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Children's Diarrhea in Hanoi, Vietnam
Importance of Enteric Pathogens

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To my parents

And to Lâm and Kiên

ABSTRACT

Diarrhea is one of the leading causes of illnesses and death among children in developing countries, where an estimated 1.3 billion episodes and 4 to 10 million deaths occur each year in children less than 5 years of age. In Vietnam, different studies have shown that the frequency of diarrhea is about three episodes for each child in one year and the death rate due to diarrhea is about 1-1.5%. The common pathogens of diarrhea are Group A rotavirus, diarrheagenic *Escherichia coli* (DEC), *Shigella* spp., enterotoxigenic *Bacteroides fragilis* (ETBF), *Salmonella* spp., *Campylobacter*, and *Vibrio cholerae*. Our objectives were to perform a detailed microbiological investigation of some potential pathogens associated with diarrhea, to characterize the isolates, and to assess clinical symptoms and the epidemiological factors related to the diarrheal disease in children less than 5 years of age in Hanoi, Vietnam.

We have successfully developed a multiplex Polymerase Chain Reaction (PCR) by combining eight primer pairs specific for enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC) to facilitate identification of these strains from stool samples in a single reaction. The prevalence of DEC was 22.5% and 12% in the diarrhea group and the control group, respectively, showing a significant difference. Among 587 fecal samples from children with diarrhea, this technique identified 68 samples (11.6%) with EAEC, 12 (2.0%) with EIEC, 39 (6.6%) with EPEC, and 13 (2.2%) with ETEC. Of the 249 age-matched controls, the distribution was 18 (7.2%) with EAEC, 11 (4.4%) with EPEC, and 1 (0.4%) with ETEC. No EHEC, *Salmonella* or *V. cholerae* were identified in either of the groups. *Shigella* spp. has been identified in 4.7% of children with diarrhea. *Campylobacter* was not investigated.

One hundred and sixty-two DEC and 28 *Shigella* strains isolated from children were tested for the minimum inhibitory concentrations following NCCLS recommendations by the agar dilution method against ampicillin, chloramphenicol, trimethoprim/sulfamethoxazole, imipenem, cefuroxime, cefotaxime, nalidixic acid, and ciprofloxacin. For *E. coli* strains, the resistance prevalence to ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole was 86.4%, 77.2%, and 88.3%, respectively. More than 89% of the *Shigella* strains were resistant to trimethoprim/sulfamethoxazole, 75% were resistant to ampicillin, and 53.6% were resistant to chloramphenicol. Multi-antibiotic resistance was detected in 89.5% of the *E. coli* and in 78.6% of *Shigella* strains.

Immunoseparation in combination with PCR assay identified ETBF in 7.3% of fecal samples from children with diarrhea and 2.4% of samples from the controls ($P < 0.01$). Within the diarrhea group, the prevalence was significantly higher in children older than 1 year of age. Three toxin subtypes in the 43 ETBF isolates have been identified with the prevalence of 67.4%, 18.6%, and 16% for *bft*-1, *bft*-2, and *bft*-3, respectively. In the controls, 5 *bft*-1 subtypes and 1 *bft*-2 subtype were identified.

Group A rotavirus was identified in 46.7% of children with diarrhea and 3.6% of the healthy ones showing a significant difference. Within the diarrhea group, the highest prevalence was seen in children from 13-24 months of age, higher in males compared to females. The symptoms of acute diarrhea caused by rotavirus were watery diarrhea, vomiting, fever and dehydration.

Of 587 children with diarrhea, 40.9% were less than 1 year of age, 71.1% were less than 2. Rotavirus, DEC, *Shigella*, and ETBF have been found in 67.3% of children with diarrhea and 17.3% of the controls ($P < 0.00001$). 13.5% of children with diarrhea and 0.8% of the controls were infected with two or more potential identified pathogens. Rotavirus infection peaked during the autumn and wintertime, whereas the bacterial infections were predominant in the summertime. The epidemiological factors such as lack of fresh water supply, unhygienic toilet, low living standard, infrequent getting information of health, and low education of parents could give rise to the morbidity of diarrhea in children.

This is the first study where several potential enteric pathogens have been identified in relation with clinical symptoms and epidemiology of diarrhea in children less than 5 years of age in Hanoi, Vietnam. The multiplex PCR has now been applied in detection of diarrheagenic *E. coli* in some microbiology laboratories. Importantly, the results of this study will contribute to the effective diagnosis, treatment, and intervention in order to decrease the morbidity and mortality of diarrhea in children less than 5 years of age.

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LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals:

I. Nguyen VT, Le VP, Le HC, Nguyen GK, Weintraub A. **Detection and characterization of diarrheagenic *Escherichia coli* from young children in Hanoi, Vietnam**. *J Clin Microbiol* 2005; 43: 755-760

II. Nguyen VT, Le VP, Le HC, Weintraub A. **Antibiotic resistance in diarrheagenic *Escherichia coli* and *Shigella* strains isolated from children in Hanoi, Vietnam**. *Antimicrob Agents Chemother* 2005; 49: 816-819.

III. Nguyen VT, Le VP, Le HC, Weintraub A. **Diarrhea caused by rotavirus in children less than 5 years of age in Hanoi, Vietnam**. *J Clin Microbiol* 2004; 42:5745-50.

IV. Nguyen VT, Le VP, Le HC, Weintraub A. **Diarrhea caused by enterotoxigenic *Bacteroides fragilis* in children less than 5 years of age in Hanoi, Vietnam**. *Anaerobe* 2005; *In press*.

V. Nguyen VT, Le VP, Le HC, Nguyen GK, Weintraub A. **Aetiology and epidemiology of diarrhoea in children in Hanoi, Vietnam**. *Submitted to the International Journal of Infectious Diseases.*

CONTENTS

ABBREVIATIONS

AA	Aggregative adherence
A/E	Attaching and effacing
bfp	Bundle forming pilus
bp	Base pair
CF	Colonization factor
CFA	Colonization factor antigen
CFU	Colony forming unit
DA	Diffuse adherence
DAEC	Diffusely adherent *Escherichia coli*
DEC	Diarrheagenic *Escherichia coli*
DNA	Deoxyribonucleic acid
*eae*A	*E. coli* attaching and effacing gene, intimin
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic *Escherichia coli*
ETBF	Enterotoxigenic *Bacteroides fragilis*
ETEC	Enterotoxigenic *Escherichia coli*
E-hly	EHEC-hemolysin
FAB	Fastidious anaerobic broth (medium)
HC	Hemorrhagic colitis
HUS	Hemorrhagic uremic syndrome
ial	Invasion-associated locus
kbp	Kilo base pairs
LEE	Locus of enterocyte effacement
LT	Labile toxin
Mab	Monoclonal antibody
ORT	Oral rehydration therapy
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
SMAC	Sorbitol-MacConkey (medium)
ST	Stable toxin
VT	Verotoxin/Verocytotoxin
VTEC	Verocytotoxin-producing *Escherichia coli*
VT1	Verocytotoxin 1
VT2	Verocytotoxin 2
WHO	World Health Organization

