O157 isolates. *Journal of Clinical Microbiology* 24: 720 -723.

McDaniel, T.K., Jarvis, K. S., Donnenberg, M. S. and Kaper, J. B. (1995). A genetic locus of enterocyte effacement conserved among diverse enterobacterial pathogens. *Proceedings of the National Academy of Science* 92: 1664 - 1668.

Mead, P. S., Slutsker, L., Dietz, V., McCain, L.F., Bresee J. S., Shapiro, C., Griffin, P. M., and Tauxe, R. V. (1999). Food - related illness and death in the United States. *Emerging Infectious Diseases* 5:607 - 625.

Mellman, A., Bielaszewska, M., Zimmerhachkl, L. B., Prager, R., Harmsen, D., Tschape. (2005). Enterohaemorrhagic *Escherichia coli* in human infection: in vivo evolution of a bacterial pathogen. *Clinical and Infectious Diseases* 41: 785-793.

Metzler, J. and Nachamkim, I. (1988). Evaluation of a latex agglutination test for the detection of *Salmonella* and *Shigella* spp by using broth enrichment. *Journal of Clinical Microbiology* 26: 2501 - 2504.

Miller, S. I., Hohmann, E. L and Pegues, D. A. (1995). *Salmonella* (including *Salmonella* Typhi). *In*: G. L. Mendel, J. E Bennett, and R Dolin(ed.) Principles and Practice of Infectious Diseases. New York N.Y. Churchill living stone, Inc. pp 2013-2033.

Miller, F.G. and Rosenstein, D.L. (2002). Reporting of ethical issues in Publications of Medical Research. *Lancet* 360: 1326-1328.

Moon, H. W., Whipp, S. C., Argenzio R. A., Lewies, M. M. and Gianella, R. A. (1998). Attaching and effacing activities of rabbit and human enteropathogenic

Escherichia coli in Pig and Rabbit intestines. *Infection and Immunity* 41: 1340 - 1351.

Morabito, S., Karch, H., Marian - Kurkdjiani, P., Schmidt, H., Minelli, F., Bingen E and Caprioli, A. (1998). Enteroaggregative Shiga toxin - producing *Escherichia coli* O111:H2 associated with an outbreak of haemolytic uraemic syndrome. *Journal of Clinical Microbiology* 36: 840 - 842.

Morgan, D., Newman, C.P. and Hutchinson, D.N. (1993). Vero toxin producing *Escherichia coli* O157 infections associated with the consumption of Yoghurt. *Epidemiology and Infection* 111:181-187.

Morgan, G.M., Newman. C. and Palmer, S.R. (1988). First recognized community outbreak of hemaorrhagic colitis due to Vero toxin-producing *Escherichia coli* O157:H7 in the UK. *Epidemiology and Infection* 101: 83 - 91.

Morris, J. S., Jnr. (1994). Non-O group 1 *Vibrio cholerae* strains not associated with epidemic disease. *In*: I. K. Wachsmuth, P. A. Blake, and O. Olsvik (ed), *Vibrio cholerae* and Cholera: Molecular to global perspectives. Washington, D.C.: ASM Press, pp 103-115.

Murray, J and Lopez A. (1996). The Global Burden, of Disease. Harvard school of Public Health, *World Health Organization*, World Bank.

Nataro, J. P. and Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiology Review* 11: 142 - 201.

Navarro, A., Eslava, C., Hernandez, U., Navarro-Henze J. L., Aviles, M., Garcia-de la and Torre, G. (2003). Antibody responses to *Escherichia coli* O157 and other lipopolysaccharides in healthy children and adults. *Clinical Diagnostic Laboratory and Immunology* 10: 797-801.

Navarro, A., Gutierrez, A., Trejo, A., Licona, D., Leon, L., Hernandez, J. M. (2004). Serologic evidence of a hete-rologous response against *Escherichia coli* O157 LPS in cattle from different farms in Mexico (abstract D-140). *In*: Abstracts, 105th General Meeting, American Society for Microbiology, New Orleans, LA, May.