1. INTRODUCTION

1.1. Diarrhea - a problem worldwide

Infectious diarrhea is one of leading causes of morbidity and mortality worldwide, affecting mainly infants in developing countries [211]. In 1980, diarrhea was the leading cause of death for the world's children, accounting for 4.6 million deaths annually from around 1 billion episodes of diarrhea every year in children less than 5 years of age [232], and the estimate in 1999 for children and adults together was 2.2 million deaths per year [53]. Most recent estimates suggest the number of death is closer to 2.5 million [133]. Diarrhea is usually defined as the passage of loose or watery stools, usually at least three times in 24 hours and the importance is put onto the change in stool consistency rather than frequency, and the usefulness of parental insight in deciding whether children have diarrhea or not [14, 53]. If diarrhea lasts less than 14 days, it is called "Acute diarrhea". "Persistent diarrhea" is diarrhea of more than 14 days. Some experts refer to diarrhea that lasts > 30 days as "chronic" [97]. There is a widening range of recognized enteric pathogens such as viruses, bacteria, and parasites that can cause diarrhea. In many studies [1, 96, 118, 276], pathogens are identified in at least about 50 to 60% of stool samples from children with acute diarrhea. Among them, rotavirus and diarrheagenic *Escherichia coli* (DEC) are the most common. Other pathogens such as *Campylobacter* spp, *Shigella* spp, *Salmonella* spp, and *Vibrio cholerae* also play an important role in many different geographic areas. Recently, enterotoxigenic *Bacteroides fragilis* (ETBF) has been regconized as an emerging enteropathogen causing diarrhea in children [162]. In the normal condition, there is a balance in absorptive and secretory functions of intestinal water and electrolytes. Diarrhea results when the balance in electrolytes and water transport is upset in favor of net secretion because of decreased absorption from the intestinal lumen or increased secretion or water loss into the lumen. These are induced by different action mechanisms of enteric pathogens. The outcome is diarrhea. In many cases, the patients have accompanying symptoms such as fever, vomiting, and abdominal pain. Dehydration in diarrhea is the consequence of diarrhea and/or vomiting. It can be a severe symptom that is life threatening for children. Diarrhea may occur alone or in combination with other symptoms leading children to hospital for treatment. The most common risks with diarrheal illness are dehydration and, in the developing countries, malnutrition. Thus, the most critical treatment must include rehydration, which can be accomplished with an oral glucose or starch-containing electrolyte solution in the vast majority of cases. Although many patients with mild diarrhea can prevent dehydration by ingesting extra fluids (such as clear juices or soups), more severe diarrhea and reduced urination signify the need for more rehydration fluids. Oral Rehydration Therapy (ORT) was probably the greatest medical innovation of the 20th century, providing an example of the transfer of technology from developing to developed countries [220]. ORT solutions contain specific concentrations of sodium, glucose, potassium, chloride, and alkali (bicarbonate or citrate) in water [97, 249, 273]. The rationale for this treatment stems from the observation that in most causes of acute infectious diarrhea, including cholera, the coupled transport of sodium to glucose or other solutes is largely unaffected [39, 95]. In some cases, antibiotic therapy could be necessary. However, because of progressing increase of antibiotic resistance of enteric pathogens, side effects of treatment with antimicrobial agents, suprainfections when normal flora are eradicated by antimicrobial agents, and the possibility of induction of disease-producing phage by antibiotics (such as Shiga-toxin phage induced by quinolone antibiotics [12, 43, 104, 165, 269, 281], any consideration of antimicrobial therapy must be carefully weighed against unintended and potentially

harmful consequences. Clinical health care providers and public health practitioners have overlapping interests in the recognition and treatment of infectious diarrhea. For clinicians, especially pediatricians, early diagnosis of acute diarrhea can lead to interventions that reduce symptoms and prevent secondary transmission. The detection of clinical symptoms of diarrhea, especially the danger signs (vomiting everything, convulsions, lethargy or unconsciousness, inability to drink or to breastfeed, blood in stool) are very important. Obtaining a thorough history, including both clinical and epidemiological features should be the first step in evaluating a patient who presents with diarrheal illness. The information of clinical features should include: (i) when and how the illness began (abrupt or gradual onset and duration of symptoms); (ii) stool characteristics (watery, bloody, mucous, purulent etc.); (iii) frequency of bowel movements and relative quantity of stool produced; (iv) presence of dysenteric symptoms (fever, tenesmus, blood and/or pus in the stool); (v) symptoms of volume depletion (thirst, tachycardia, decreased urination, lethargy, decreased skin turgor); and (vi) associated symptoms and their frequency and intensity (nausea, vomiting, abdominal pain, cramp, headache, myalgias, altered sensorium). For public practitioners, prompt notification of pathogen-specific diagnoses and subtyping of enteric isolates, as well as identification of epidemiological factors related to diarrhea can lower rates of transmission and lead to timely detection and control of outbreaks. It has been shown that there are several factors that contribute to the development of diarrhea. They can be living standard, environmental conditions, and people's behaviors. It is clear that diarrhea diseases are more prevalent in poor people, poor communities, and poor countries. It is due to the fact that they do not have enough facilities for living and for making their environmental conditions better. The "F-diagram" of Wagner and Lanoix in 1958 [52] schematizes the routes that fecal pathogens take through the environment to reach a new host (**Figure 1**).

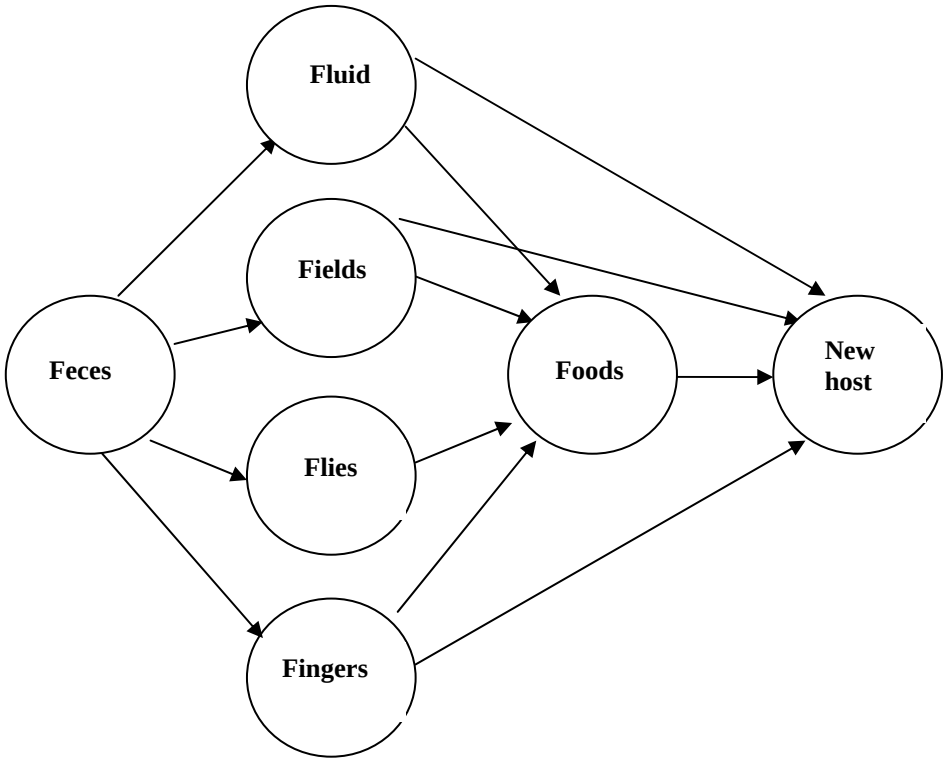


Figure 1. "F-diagram"

It is now widely accepted that water supplies and sanitation, though necessary for the prevention of diarrhea in young children, are not sufficient unless they are accompanied by changes in domestic hygiene behavior [155]. It is likely that improving hygiene practices is potentially one of the most effective means of reducing the global burden of diarrheal diseases in children.

Vietnam is located in Southeast Asia bordering the People's Republic of China to the north, the People's Democratic Republic of Laos and the Kingdom of Cambodia to the west, and the Pacific Ocean to the east. The country is divided into 61 provinces and cities directly belonging to the central government. There are three administrative levels in Vietnam: provinces, districts, and communes. At present, there are 600 administrative units at district level (districts, urban districts, cities belonging to provinces, and towns) and about 11,000 administrative units at commune level or equivalent. The population of Vietnam was estimated to be around 79.7 million persons according to the National census in 2002 [171]. By the end of 2004, it was around 82 million (information of National committee for Population, Family and Children of Vietnam). Hanoi is the capital of Vietnam with a population of 3 million persons including 7 urban districts and 5 suburban districts (General Statistic Office of Vietnam, 2002). Vietnam started launching National Program for Prevention for Diarrhea in 1982 when every year, the prevalence of at least two episodes of diarrhea occured each child less than 5 years of age. This program has brought promising results when the diarrhea episodes per child per year have been decreasing from 2.2 in 1985, to 1.8 in 1990, and to 1.3 in 2003. The death rate due to diarrhea was decreasing with 2.4%, 1.4%, and 0.7% in 1986-1987, 1990-1991, and 2003, respectively [127]. The Ministry of Health utilizes both preventive as well as curative strategies to minimize the effect of diarrhea on children health. The Ministry emphasizes health education programs to reduce the incidence of diarrhea among children, and promotes the use of oral rehydration therapy mostly through ORS.

1.2. Enteric pathogens

1.2.1. Diarrheagenic *Escherichia coli* and *Shigella*

1.2.1.1. *Escherichia coli*

E. coli is the type species of the genus *Escherichia* that contains mostly motile Gram-negative bacilli that fall within the family Enterobacteriaceae. It is the predominant facultative anaerobe of the human colonic flora. The organism typically colonizes the infant gastrointestinal tract within hours of life, and, thereafter, *E. coli* and the host derive mutual benefit for decades [120]. However, there are several highly adapted *E. coli* clones that have acquired specific virulence factors, which increase ability to adapt to new niches and allow them to cause a broad spectrum of diseases. Three general clinical syndromes can result from infection with pathogenic *E. coli* strains: enteric/diarrheal disease; urinary tract infection; and sepsis/meningitis [165]. As long as these bacteria do not acquire genetic elements encoding for virulence factors, they remain benign commensals. Strains that acquire bacteriophage or plasmid DNA encoding enterotoxins or invasion factors become virulent and can cause either a plain, watery diarrhea or an inflammatory dysentery. These diseases are most familiar to tourists as traveller's diarrhea, but they are also major health problems in endemic countries, particularly among infants. Among the *E. coli* causing intestinal diseases, there are six well-described categories: enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* (DAEC), enterohemorrhagic *E. coli* (EHEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E.*

coli (ETEC) [165]. These categories have virulence attributes that help bacteria to cause diseases by different mechanisms (**Figure 2**):

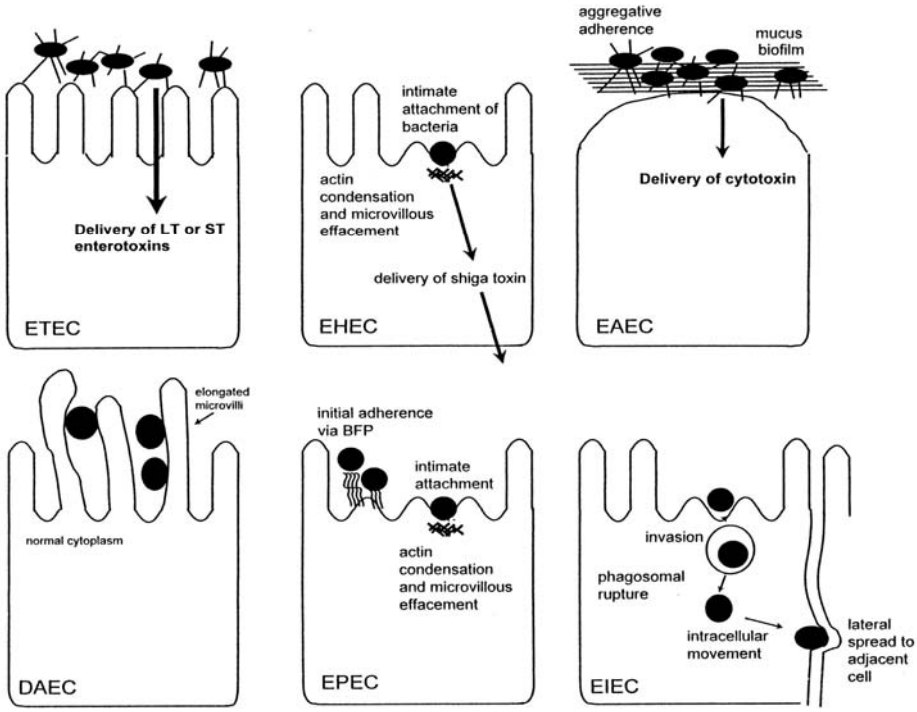


Figure 2. Pathogenic schemes of diarrheagenic *E. coli*. The six recognized categories of DEC have unique features in their interaction with eukaryotic cells. Here, the interaction of each category with a typical target cell is schematically represented. It should be noted that these descriptions are largely the result of *in vitro* studies and may not completely reflect the phenomena occurring in infected humans. (Reproduced with the permission from [165]).

EAEC is now defined as *E. coli* that do not secrete heat-labile (LT) or heat-stable (ST) enterotoxins and adhere to HEp-2 cells in an aggregative (AA) pattern [165, 168]. The basic strategy of EAEC seems to comprise colonization of the intestinal mucosa, probably predominantly that of the colon, followed by secretion of enterotoxins and cytotoxins [168]. Studies on human intestinal specimens indicate that EAEC induces mild, but significant, mucosal damage [102]. EAEC strains characteristically enhance mucus secretion from the mucosa, with trapping of the bacteria in a bacterium-mucus biofilm [165]. Two further observations support a role of mucus in EAEC pathogenesis: EAEC bound rabbit mucus avidly in an *in vitro* model [257] and volunteers fed EAEC strains excrete mucoid stools [164]. The formation of a heavy biofilm may be related to the diarrheagenicity of the organisms and, perhaps, to its ability to cause persistent colonization and diarrhea. In addition to forming mucous biofilm, many EAEC strains induce cytotoxic effects on the intestinal mucosa. In animal models infected with EAEC in rabbit and rat ileal loops, light microscopy showed a destructive lesions [266]. EAEC also induced shortening of the villi, hemorrhagic necrosis of the villous tips, and a mild inflammatory response with edema and mononuclear infiltration of the submucosa. Both light and electron microscopy showed adherent bacteria without the attaching and effacing lesion, which is characteristic of EPEC. The clinical features of EAEC diarrhea are increasingly well defined in outbreaks, sporadic cases, and the volunteer model. Typical illness is characterized by a watery, mucoid, secretory diarrhea with low grade fever and

little to no vomiting [19, 194]. However, up to one third of patients with EAEC diarrhea have had grossly bloody stools [51]. A growing number of studies have supported the association of EAEC with diarrhea in developing countries, most prominently in association with persistent diarrhea [18-20, 68, 142]. Previous studies in children less than 5 years of age with all diarrhea or acute diarrhea have shown the significant difference of EAEC identification prevalence compared to the controls: 33% vs. 15%; 12% vs. 5%; 21% vs. 4%; 32% vs. 17%; and 27% vs. 15% according to Nataro et al., Cravioto et al., Bhatnagar et al., Bouzari et al., and Gonzalez et al., respectively [21, 32, 51, 92, 166]. EAEC and persistent diarrhea syndrome have been consistently associated [68, 142, 258]. The increasing number of such reports and the rising proportion of diarrheal cases in which EAEC is implicated suggest that EAEC is important emerging agent of pediatric diarrhea.

Unlike EAEC that often cause persistent diarrhea, ETEC is associated with acute diarrhea among children in developing countries and diarrhea in travelers to developing countries [120]. ETEC is a common cause of infectious diarrhea [27], especially in tropical climates, where uncontaminated water is not readily available. Most of the illnesses, in terms of both numbers of cases and severity of symptoms, occur in infants and young children after weaning. This pathogen may express an LT only, an ST only, or both. LTs are a class of enterotoxins that are closely related in structure and function to cholera enterotoxin (CT), which is expressed by *Vibrio cholerae* [229]. Genes encoding LT and ST are carried on plasmids. ETEC colonizes the surface of the small bowel mucosa and elaborates enterotoxins, which give rise to intestinal secretion. Colonization is mediated by one or more proteinaceous fimbrial or fimbrillar Colonization Factors (CFs), which are designated by CFA (Colonization Factor Antigen), CS (Coli Surface Antigen) or PCF (Putative Colonization Factor) followed by a number [120]. A single plasmid often carries a toxin and CFA, for example, CFA/I and ST [152, 159, 206], CFA/II and LT and ST [195, 231], and CFA/IV and ST [250]. The clinical features of ETEC diarrhea are constant with the pathogenetic mechanism of ETEC enterotoxins. The similarity has been seen in both volunteers and clinical cases. Diarrhea caused by ETEC result from the ingestion of rather large inocula of bacteria that then colonize the small intestine and produce toxins that cause net secretion into the intestinal lumen. The illness is typically abrupt. The diarrhea is usually watery, without blood, mucus, or pus, fever and vomiting present in a minority of patients [58, 138]. ETEC diarrhea may be mild, brief, and self-limiting or may result in severity similar to that seen in *V. cholerae* infection [139, 271]. ETEC are associated with two clinical syndromes: weanling diarrhea among children in developing countries and traveler's diarrhea. The percentage of ETEC diarrhea in children varies from 10 to 30% [2, 77, 108, 150]. Several studies suggest that 20 to 60% of travelers from developed countries visiting the areas where ETEC infection is endemic, experience diarrhea, 20 to 40% of cases are due to ETEC [7, 28, 57].