Navarro, A., Gutierrez, A., Trejo, A., Licona, D., Leon, L., Hernandez, J. M. (2005). The low isolation frequency of *Escherichia coli* O157 in Mexican cattle is

associated with a heterologous immune response to cross reacting LPS antigens (abstract D-186). *In*: Abstracts, 105th General Meeting, American Society for Microbiology, New Orleans, LA, May.

Neil, M. A. (1998). Treatment of disease due to shiga toxin-producing *Escherichia coli*: Infectious Disease Management. *In*: J. B. Kaper and A. D. O'Brien, (eds.): *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli*. Washington, D C, ASM Press, 1998.

Nelson, M.R. Shanson, D.C. and Hawkins, D.A. (1992). *Salmonella, Campylobacter* and *Shigella* in HIV- Seropositive patients. *AIDS* 6: 1495-1498.

O'Brien, A. D. and Holmes, R. K. (1987). Shiga and Shiga-like Toxins. *Microbiological Reviews* 51 (2) : 206 – 220.

Obrig, T. G., Louise, C.B., Lingwood, C.A, Boyd, B., Barley-Mahoney, L. and Daniel, T. O. (1993). Endothelial heterogenicity in shigatoxin receptors and responses. *Journal of Biological Chemistry* 268:15484 – 15488.

Okude, J., Ishibashi, M., Hayakawa, E., Nishino, T., Takeda, Y., Mukhopadhyay A. K., Garg, S., Bhattacharya, S. K., Nair, G. B. and Nishibuchi, M. (1997). Emergence of a unique 03:K6 clone of Vibrio parahaemolyticus in Calcutta, India and isolation of strains of the same clonal group from South East Asian travellers arriving Japan. *Journal of Clinical Microbiology* 35: 3150 - 3155.

Olsen, S. J., Mackenon, L. C., Goulding, J. S., Bean, N. H. and Slutsker, L. (2000). Surveillance for foodborne disease outbreak – United States, 1993 – 1997. CDC Surveillance Summarries. *Morbidity and Mortality Weekly Report* 49:1-62.

Ostroff, S.M., Kobayashi, J.M. and Lewis, J.H. (1989). Infections with *Escherichia coli* O157:H7 in Washington State: The first year of statewide disease surveillance. *Journal of American Medical Association* 262: 355 - 359.

Oswald, E., Caprioli, A, Schmidt, H., Morabito, S., Marches, O. and Karch, H. (2002). Intimin gene typing of enteropathogenic and enterohaemorrhagic *Escherichia coli:* Characterization and detection of intimin ε. Culled from the Internet A:\INTIMI~1.HTM.

Padhye, N.V. and Doyle, M.P. (1991). Rapid procedure for detecting enterohaemorrhagic *Escherichia Coli* O157 in food. *Applied Environmental Microbiology* 57: 2693 - 2698.

Padhye, N.V. and Doyle, M.P. (1992). *Escherichia coli* O157:H7, Epidemiology, Pathogenesis, and Methods of detection in food. *Journal of Food Protection* 55: 555 - 565.

Pai, C.H., Ahmed, N., Lior, H., Johnson, W.M., Sims, H.V. and Woods, N.E. (1988). Epidemiology of sporadic diarrhoea due to verocytoxin-producing *Escherichia Coli*: a two-year prospective study. *Journal of Infectious Diseases* 157: 1054 - 1057.

Pai, C.H., Gordon, R., Sims, H. V. and Bryan L. E. (1984). Sporadic cases of haemorrhagic colitis associated with *Escherichia coli* O157:H7: Epidemiologic and bacteriologic features. *Annals of Internal Medicine* 101: 738 - 742.

Passaro, D. J., Reporter, R., Maseda, L., Kilman, L., Malcolm, G. B., Rolka, H., Werner, S.S. and Vugia, D. J. (1996). Epidemic *Salmonella enteritidis* infection in Los Angeles County, California. The Predominance of phage type 4. *Western Journal of Medicine* 63:126 -130.

Paton, J. C. and Paton, A. W. (1998). Pathogenesis and Diagnosis of shiga toxin-producing *Escherichia coli* Infections. *Clinical Microbiology Review* 11: 450 - 479.

Paton, A. W., RatCliff, R.M. and Doyle, R.M. (1996). Molecular Microbiological investigation of an outbreak of haemolytic-uraemic syndrome caused by dry fermented sausage contaminated with shiga-like toxin-producing *Escherichia coli* O157:H7. *Journal of Clinical Microbiology* 34: 1622 - 27.

Pauwels, A., Meyohas, M.C. and Eliaszewica, M. (1992). *Toxoplasma* colitis in the Acquired Immunodeficiency Syndrome. *American Journal of Gastroenterology* 87: 518-519.

Pavia, A. T., Nichols, C. R. and Green, D. P. (1990). Haemolytic uraemic syndrome during an outbreak of *Escherichia coli* O157:H7 infections in institutions for mentally retarded person: Clinical and Epidemiological Observations. *Journal of Pediatrics* 116: 544 - 551.

Pavos, M., Tarr, P.I. and Kim, H. (1993). A comparison of human and bovine *Escherichia coli* O157:H7 isolates by toxin genotype, plasmid profile and bacteriophage lambda – restriction fragment length polymorphism profile. *Journal of Infectious Diseases* 168: 1300 - 1303.

Pedersen, K., Verdonck, L., Austin, B., Austin, D. A., Blanche, A.R., Grimoi, P. A. D., Jofre, J., Koblari, S., Larsen, J. L., Tiavio, T., Vigneulle, M. and Swings, J. (1998). Taxonomic evidence of *Vibrio carchariae* Grimes *et. al*., 1985 is junior synonym of *Vibrio harveyi* (Johnson and Shunk, 1936). Baumann *et. al.*. 1981. *International Journal of Systematic Bacteriology* 48: 749 - 758.

Perma, N T., Mayhew, S. F., Posfai, G., Elliot, S., Donnenberg. M. S., Kaper, J. B. and Blattner, F. R. (1998). Molecular evolution of a pathogenicity Island from enterohaemorrhagic *Escherichia coli* O157:H7. *Infection and Immunity* 66: 3810 - 3817.

Pierard, D., Steves, D., Moriau, L., Lior, H. and Lauwers, S. (1997). Isolation and Virulence factors of Verocytotoxin-producing *Escherichia coli* in human stool samples. *Clinical Microbial Infection* 3: 531 - 540.

Polland, D.R., John, W. M. and Lior, H. (1990). "Rapid and specific Detection of Verotoxin

genes in *Escherichia coli* by the PCR. *Journal of Clinical Microbiology* 28: 540-554.

Polotsky, Y., Nataro, J. P., Kotler, D., Barrett, T. J. and Orenstein, J. M. (1997). Hep-2 cell adherence patterns, serotyping, and DNA analysis of *Escherichia coli* Isolates from eight Patients with AIDs and chronic diarrhoea. *Journal of Clinical Microbiology* 35: 1952-1958.

Popoff, M. Y., Bockmuhl, F., Brenner, W. and Gleesling, L. L., (2001). Supplement 2000 (No. 44) to the Kauffman-White scheme. *Resources in Microbiology* 152: 907-909.

Proulx, P., Turgeon, J P, Delage, G., Lafleur L and Chicanel (1992). Randomized Controlled trial of antibiotic therapy for *Escherichia coli* O157:H7 enteritis. *Journal of Pediatrics* 121: 299 - 303.

Ratnam, S. and March, S.B. (1986). Sporadic occurrence of hemorrhagic colitis associated with *Escherichia Coli* O157:H7 in Newfoundland. *Canadian Medical Association Journal* 134: 43 - 49.

Reeves, M. W., Elvis, G. M., Heiba, A. A., Plikaytis, B. D. and Farmer III, J. J. (1989). Clonal nature of *Salmonella typhi* and its genetic relatedness to other *Salmonellae* as shown by multilocus enzyme electrophoresis and proposal of *Salmonella bongori*. *Journal of Clinical Microbiology* 27: 313-320.