EPEC was the first pathotype of *E. coli* to be described. Large outbreaks of infant diarrhea in United Kingdom led Bray, in 1945, to describe a group of serologically distinct *E. coli* strains that were isolated from children with diarrhea but not from healthy children [120]. The hallmark of infections due to EPEC is the attaching-and-effacing (A/E) histopathology, which can be observed in intestinal biopsy specimens from patients or infected animals [165]. The bacteria intimately attach to intestinal epithelial cells and cause striking cytoskeletal changes, including the accumulation of polymerized actin directly beneath the adherent bacteria. The microvilli of the intestine are effaced and pedestal-like structures on which the bacteria perch frequently rise up from the epithelial cell. This ability is encoded by genes on a 35-kb pathogenecity island (PAI) called the locus of enterocyte effacement (LEE). The LEEs have a gene *eae*A encoding a protein called

intimin, which mediates the intimin attachment of EPEC to epithelial cell [115, 153]. EPEC also has other virulence factors such as a protein named lymphostatin, which inhibits lymphocyte activation [130]. Diarrhea probably results from multiple mechanisms, including active ion secretion, increased intestinal permeability, intestinal inflammation and loss of absorptive surface area resulting from microvillus effacement. A consensus definition was achieved at the Second International Symposium on EPEC: A/E, Stx-negative strains possessing the EAF plasmid would be called "typical EPEC", while such strains that do not possess the EAF plasmid would be called "atypical EPEC" [165]. The most special feature of the epidemiology of disease due to EPEC is the striking age distribution. EPEC infection is primarily a disease of infants younger than 2 years [165]. EPEC causes primarily acute diarrhea, although many cases of persistent EPEC diarrhea have also been reported [165, 223]. In addition to watery diarrhea, vomiting and low-grade fever are common symptoms of EPEC infection. As compared to the developed countries, EPEC plays more important role in developing countries since it is the major cause of diarrhea. Many case-control studies have found EPEC to be more frequently isolated from children with diarrhea than from the controls, even studies in Brazil, Mexico, South Africa have shown that 30 to 40% of infant diarrhea can be attributed to EPEC [49, 50, 90, 91, 210]. EPEC also causes diarrheal diseases in many settings: nosocomial outbreaks, outpatient clinics, patients admitted to hospitals, urban and rural areas.

EHEC is an etiological agent of diarrhea with life-threatening complications. EHEC belongs to a group of *E. coli* called VTEC ("verotoxigenic *E. coli*" or "Vero cytotoxin-producing *E. coli*") or STEC ("Shiga toxin-producing *E. coli*"), formerly SLTEC ("Shiga – like toxin-producing *E. coli*"). Most EHEC strains are acid resistant, which allows them to survive the acid conditions of the stomach. It is believed that they adhere to the colon and distal small intestine; however, typical lesions have not been demonstrated [124]. The best-characterized adherence phenotype is the intimate or attaching and effacing adherence mediated by *eae*A gene. STEC isolates that possess the *eae*A gene are capable of producing diarrhea. However, the pathological lesions associated with HC (Hemorrhagic Colitis) and HUS (Hemorrhagic Uremic Syndrome), are due to the action of Shiga toxin (Stx) with endothelial cells. Sxt is translocated from the apical surface of the enterocyte to the basolateral side without damage to the enterocyte [165]. In the bloodstream, stx targets the tissues, the intestine, the central nervous system, and the kidneys. It damages renal endothelial cells and occludes the microvasculature through a combination of direct toxicity and induction of local cytokine and chemokine production, resulting in renal inflammation [6]. Stx also induces apoptosis in intestinal epithelial cells and mediates local damages in the colon, which results in bloody diarrhea, hemorrhagic colitis, necrosis and intestinal perforation. [116]. The term "enterohemorrhagic *E. coli*" (EHEC) was originally coined to denote strains that cause HC and HUS, express Stx, cause A/E lesions on epithelial cells, and possess a ca 60-MDa plasmid [138, 140]. Thus, EHEC denotes a subset of STEC and includes a clinical connotation that is not implied with STEC. Whereas not all STEC strains are believed to be pathogens, all EHEC strains by the above definition are considered to be pathogens. EHEC is an emerging pathogen that has stimulated worldwide interest in several large food-borne outbreaks. EHEC can cause non-bloody diarrhea, bloody diarrhea, and HUS in all age groups, but the young and the elderly are most susceptible. The most notorious *E. coli* serotype associated with EHEC is O157:H7, which has been the cause of several large outbreaks of disease in North America, Europe, and Japan [33, 67, 94, 119, 183]. The principle reservoir of EHEC is the bovine intestinal tract and initial outbreaks were associated with consumption of undercooked hamburgers. However, many others items have been associated with EHEC infection such as sausages, unpasteurized milk, lettuce [262], apple juice. EHEC has also

transmitted via recreational and municipal drinking water, petting zoo, and farm visitation [120].

EIEC is a pathogenic form of *E. coli* that can cause dysentery [165]. EIEC strains are biochemically, genetically and pathogenically closely related to *Shigella* spp. The precise pathogenetic scheme of EIEC has yet to be elucidated. However, pathogenesis studies of EIEC suggest that its pathogenetic features are virtually identical to those of *Shigella* spp. [89, 188]. Genes necessary for invasiveness are carried on a 120-MDa plasmid in *S. sonnei* and a 140-MDa plasmid in other *Shigella* serotypes and in EIEC [16, 221, 230]. EIEC penetrates the intestinal mucosa, predominantly that lining the large intestine, to cause inflammation and mucosal ulceration that are charateristic of bacillary dysentery. Although EIEC is prototype of invasive bacteria, that are unable to penetrate enterocytes via their luminal aspect. Instead, it passes through M cells, which are antigen-sampling cells that are a major constituent of the specialized epithelium overlying the lymphoid follicles in the small and large intestine [219].

The most severe manifestation of infection with *Shigella* spp. and EIEC is bacillary dysentery, a syndrome characterized by frequent small-volume stools with blood and mucus. Bacillary dysentery is responsible for a substantial proportion of acute diarrheal diseases worldwide. Both organisms have been shown to invade the colonic epithelium, a phenotype mediated by both plasmid and chromosomal loci. However, most persons infected with *Shigella* spp. or EIEC present with watery diarrhea that may or may not be followed by dysentery [165, 233, 248]. In addition, both EIEC and *Shigella* spp. elaborate one or more secretory enterotoxins that may play roles in diarrheal pathogenesis [165, 167]. In most of cases, EIEC elicits watery diarrhea that is indistinguishable from that due to infection by other *E. coli* pathogens [165]. EIEC can cause outbreaks of gastroenteritis. In sporadic cases, many EIEC are probably misidentified as *Shigella* spp. or nonpathogenic *E. coli* strains. EIEC outbreaks are usually food-borne or waterborne [165].

DAEC is a category of DEC that produces the diffuse adherence on Hep-2 cell model [168]. Little is known about the pathogenesis of DAEC. A surface of fimbria that mediates DA (Diffuse Adherence) phenotype has been cloned and characterized [22-24, 125]. The gene encoding the fimbria can be found on either the bacterial chromosome or a plasmid. Few epidemiological and clinical studies have been carried out to adequately describe the epidemiology and clinical aspect of diarrhea caused by DAEC. In the study by Poitrineau et al. [197], the patients with DAEC had watery diarrhea without blood and fecal leukocytes. The association of DAEC with diarrhea has been pointed out in some studies [87, 113, 141] but not in the others [84, 98, 222].

There have been several available assays to identify all categories of diarrheagenic *E. coli*. Isolation and identification of *E. coli* based on the biochemical properties are widely used in most of microbiological laboratories since it does not require many advanced equipments and complicated protocols. *E. coli* can be easily recovered from clinical samples on general or selective media at 37^{o}C under aerobic conditions. *E. coli* are usually identified via biochemical reactions that can be done in individual culture tubes or by using test "strips". These tests are commercially available and either method has satisfactory results. In general, DEC cannot be identified based on biochemical criteria alone, as in most cases they are indistinguishable from nonpathogenic *E. coli*.

In addition to the biochemical tests, serology has been commonly used. It is based on Kauffman's scheme for the serologic classification of *E. coli*. *E. coli* are serotyped on the basis of their O (somatic), H (fragellar), and K (capsular) surface antigen profile. At least 180 O, 60 H, and 80 K antigens have been proposed [209, 264]. Each O antigen defines a

serogroup. *E. coli* of specific serogroups can be associated with certain clinical syndromes [36, 165]. A specific combination of O and H antigens defines the ‘‘serotype’’ of an isolate. One DEC type can be in many serogroups and one serogroup may belong to several DEC and even, avirulent *E. coli* [36, 165]. Due to the limited sensitivity and specificity, and the various combinations of antigens, serotyping is tedious and expensive and is performed reliably only by a small number of reference laboratories.

Among the most useful methods to diagnose DEC are phenotypic assays, which are based on the virulence characteristics. Of them, the Hep-2 adherence assay is useful to identify the adherence patterns of diarrheagenic *E. coli*. It remains the “Golden standard” for the diagnosis of EAEC and DAEC [56, 165, 265]. Identification of ETEC has relied on the detection of the enterotoxins LT and/or ST. This assay was initially detected in a rabbit ligated ileal loop assay [66]. The expense and lack of standardization caused this test to be replaced by the suckling-mouse assay [85]. The classical phenotypic assay for EIEC identification is the Sereny (guinea pig keratoconjunctivitis) test which correlates with the ability of the strain to invade epithelial cells and spread from cell to cell [132]. The methods based on phenotype are often expensive, require special expertise, and employ various detection systems (e.g., cell culture, cytotoxicity assays). Applying such assay to enteric microbiologic diagnosis is cumbersome.

Molecular methods remain the most popular and most reliable techniques for differentiating diarrheagenic strains from nonpathogenic members of the stool flora and distinguishing one category from another. The assays based on Nucleic acid probes and Polymerase Chain Reaction (PCR) has been intensively used. Of them, PCR is a powerful molecular biology technique. PCR has its advantages such as great sensitivity in detection of target templates and it gives reliable, rapid results and well as it is highly sensitive and specific [59, 86, 117, 201, 204, 224, 238]. However, there are some substances in stools that inhibit PCR reaction resulting in decreasing its sensitivity [238]. Employing isolated colonies and/or extraction and partial DNA purification can solve this obstacle. In addition, the single PCR applied for certain virulent factor could be insufficient, time-consuming, and labor intensive when processing a number of samples with different targets. Multiplex PCR by combining different primers specific for the target genes can simultaneously detect numerous target genes in a single reaction.

1.2.1.2. *Shigella*

Shigella are Gram-negative, non-motile, facultatively anaerobic, non-spore-forming rods. *Shigella* are differentiated from the closely related *E. coli* on the basis of pathogenicity, physiology (failure to ferment lactose or decarboxylate lysine) and serology [216]. The genus is divided into four serogroups with multiple serotypes: A (*S. dysenteriae*, 12 serotypes); B (*S. flexneri*, 6 serotypes); C (*S. boydii*, 18 serotypes); and D (*S. sonnei*, 1 serotype) [216]. Spent medium from *S. flexneri* or EIEC cultures elicit fluid accumulation in rabbit ligated ileal loops and ion secretion in isolated ileal tissue. Using these assays, enterotoxins designated ShET1 and ShET2 (*Shigella* enterotoxin 1, and *Shigella* enterotoxin 2, respectively) have been identified, and the genetic loci encoding these toxins have been localized to the chromosome and plasmid, respectively. ShET1 is neutralized by convalescent sera from volunteers challenged with *S. flexneri* 2a, suggesting that this toxic moiety is expressed by *Shigella* growing in the human intestine. The ShET1 locus is present on the chromosome of *S. flexneri* 2a, but it is only occasionally found in other serotypes. In contrast, ShET2 is more widespread and detectable in 80% of *Shigella*

representing all four species. These enterotoxins may elicit the diarrheal prodrome that often precedes bacillary dysentery; however, their role in the disease process remains to be defined by controlled challenge studies using toxin-negative mutants. *S. dysenteriae* serotype 1 expresses Shiga toxin, an extremely potent, ricin-like, cytotoxin that inhibits protein synthesis in susceptible mammalian cells. This toxin also has enterotoxic activity in rabbit ileal loops, but its role in human diarrhea is unclear, since *Shigella* apparently express a number of enterotoxins. Experimental infection of rhesus monkeys with *S. dysenteriae* 1, and with a Shiga toxin-negative mutant, suggests that this cytotoxin causes capillary destruction and focal hemorrhage that exacerbates dysentery. More importantly, Shiga toxin is associated with HUS, a complication of infections with *S. dysenteriae* 1. Closely related toxins are expressed by EHEC including the potentially lethal, food-borne O157:H7 serotype [216].

The four *Shigella* species cause varying degrees of dysentery. This is characterized by fever, abdominal cramps and diarrhea containing blood and mucous. Shigellosis is endemic in developing countries where sanitation is poor. Typically 10 to 30% of enteric disease [1, 111, 182, 247], and 50% of the bloody diarrhea or dysentery of young children [61, 247], can be characterized as shigellosis, and the prevalence of these infections decreases significantly after 5 years of life. In developed countries, single-source, food or water-borne outbreaks occur sporadically, and pockets of endemic shigellosis can be found in institutions and in remote areas with substandard sanitary facilities. Isolation and identification of *Shigella* spp. is usually based on the culture, biochemical tests, and serotyping. Molecular methods can be used to determine some target genes.

1.2.1.3. Antibiotic resistance of DEC and *Shigella*

Antimicrobial therapy is indicated for moderate to severe disease to reduce the duration of illness for gastroenteritis [63, 64]. The progressive increase in antimicrobial resistance among enteric pathogens in developing countries is becoming a critical area of concern. Among these bacteria, strains of different diarrheagenic categories of *E. coli*, such as EAEC, EHEC, EIEC, EPEC, and ETEC; and *Shigella* are among the most important causes of acute enteritis and subsequent morbidity and mortality in children in developing countries [165]. In these countries, ampicillin, chloramphenicol, trimethoprim/ sulfamethoxazole are widely used to treat diarrhea because of their low cost and ready availability [45, 215, 256]. Many studies have shown high prevalence of resistance in enteric pathogens, especially DEC and *Shigella* [12, 112, 228, 269]. There are several factors that have contributed to the increasing resistance of the pathogens. Among them, the overuse and misuse of antibiotics in the treatment of infectious diseases, especially in diarrhea could lead to an increase of antibiotic resistance. A community study in Vietnam showed that 75% of the children had been treated with antibiotics during the month preceding the study; of these, 80% had been purchased from private drug outlets [137]. Another study in Vietnam in 1997 mentioned that ampicillin, chloramphenicol, tetracycline, and trimethoprim/sulfamethoxazole were the antimicrobial agents that were the most commonly dispensed over counter [256]. According to some national surveys in Vietnam, *E. coli* and *Shigella* are among the most frequently isolated bacteria in clinical specimens, 16% and 1.5%, respectively [268]. They are increasingly resistant not only to commonly used antibiotics such as ampicillin, chloramphenicol, and trimethoprim/ sulfamethoxazole, but also to quinolones, which are the alternatives for treatment of severe diarrhea. The issues mentioned above raised a necessity of updated information and surveillance on antibiotic resistance of DEC and *Shigella*.