Reischl, U., Youssef, M. T., Kilwinski, J., Lehn, N., Zhang, W. L., Karch, H. and Strockbine, N. A. (2002). Real-time fluorescence PCR assays for detection and characterization of Shiga toxin. Intimin and enterohemolysin genes from Shiga toxin-producing *Escherichia coli. Journal of Clinical Microbiology.* 40: 2555-2565.

Reischl, U., Youssef, M. T., Wolf, H., Hyytiä-Trees, E. and Strockbine, N. A. (2003). Real-time fluorescence PCR assays for the detection and characterization of heat labile I and heat-stable I enterotoxin genes from enterotoxigenic *Escherichia coli*. Manuscript.

Rennie, D and Yank, V. (1997). Disclosure to the reader of Institutional Review Board approval and informed consent. *Journal of American Medical Association* 277: 922-923.

Renwick, S.A., Wilson, J.B. and Clarke, R. C. (1993). Evidence of direct transmission of *Escherichia coli* O157:H7 infection between calves and a hum. (Lett). *Journal of Infectious Diseases* 168: 792 - 793.

Rhode, R. (1979). Serological Integration of all known *Arizona* species into the Kauffman - White scheme. *Zentblatt Bacteriologie Parasitelogie Infectiologie* 243: 148 - 176.

Rice, D.H., Hancock, D. D., Besser, T. E. (1995). Verocytotoxigenic *E.coli* O157 colonization of wild deer and Range Cattle. *Veterinary Record*: 137:524.

Ries, A., Griffin, P. and Greene, K. (1993) Sources of sporadic *Escherichia Coli* O157:H7 infection in the United States a nation-wide case control study. Abstracts of the 33rd interscience conference on Antimicrobial Agents and chemotherapy, New Orleans, Louisiana.

Riley, L. W., Remis. R.S., Helgerson, S.D, Mc Gee, H.B., Wells, J. G., Davis, B. R., Hebert, R. J., Olcott, E.S., Johnson, L.M, Hargrett, N. T., Blake, P. A. and Cohen, M. L. (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New England Journal of Medicine* 308: 681 - 685.

Riley, T.R. and Riley, D.K. (2003). Infectious colitis in HIV - infected Patients. From Infections in Medicine. Culled from the Internet on 14 Oct. 2003.

Robson, W.L., Leung, A.K. and Kaplan, B.S. (1993). Haemolytic uraemic syndrome. *Current Problems in Pediatrics* 23: 16-33.

Rondeau, E. and Peraldi, M. N. (1996). *Escherichia coli* and the Hemolytic-uremic syndrome. *New England Journal of Medicine* 335: 660-662.

Rovozzo G. C and Burke, C. N. (1973). A manual of Basic Virological Technique. Prentice- Hall Inc., Engel wood cliffs, New Jersey. pp 287.

Rowe, P.C., Orbine, E., Lior, H., Wells, G. A, Melanie, P.N. (1993). A prospective study of exposure to Vero toxin-producing *Escherichia coli* among Canadian children with haemolytic uraemic syndrome.' *Epidemiology and infection* 110: 1-7.

Sack, R. B., Rhaman, M., Yunus, M., Khan, E. H. (1997). Antimicrobial resistance in organisms causing diarrhoeal disease. *Clinical Infectious Diseases* 24 (1): S 102 - 5105.

Samadpour, M. (1995). Molecular epidemiology of *Escherichia coli* O157:H7 by Restriction fragment length polymorphism using shiga-like toxin genes. *Journal of Clinical Microbiology* 33: 2150 - 2154.

Samadpour, M., Grimm, L. M. and Desai, B. (1993). Molecular epidemiology of *Escherichia coli* O157:H7 strains by bacteriophage lambda (λ) restriction fragment length polymorphism analysis: application to a multistate food borne outbreak and a day-care center cluster. *Journal of Clinical Microbiology* 31: 3179 -3183.

Sansonetti, P.J. (1992). *Escherichia coli*, *Shigella*, antibiotic-associated diarrhoea, Prevention, and Treatment of Gastroenteritis. *Current Opinion in Infectious Diseases* 5: 66-73.

Scheutz, F., Beutin, l., and Smith, H. R. (2000). Characterization of non-O1547 verotoxigenic *E. coli* (VTEC) isolated from patients with haemoltic uraemic

syndrome (HUS) worldwide from 1982-2003 [abstract203]. *In*: Abstracts from the 4th International Symposium and Workshop on Shiga toxin (Verocytotoxin)-Producing *Escherichia coli* infections, Kyoto, Kyoto: VTEC 2000 Organizing Committee, 2000.

Schoenii, J. L. and Doyle, M.P. (1994). Variable colonization of chickens peritoneally innoculated with *Escherichia coli* O157:H7 and subsequent contamination of eggs. *Applied Environmental Microbiology* 60: 2958 - 2962.

Schumacher, G., Ljungli, A. and Sandstedt, B. (1995). Inflammatory bowel disease or infectious colitis: Clinical Picture, Microbiology, and Histology offer guidelines. *Lakartidningen* 92: 3885.

Scotland, S. M., Cheasty, T., Thomas, A., and Bowe, B. (1991). Beta-glucuronidase activity in verocytotoxin-producing strains of *Escherichia coli* including serogroup O157 isolated in the United Kingdom. *Letters of Applied Microbiology* 13: 42-44.

Scotland, S. M., Dry, N. P. and Rowe, B. (1980). Production of a cytotoxin affecting Vero cell by strains of *Escherichia coli* belonging to traditional Enteropathogenic serotypes. *FEBS Microbial letters* 7: 15-17.

Scotland, S. M. and Smith, H.R. (1997). Verocytotoxins. *In*: *Escherichia coli*: Mechanisms of virulence. Cambridge University Press. pp. 257-280.

Siegler, R.C. (1988). Management of haemolytic uraemic syndrome. *Journal of Pediatrics* 112: 1014 - 1020.

Siegler, R.L., Griffin, P.M., Barrette, T.J. and Strockbine N A. (1993). Recurrentment haemolytic uraemic syndrome secondary to *Escherichia coli* O157:H7 infection. *Prediatrics* 91: 666 -668.

Smith, H, R., Rowe, B., Gross, R. J., Fry, N. K. and Scotland, S. M. (1987). Haemorrhagic colitis and vero-cytotoxin-producing *Escherichia coli* in England and Wales. *Lancet*: 1062 -1065.

Smith, H. R., Willshaw, G. A. and Cheast, T. (2002). Typing of Vero cytotoxin-producing *Escherichia coli* for Epidemiological investigation. Culled from the Internet A:\TYPING~1.HTM.

Smith, P. J., Illingworth, D. S and Coates D. (1996). Evaluation of the Eiafoss Assay for the detection of *Escherichia coli* O157 in foods, faeces and environmental samples. Culled from the Internet A:\EVALUA~1.HTM.

Smith, P. D., Lane, H.C. and Gill, V.J. (1988). Intestinal Infections in patients with the Acquired Immunodeficiency Syndrome (AIDS). *Annals of Internal Medicine* 108: 328-333.

Snyder, J. D. and Merson, M. H. (1982). The magnitude of the global problem of acute diarrhoeal disease, a review of active surveillance data. *Bulletin of the World Health Organisation* 60: 605 - 613.

Spika, J. S., Parsons, J. E., Nordenberg, D., Wells, J. G., Gunn, R.A. and Blake, P. A. (1986). Haemolytic uraemic syndrome and diarrhoea associated with *Escherichia coli* O157:H7 in a Day Care Center. *Journal of Pediatrics* 287 - 291.

Stavric, S., Buchanan, B., Glaeson, T. M. (1993). Intestinal colonization of Young chicks with *Escherichia coli* O157:H7 and other verotoxin-producing serotype. *Journal of Applied Bacteriology* 74: 557 – 563.