1.2.2. Enterotoxigenic *Bacteroides fragilis*

The members of *Bacteroides fragilis* group are Gram-negative anaerobic, non-spore forming rods. They are members of the normal flora in the human intestine. There are ten species within the *Bacteroides* group: *B. caccae*, *B. distasonis*, *B. eggerthi*, *B. fragilis*, *B. thetaiotaomicron*, *B. vulgatus*, *B. ovatus*, *B. stercoris*, *B. uniformis* and *B. merdae* [235, 255]. Recently, two new species, *Bacteroides nordii*, and *Bacteroides salyersae*, have been proposed to honor Carl Erik Nord and Abigail Salyers, who have contributed so much to our knowledge of intestinal bacteriology and anaerobic bacteriology in general [234]. Although, *B. fragilis* makes up about 1 to 2% of the normal flora, it accounts for roughly one-fourth of all anaerobic bacteria isolated from clinical specimens [157]. There are several clinical syndromes that are associated with *Bacteroides* infections such as skin and soft tissue infections, abscesses, bacteremia, intra-abdominal infections and gynecological infections, especially after surgery or trauma. Recently, a new potential pathogen for diarrheal disease has been discovered. This pathogen, enterotoxigenic *B. fragilis* (ETBF) may contribute to the burden of diarrheal disease [35, 121, 123, 154, 160-162, 213, 214, 217, 277]. These organisms were first identified in diarrheal stools of young domestic animals in 1984 [160] and later, in 1987, also in the stools of children and adults with acute and chronic diarrhea [162]. Controlled studies of ETBF showed an association of ETBF with diarrheal disease in children between the ages of 1 and 5 years [35, 121, 154, 213, 214, 217], especially, a strong association with acute diarrhea in children older than 1 year of age [213, 214, 217]. In the study by Sack et al. [214], the prevalence of ETBF was 12% in patients with diarrhea of unknown etiology compared to 6% of controls. However, in children between ages of 1 to 3 years old, 20-25% of diarrheal illnesses were associated with ETBF vs. an 8-9% ETBF carriage rate in healthy children. The prevalence of ETBF detection was 6.1% of children with diarrhea compared to 1.2% in controls in another study [213]. In a recent study in adults with diarrhea, ETBF was detected in 27% of the patients and 12% controls, with the highest prevalence in adults > 60 years old [277]. However, limited details of the clinical illnesses associated with ETBF infection are available from the studies conducted to date, which have focused solely on microbiologic evaluations. When discussed, the clinical syndrome associated with ETBF infection has predominantly been acute, self-limited watery diarrhea seemingly non-inflammatory in nature.

The ETBF strains are distinguished from non-toxigenic by the secretion of a 20-kDa metalloprotease toxin (*B. fragilis* toxin or BFT), the first recognized and only established toxin to date for *B. fragilis* [225]. Enterotoxin produced by *B. fragilis* causes fluid to accumulate in the ligated lamb intestine [160] and generates reversible morphological changes of HT29/C1 cell monolayers [260]. In addition, this toxin has been shown to produce extensive tissue damage in the intestinal mucosa *in vivo* [178], to increase bacterial internalization by enterocytes [263], and to modify the actin cytoskeleton of sensitive cells [55, 134]. Recently, the terminology *B. fragilis* toxin (BFT) or fragilysin has come to replace enterotoxin [122]. The nucleotide sequence of the gene, termed *bft* encoding BFT has been determined and these studies indicate that there are three subtypes of the *bft* gene, *bft*-1, *bft*-2 and *bft*-3 [78, 122, 131].

The *Bacteroides fragilis* group is more resistant to antimicrobial agents than most other anaerobic bacteria [69]. Majority of the *B. fragilis* isolates are resistant to β-lactam antimicrobial agents as well as other antibiotics [83, 254]. The *B. fragilis* species unexplainably remains more susceptible to many antimicrobial agents than the other species within the groups [4, 75, 246]. However, the heavy use of certain antibiotics may

result in the selection and spread of resistant strains. It is well known that the level of antimicrobial resistance can differ from different settings due to the usage of certain antimicrobials [69, 74, 83, 254].

So far, there have been several methods for identification of *B. fragilis* from clinical samples. These include biochemical assay, gas liquid chromatography analysis, imunological methods, DNA probes, and PCR [47, 105, 135, 136, 143, 145, 193, 261, 275]. They contribute not only to diagnosis of *B. fragilis* infection in clinical aspect, but also to epidemiological studies.

1.2.3. Rotavirus

Rotavirus is a leading course of infantile gastroenteritis worldwide. Each year, rotavirus causes approximately 111 million episodes of gastroenteritis requiring only home care, 25 million clinic visits, 2 million hospitalizations, and 352,000-592,000 deaths in children less than 5 years of age [187]. The death rate due to rotavirus infection is responsible for 21% for low-income countries, 17% for low-middle income countries, 9% for high-middle income countries, and 1% for high-income countries [187]. These figures demonstrate the tremendous amount of global illness and deaths caused by rotavirus.

The wheel-like (Latin *rota* = wheel) particles of rotavirus were first identified as a human pathogen in 1973 by Bishop et al. when characteristic particles were observed in the cytoplasm of duodenal epithelial cells obtained from young children admitted to the hospital for treatment of acute diarrhea [26]. Rotavirus belongs to the Reoviridae family and is characterized by the segmented (11 segments) double-stranded RNA genome. The segments encode six viral capsid proteins (VP1, 2, 3, 4, 6 and 7) and six nonstructural proteins (NSP1-6) [46]. Rotavirus is classified into seven serogroups A-G based upon the antigenic properties of VP6. Only groups A, B, and C have been shown to infect humans and most animals. Rotavirus disease is mainly caused by group A. Furthermore, it is possible to determine the subgroup I (SG I) and II (SG II) specificities associated with the major inner capsid protein VP6 [30, 38, 128, 146, 186]. Within these groups, viruses are classified into serotypes based on differing outer capsid antigens VP7 and VP4. To date, 15 group A VP7 antigens (termed G types, G1-G15) and 20 VP4 antigens (termed P types, P1-P20) have been identified among human and animal strains [46]. The circulating serotypes of rotavirus differ in different geographical areas. However, the most prevalent rotavirus types are G1 to G4 [17, 25, 48, 149, 176, 226].

Rotavirus infects the mature enterocytes in the mid and upper part of the villi of the small intestine, which ultimately leads to diarrhea. Four hypotheses have been proposed with regard to virus-evoked intestinal secretion of fluid and electrolytes [146]. The first one is the diminished absorptive capacity of the intestinal epithelium. The experimental evidence for a diminished absorptive capacity of fluid, electrolytes, glucose and amino acids in rotavirus-infected intestines was reviewed when describing the morphological and functional effects of the virus [37, 106, 148, 180, 208]. While making an antiserum against NSP4, one of the non-structural proteins of rotavirus, Ball et al. [13] noted that giving the protein intraperitoneally induced diarrhea in newborn mice. This observation initiated a series of studies to elucidate if NSP4 might represent a virus enterotoxin. Some studies in animal models have showed that a part of NSP4 may function as an enterotoxin. It influences intracellular calcium concentration in colonic crypt cells [101, 245, 279, 280]. In addition to the two proposed hypotheses mentioned above, several observations made during rotavirus enteritis in neonatal mice suggested that the secretory response is in part

explained by an activation of the enteric nervous system (ENS). The involvement of the ENS may explain how the comparatively few cells at the villus tips infected by the virus can influence the intestinal crypts to augment its secretion of electrolytes and water [146]. The final hypothesis is about the deranged intestinal microcirculation. It refers that the invasion of the villus tip cells by rotavirus triggers release of "neuroactive/hormonal substances" which cause a villus ischemia and subsequent shortening of the villi and, hence, a decreased absorptive capacity [180, 236, 239, 240].

A symptomatic infection with rotavirus stimulates a strong humoral IgG immune response, which lasts for lifetime. While the IgG responses are easily recorded, it is generally thought that local IgA antibodies mediate protection from rotavirus disease. Several studies have shown that local IgA is important or at least a good predictor of protection [259].

Rotavirus infections may occur repeatedly in humans from birth to old age. Contrasting with the capacity of rotavirus to cause infection at any age, the clinical consequences of infection appear to be strongly influenced by age. The relative resistance to clinical symptoms that is observed in older children and adults is more likely due to active immunity, reinforced by repeated infections throughout life [146]. In addition to the different distribution of rotavirus infection over age, several differences in the epidemiology of rotavirus between developing countries and developed countries in temperature climates have been shown. In tropical countries, rotavirus occurs year round, but winter peaks and summer decreases of infection are seen in developed countries [9, 71, 147, 174, 282]. The seasonal pattern of infection could be useful for immunization against infection in infants.

The method of choice for diagnosis of rotavirus is PCR of the stool [30, 70, 128, 198]. This is more sensitive than microscopy and serological methods, and is also less time-consuming. However, it costs and requires the advanced technique. Rotaviruses are routinely detected by ELISA [109, 114, 176] because of their sensitivity, specificity, simplicity, and low cost. Novel methods for typing, including microarray, have been shown to be of use [144].