Strockbine, N. A., Marques, L. R., Newland, J. W., Smith, H. W., Holmes. R. K. and O'Brien, A. D. (1986). Two toxins-converting phages from *Escherichia coli* O157:H7 strain 933 encode antigenically distinct toxins with similar biologic activities. *Infection and Immunity* 53:135-140.

Strockbine, N. A., Wells, J. G., Bopp, L. A. and Barret, J. J. (1998). Overview of detection and subtyping methods. *In*: J. B. Kaper and A.D. O' Brien (eds). *Escherichia coli* O157:H7 and other shiga toxin-producing *E.coli* strains. ASM. Washington DC. pp 331-356.

Swerdlow, D.L., Woodruff, B. A., Brady, R. C., Griffin, P.M., Tippen, S., Donnell, H. D. J., Geldrachi, E., Payne. B. J., Meyer, A. J. and Wells, J. G. (1992). A water borne outbreak in Missouri of *Escherichia coli* O157:H7 associated with bloody diarrhoea and death. *Annals of Internal Medicine* 117: 812 - 819.

Synge, B. A., Hopkins, G. E. and Riley, W. J. (1993). Possible link between Cattle and *E.coli* O157:H7 infection in a human. (Lett). *Veterinary Record 1*33: 507.

Szych, J., Cieslik, A., Paciorek, J and Kaluzewski, S. (2001). Antibiotic resistance in *Salmonella enterica* subsp. Enterica strains isolated in Poland from 1998 to 1999. *International Journal of Antimicrobial Agents* 18: 37-42.

Tarr, P. I., Fouser, L.S., Stapleton, A. E., Wilson, R. A., Kim, H. H., Vary, J. C. and Clausen, C. R. (1996). Haemolytic uraemic syndrome in a six - year old girl after a urinary tract infection with shiga-toxin-producing *Escherichia coli* O103:H2. *New England Journal of Medicine* 335: 635 - 638.

Tarr, P. I., Neill, M. A., Clausen, C. R., Vatkins, S. L., Christine, D. L. and Hickman, R. O. (1990). *Escherichia coli* O157:H7 and the haemolytic uraemic syndrome: Importance of early cultures in establishing aetiology. *Journal of Infectious Diseases* 162: 553-556.

Tarr, P. I. and Neill, M. A. (2001). *Escherichia coli* O157:H7. *Gastroenterology clinics of North America* 30 (3) : 735 – 751.

Thomson, R. B. and Miller, J. N. (2003). Specimen collection, Transport and processing: Bacteriology. *In*: P. R. Murray, E. J Baron, J. A Jogensen , M. A. Pfaller and R. H. Yolken, (eds). Manual of Clinical Microbiology (8 edn) pp 286-330

Threfall, E.J. and Ward, L.R. (2001) Decreased susceptibility to Ciprofloxacin in *Salmonella enterica* serotype Typhi in United kingdom. *Emerging Infectious Diseases*. 7: 448-450.

Tilden, J., Young, A. M. and Mc Namara, M. C. (1995). New routes of transmission of an Emerging Pathogen, Investigation of an outbreak of *E. coli* O157:H7 associated with dry cured salami. *In*: Abstract of the 35th Inter- science conference on Antimicrobial Agents and Chemotherapy. San Francisco, California. September 17 - 20. Washington DC. American Society for Microbiology: 314.

Tozzi, A.E., Nicolini, A. and Caprioli, A. (1994). A community outbreak of haemolytic uraemia syndrome in children occurring in a large area of Northern Italy over a period of several months. *Epidemiology and Infection* 113: 201 - 219.

Tyler, K. D., Wong, G., Tyler, S. D. and Johnson, W. M. (1997). Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. *Journal Clinical Microbiology* 35: 331 - 346.

Upton, P. and Coia, J. E. (1994). Outbreak of *Escherichia Coli* O157 infection associated with Pasturized Milk supply (Lett). *Lancet* 344:1015.

Van Loovern, M., Chasseur-Libotte, M.L., Gordard, C., Lammens, C., Wydooghe, M., Peeters, L. and Goosens, H. (2001). Antimicrobial Susceptibility in non-typhoidal *Salmonella* isolated from humans in Belgium*. Acta Chirurgica Biologica* 56:180-186.

Vidyya Medical News Service (2005) Known Pathogens. RAmEx Ars Medica, Inc. www.vidyya.com/vol5/v5i149-3.htm.

Voravuthikunchai, S. P., Chaowana, C., Perepat, P., Lida, T., and Honda, T. (2005). Antibodies among healthy population of developing of developing countries against enterohaemorrhagic *Escherichia coli* O157:H7. *Journal of Health, Population and Nutrition* 23: 305 - 310.

Wachsmuth, I. K., Blake, P. A. and Olsvik, O. (1994) *Vibrio cholerae* and Cholera: Molecular to global perspectives. Washington DC: ASM Press.

Waddell, T., Cohen, A., and Lingwood, C. A. (1990). Induction of verotoxin sensitivity in receptor-deficient cell lines using the receptor glycolipid globotriosylceramide. *Proceedings of the National Academy of Science* USA 87: 7898 - 7901.

Waters, J.R., Sharp, J. C. and Dev V.J. (1994). Infection caused by *Escherichia coli* O157:H7 in Alberta, Canada and in Scotland: a Five-year Review, 1987 – 1991. *Clinical Infectious Diseases* 19: 834-843.

Weiler, L. H., McDonald, T. K., Whittam, T. S. and Kaper, J. B. (1997). Insertion site of the locus of enterocyte effacement in enteropathogenic and enterohaemorhagic *Escherichia coli d*iffers in relation to the clonial phylogeny of the strains. *FEMS Microbiological Letters* 156: 49 - 53.

Weintraub, A., Widmalm, G., Jansson, P. E., Jansson, M., Huttenby, K. and Albert, M. J. (1994). *Vibrio cholerae* O139 Bengal possesses a capsular polysaccharide, which confers increased virulence. *Microbial Pathology* 16: 235 - 241.

Weisman, J.B., Schmarler, A. and Weiter, P. (1974). The role of Preschool Children in Day-Care Centers in the spread of Shigellosis in Urban Communities. *Journal of Pediatrics* 84: 797- 802.

Wells, J. G., Shipman, L. D., and Greene. K. D. (1991). Isolation of *Escherichia Coli* serotypes O157:H7 and other shiga-like-toxin-producing *E.coli* from dairy cattle. *Journal of Clinical Microbiology* 29: 985 - 989.

Willshaw, G. A., Thirlwell J, Jones, A. P., Parry, S., Salmon, R. L. and Hickey M. (1994). Verocytotoxin - producing *Escherichia coli* O157 in beef burgers linked to an outbreak of diarrhoea, haemorrhagic colitis and the haemolytic uraemic syndrome in Britain. *Letters in Applied Microbiology* 19: 304 - 307.

Wong, C. S., Jelacic, S., Habeeb, R. L., Watkins, S. L. and Tarr, P. I. (2000). The Risk of the haemolytic uraemic syndrome after antibiotic treatment of *Escherichia coli* O157:H7 infections. *New England Journal of Medicine* 342 (l): 1930 - 1936.

World Health Organization (2000). Health Systems. Improving Performance. World Health Report. World Health Organization, General, Switzerland.

Wray, C., Mclaren, I. M. and Carroll, P. J. (1993). *Escherichia coli* Isolated from farm animals in England and Wales between 1986 and 1991. *Veterinary Record* 133: 439 - 442.

Wright, D. J., Chapman. P. A. and Siddons, C. A. (1994). Immunomagnetic Separation as a Sensitive Method for Isolating *Escherichia coli* O157 from food samples. *Epidemiology and Infection* 113: 31 - 39.

Zadik, P. M., Chapman, P. A. and Siddons, C. A. (1993). Use of tellurite for the selection of verocytotoxigenic *Escherichia coli* O157. *Journal of Medical Microbiology* 39: 155 - 158.