Previous studies have shown the burden of rotavirus diarrhea in many parts of the world [30, 70, 129, 147, 282]. In Vietnam, the investigations carried out from 1994 to 1999 clearly demonstrated that diarrhea due to rotavirus in Vietnamese children is substantial [174, 176]. Therefore, the updated information about rotavirus infections in correlation with clinical symptoms, epidemiological factors, and especially with co-infections with other pathogens is very important for the pediatricians and healthcare workers. It will help not only to improve the diagnosis and treatment of children's diarrhea but also to provide useful information in prophylaxis by vaccine in the near future.

There are few published studies on diarrhea in Vietnam [42, 111, 172-174, 176, 274]. Diarrhea accounts for almost 10,000 deaths per year, which is approximately 15% of all deaths among Vietnamese children less than 5 years old, or 6.5 deaths per 1,000 children [174]. Isenbarger et al. [111] carried out a prospective study in diarrhea incidence among 1,655 children less than 5 years of age in northern Vietnam for 1 year. Overall; 2,160 cases occurred (1.3 cases/child per year). Peak rates of diarrhea occurred in children less than 12 months old. Rates ranged from 3.3 cases/child per year in children less than 1 year old, to 0.7 cases/child per year in 4-year-olds. *Campylobacter*, *Shigella* and ETEC were most commonly isolated. Other studies [174, 176] looking at the burden of rotavirus diarrhea showed that about 50% of the diarrhea-related deaths in Vietnam were due to rotavirus, the number would represent 4-8% of all deaths among children less than 5 years of age; 2,700-

5,400 rotavirus-related deaths per year, and 1 death per 280-560 children during the first 5 years of life. Diarrhea, in general, and diarrhea caused by some common pathogens such as rotavirus, *Shigella*, DEC is substantial. Detailed knowledge on enteric pathogens, clinical symptoms, and epidemiological factors related to infectious diarrhea will contribute to effective diagnosis, treatment, and intervention of diarrhea in Vietnamese children.

2. AIMS OF THE STUDY

1. To identify and characterize diarrheagenic *Escherichia coli* and some other enteric pathogens from fecal samples in children less than 5 years of age in Hanoi, Vietnam.

2. To assess the antibiotic susceptibility of isolated diarrheagenic *Escherichia coli* and *Shigella* strains in these children.

3. To determine the role of Group A rotavirus in causing diarrhea in children in Hanoi.

4. To determine the role of enterotoxigenic *Bacteroides fragilis* in causing diarrhea in children in Hanoi.

5. To relate the etiology and epidemiology of diarrhea in children in Hanoi.

3. MATERIALS AND METHODS

3.1. Study subject (Paper I, III, IV, V)

A total of 836 children from 0 to 60 months of age living in Hanoi, Vietnam were included in the study. The children were enrolled in the study during a 1-year period starting in March 2001 and ending in April 2002. This consisted of 587 children with diarrhea attending three different hospitals and 249 age-matched healthy controls enrolled from one day care center and one health care center in Hanoi. Diarrhea was characterized by the occurrence of three or more loose, liquid, or watery stools or at least one bloody loose stool in a 24-h period. An episode was considered resolved on the last day of diarrhea followed by at least 3 diarrhea-free days. An episode was considered persistent if it continued for ≥ 14 days [14]. The healthy children did not have diarrhea at least 1 month before the collection of fecal sample. Vomiting was defined as the forceful expulsion of gastric contents occurring at least once in a 24-h period. Fever was defined as an under-arm measured temperature > 37.2^{o}C (99 degrees Fahrenheit). Thresholds of 37.2^{o}-39^{o} and > 39^{o}C were moderate and high fever, respectively. Dehydration level was assessed following the recommendations of WHO Program for Control of Diarrhoeal Diseases and was done by the pediatricians [273]. After the informed consent was obtained, a pediatrician specifically assigned to the study examined each patient and filled out the demographic data and information on clinical symptoms, illness onset, on a standardized questionnaire. The health care workers also obtained the similar information from the controls. Some other factors related to the demography and socio-economy of the children's parents have been obtained. Education of the parents was assessed as high and lower level based on whether they were officers (persons finishing at least college or university) and workers or farmers, or free labors (persons learning up to high school), respectively. Living standard of the child's family was evaluated in monthly income of the whole family in Vietnamese Dong (VND) and ranked into five levels (very poor, poor, middle, fair, and rich). Water sources were divided into hygienic (pipe water) and unhygienic resource (pool or well, or rainwater). Flush toilet was considered the hygienic convenience. Getting information of health and sanitation from any sources had been assessed according to whether the child's family had access to this kind of information often (daily and weekly) or less often (monthly, rarely, or almost never).

3.2. Sample collection

Fecal samples (one from each subject), from children without diarrhea were collected by their parents and teachers at day care center. From the children with diarrhea, the nurses collected stools as soon as the children were admitted to the hospital. Each stool specimen was collected in a special container with Cary-Blair transport medium, kept at 4^{o}C, and transferred to the microbiology laboratory within 24 hours for analysis. The residual of each sample after the first culture on the media was kept at –70^{o}C for further work.

The collection of samples stopped for 2 weeks for Tet holidays in Vietnam in February 2002.

3.3. Bacterial strains (Paper I-IV)

Bacterial strains used for PCR for DEC (Study 1), antibiotic testing (Study 2), and PCR for enterotoxigenic *B. fragilis* (Study 4) are listed in **Table 1**.

Table 1. Reference strains used in the studies

Category	Reference strain	Target gene	Study
ETEC	ATCC 35401	*elt*B, *est*A	1
EHEC	ATCC 43890	*vt*1, *eae*A	1
EHEC	ATCC 43889	*vt*2, *eae*A	1
EPEC	ATCC 43887	*eae*A, *bfp*A	1
EIEC	ATCC 43893	*ial*	1
EAEC	97R*	pCVD432-harboring strain	1
E. coli (negative control)	ATCC 11775	No virulence gene	1
E. coli	ATCC 25922		2
Staphylococcus aureus	ATCC 29213		2
ETBF	D-94	Harboring *bft* gene**	4
B. fragilis (negative control)	NCTC 9343	No *bft* gene	4

* This strain was verified by other methods and was kindly provided by the Collaboration Project between Sweden and Nicaragua on Diarrhea.

** This strain was used in the previous study [278].

Two hundred and thirty-eight verified strains of different *E. coli* categories from the Swedish Center for Control of Infectious Diseases, Culture Collection of University of Gothenburg, and the strain collection at the Division of Clinical Bacteriology (Karolinska University Hospital, Huddinge, Karolinska Institutet) were used to evaluate the multiplex PCR assay. These included 139 non-DEC and 99 DEC, divided into 18 strains of EAEC, 17 strains of EIEC, 15 strains of EHEC, 17 strains of EPEC, and 32 strains of ETEC.

3.4. Methods

3.4.1. Bacterial culture condition (Paper I, II, IV)

Stool samples collected in Cary-Blair transport medium were cultured on the surface of Sorbitol-MacConkey Agar (SMAC) (Labora, Stockholm, Sweden) for selection of *E. coli* isolates and on other media, such as TCBS Cholera Medium (Labora) for *Vibrio;* Deoxycholate Citrate Agar (Sigma-Aldrich, Stockholm, Sweden) for *Shigella*, *Salmonella* followed by overnight incubation at 37^{o}C.

DEC and *Shigella* strains were cultured according to the recommendations of National Committee for Clinical Laboratory Standards (NCCLS) for antibiotic susceptibility testing.

For identification of ETBF, one milliliter of fecal sample suspended in PBS (1g/5ml) was inoculated into Fastidious Anaerobe Broth (FAB) medium (LAB 71, LAB M, International Diagnostic Group, Bury, UK) containing 0.05% (wt/vol) kanamycin (Sigma Chemical Company, St. Louis, Mo, USA). The mixture was anaerobically incubated at 37°C for 48 h.

3.4.2. Multiplex PCR for identification of DEC (Paper I)

3.4.2.1. Setting up Multiplex PCR

A smear of bacteria from the first area of a SMAC plate was inoculated into 5 ml PBS tube to a density of MacFarland 4 (10^9 – $5x10^9$ bacteria/ml). The 5 ml tube was boiled for 20 minutes, followed by centrifugation at 2,500 x g for 10 minutes to pellet cell debris (Sorvall RT 6000 B Refrigerated Centrifuge, Dupont, USA). The supernatant was used for PCR.