Zhao, S., Mitchell, S. E. and Mong, J. (1997). Genomic typing of *E.coli* 0157:H7 by semi automated fluorescent AFLP analysis, abstract. V5/V1. *In*: VTEC 97: 3rd International Symposium and workshop on Shiga Toxin (Verocytotoxin) - producing *Escherichia coli* Infections. pp 94.

Zimmerhackl, L. B. (2000). *E. coli*; Antibiotics and the Haemolytic Uremic Syndrome. *New England Journal of Medicine* 342: 1990 - 1994.

Zoja, C., Corn, D. and Farina, C. (1992). Verotoxin glycolipid receptors determine the localization of microangiopathic process on rabbits given verotoxin-1. *Journal of Laboratory and Clinical Medicine* 120: 229 - 238.

Appendix A1

QUESTIONNAIRE

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

A. Characteristics of patients:

1. Name……..
2. Clinic/Hospital…….. Hosp. No:……
3. Sex…….
4. Age…….
5. Race or Ethnic Group……
6. Location of Residence……
7. Clinical Features Yes No
   - Nausea
   - Abdominal Cramps
   - Watery Diarrhoea
   - Bloody Diarrhoea
   - Vomiting
   - Fever (note exact temp)

8. Complications:
   - Haemolytic Uraemic Syndrome (HUS)……..
   - Thrombotic Thrombocytopaenic Pupurae (TTP)……

9. Date, Month, Year of Illness……..

10. Educational Levels:……..

11. Occupation:……

B. Duration of illness in patients:

i. Illness before presenting to the hospital……

ii. Diarrhoea……

iii. Illness before bloody diarrhoea……..

iv. Bloody diarrhoea……

v. Hospitalisation……..

- Without complications……
- With complications……..

vi. Illness from onset to cure……..

C. Others:

i. Possible food eaten before onset of infection and the time lapse between them……

ii. Food history for 7 days before the onset of symptoms……

(i.e. Regularly consumed food, for children include

breast milk or infant weaning formular).

iii. Drugs taken since onset of symptoms……..

(include before visit to the hospital).

D. Exposure Variables:

i. Diarrhoeal illness in family members……

ii. Diarrhoeal illness in close contact (for children, day-care and schools are to be included)…….

E. Travel History:

i. Any recent travels……

ii. If yes:

a. Indicate where to……

b. When……..

F. 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.

ii. *E.coli* ATCC 25922

iii. *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:

a. Sterility
b. Ability to support the growth of target pathogens
c. Ability to produce appropriate biochemical reactions
d. pH

b and c above are known as Performance Testing.

a. 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.

b. 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.

c. 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.

d. 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 121^{o}C 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 50^{o}C. 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. H_2S 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 H_2S, *Salmonella typhi* gave an acid butt, alkaline slant, production of a little gas and H_2S 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 121^{o}C for 15 minutes. It was allowed to cool to about 50^{o}C 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 w*as 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

i. Buffered peptone water (Oxoid, CM 809).

ii. 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 50^{o} 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 μm 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 0.22μm 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 (10^6 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 100μg/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 $0.1mm^3$ (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 $0.1mm^3$. 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 μg/μl

To prepare 200 μl of 25 μM 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 μM to μg/μl using the molecular weight of the primer:

1 M = 6600 g/l; 1μM = 0.0066 μg/μl; 25 μM = 25 x 0.0066 μg/μ = 0.165 μg/μl

The amount of primer stock needed (Y) is calculated by using equation

vol_1 x $conc_1$ = vol_2 x $conc_2$:

200 μl x 0.165 μg/μl = Y x 0.64 μg/μl

Y = 51.56 μl

The amount of water needed:

200 μl - 51.56 μl = 148.44 μl

An example of how to calculate working concentrations for probes:

STECI-HP-1:

Concentration of the stock: 135.17 μM

To prepare 200 μl of 5 μM working stock:

The amount of primer stock needed (Y):

200 μl x 5 μM = Y x 137.17 μM

Y = 7.40 μl

The amount of water needed:

200 μl - 7.40 μl = 192.60 μl

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 μM. In order to prepare a 100 μM 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 μl of dH_2O → 100 μM 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