The DNA templates were subjected to multiplex PCR using specific primers (**Table 2**), as previously described [244] for the detection of the following virulence markers: *eae*A (structural gene for intimin of EHEC/EPEC), *bfp*A (structural gene for the bundle-forming pilus of EPEC), *vt*1 and *vt*2 (Shiga toxins 1 and 2 of EHEC), *elt*B and *est*A (enterotoxins of ETEC), *ial* (invasion-associated locus of the invasion plasmid found in EIEC/*Shigella*), and pCVD (the nucleotide sequence of the EcoRI-PstI DNA fragment of pCVD432 of EAEC). The primer sequences selected for the amplification completely matched the sequences of the corresponding genes of EAEC, EHEC, EIEC, EPEC, and ETEC in the GenBank/EMBL database libraries.

PCRs were performed in a 20-µl reaction mixture containing 2 µl of template DNA, 2 µl of 10x PCR buffer II, 1.6 µl of 1.25 mM dNTPs mixture, 1.6 µl of 25 mM $MgCl_2$, 0.1 µl of 5U/µl AmpliTaq Gold DNA polymerase (Perkin-Elmer, New Jersey, USA), and 0.2 µM of each primer, except VT1 0.4 µM (INTERACTIVA Biotechnologie GmbH, Ulm, Germany). Thermocycling conditions with a Gene Amp PCR System 9700 (AB Biosystem, Stockholm, Sweden) were as follows: 96°C for 4 minutes, 94°C for 20s, 55°C for 20s, and 72°C Applied for 10s, for 30 cycles, ending with a 7-min extension at 72°C. PCR products (10 µl) were evaluated with a 1.5% (wt/vol) agarose gel (Gibco Life Technologies, Paisley, UK) at 120 mV for 30 min. A molecular marker (1 Kb DNA Ladder; Gibco/BRL) was run concurrently. The DNA bands were visualized and photographed under UV light after staining with ethidium bromide.

Firstly, multiplex PCR was set up with reference strains of EAEC, EIEC, EHEC, EPEC, ETEC, and non-diarrheagenic *E. coli*. Then, the sensitivity of this diagnostic method was determined by the number of reference strains of DEC (in cfu/ml) spiked into each ml of stool sample that could be detected. Finally, 238 strains of various categories of *E. coli* were tested to evaluate multiplex PCR.

Table 2. Primers used in the multiplex PCR for detection of diarrheagenic *E. coli*

Primer	Target gene	Accession number	Primer sequence	Amplimere (bp)
LT	*elt*B	S60731	$^{5'}$TCTCTATGTGCATACGGAGC$^{3'}$ $^{5'}$CCATACTGATTGCCGCAAT$^{3'}$	322
ST	*est*A	M34916	$^{5'}$GCTAAACCAGTA$^{G}_{A}$GGTCTTCAAAA$^{3'}$ $^{5'}$CCCGGTACA$^{G}_{A}$GCAGGATTACAACA$^{3'}$	147
VT1	*vt*1	AF461172	$^{5'}$GAAGAGTCCGTGGGATTACG$^{3'}$ $^{5'}$AGCGATGCAGCTATTAATAA$^{3'}$	130
VT2	*vt*2	AY143337	$^{5'}$ACCGTTTTTCAGATTTT$^{G}_{A}$CACATA$^{3'}$ $^{5'}$TACACAGGAGCAGTTTCAGACAGT$^{3'}$	298
eae	*eae*A	AE005595	$^{5'}$CACACGAATAAACTGACTAAAATG$^{3'}$ $^{5'}$AAAAACGCTGACCCGCACCTAAAT$^{3'}$	376
SHIG	*ial*	Ref. [79]	$^{5'}$CTGGTAGGTATGGTGAGG$^{3'}$ $^{5'}$CCAGGCCAACAATTATTTCC$^{3'}$	320
bfpA	*bfp*A	U27184	$^{5'}$TTCTTGGTGCTTGCGTGTCTTTT$^{3'}$ $^{5'}$TTTTGTTTGTTGTATCTTTGTAA$^{3'}$	367
EA	pCVD	X81423	$^{5'}$CTGGCGAAAGACTGTATCAT$^{3'}$ $^{5'}$CAATGTATAGAAATCCGCTGTT$^{3'}$	630

3.4.2.2. Multiplex PCR for identification of DEC from fecal samples

Multiplex PCR for identification of DEC was done as mentioned above for DNA preparation from the smear taken from the first area of the SMAC plate. If the result was negative, the sample was considered as negative for diarrheagenic *E. coli*. If the multiplex PCR was positive, the sizes of bands on the gel were compared with the marker, in order to identify the suspected DEC in the stool sample. Another smear of the same area was taken and cultured on another fresh SMAC plate to get separate colonies. After incubation at 37^{o}C overnight, five to ten colonies with typical *E. coli* morphology were streaked on the fresh plates. Each colony was independently tested by PCR with primer specific for the suspected DEC from the multiplex PCR.

Minimum criteria for determination of DEC were defined as follows: the presence of *elt*B and/or *est*A for ETEC; the presence of *vt*1 and/or *vt*2 for EHEC; (the presence also of *eae*A confirms the diagnosis of a typical EHEC); the presence of *bfp*A and *eae*A for typical EPEC, (only *eae*A for atypical EPEC); the presence of *ial* for EIEC/*Shigella*; and the presence of pCVD for EAEC.

3.4.3. Identification of *Vibrio cholerae*, *Salmonella*, and *Shigella* (Paper I)

Conventional methods based on culture, biochemical tests, and serotyping were used to identify *Vibrio cholerae*, *Salmonella*, and *Shigella*. Since *Shigella* and EIEC could be detected by PCR by the presence of *ial* gene. To verify *Shigella*, agglutination with antisera specific to *Shigella* species was performed for those strains positive with PCR for the primer specific for *ial*. A strain was identified as EIEC if it was positive with PCR with the primer targeting *ial* gene and negative with *Shigella* antisera.

3.4.4. Immunoseparation in combination with PCR for identification of enterotoxigenic *B. fragilis* (Paper IV)

After incubation (as described in 3.4.1), the broth medium was centrifuged at 600 x g for 10 minutes. The supernatant was collected and centrifuged at 3,000 x g for 10 minutes, and the pellet was suspended in 80 µl of PBS for incubation with coated magnetic beads. The procedures were basically carried out as described in the previous study [278] with some minor changes. In the original protocol, two monoclonal antibodies (mAb C3 and mAb 4H8) were used. Due to the loss of the cell-line for mAb 4H8, only the mAb C3 antibody was used and a double volume (50 µl) of the coated beads has been transferred to a well of the 96-well microtiter plate (Techne, Cambridge, UK).

The PCR assay for ETBF detection was accomplished using a primer pair, GBF-201 and GBF-210 specific (**Table 3**) for the enterotoxin gene [122].

Table 3. Primers used for PCR amplification to detect *bft* gene and subtypes of *bft*

Primer	Sequence (5'-3')	Description
GBF-201	GAACCTAAAACGGTATATGT	Forward primer for *bft* gene
GBF-210	GTTGTAGACATCCCACTGGC	Reverse primer for *bft* gene
GBF-312	CCTCTTTGGCGTCGC	Reverse primer for *bft*-1
GBF-322	CGCTCGGGCAACTAT	Reverse primer for *bft*-2
GBF-334	TGTCCCAAGTTCCCCAG	Reverse primer for *bft-3*

Thirty microliters of master mix consisted of 3 µl of PCR Gold Buffer (10x), 3 µl of $MgCl_2$ (25mM) (Applied BioSystems, Roche, CA, USA), 2.4 µl of nucleotide mixture (2.5 mM each dATP, dCTP, dGTP, dTTP) (Pharmacia Biotech, Uppsala, Sweden), 3 µl of each primer (1 µM) (Thermo Electron GmbH, Ulm, Germany), 0.03 µl of Ampli TaqGold (5 UI/µl) (Applied BioSystems, Roche, CA, USA), and 0.5 µl of DNA template.

To determine the subtypes of the enterotoxin, a primer mixture of one forward primer, GBF-201, and three reverse primers (GBF-312 for *bft*-1, GBF-322 for *bft*-2 and GBF-334 for *bft-3*) (**Table 3**) were used in the same amplification reaction [122]. Thirty microliters of the reaction mixtures were prepared with the same amount of PCR Buffer, dNTP, $MgCl_2$, Taq polymerase, and DNA template as the PCR assay for ETBF detection described above. The primers GBF-201, GBF-312, GBF-322 and GBF-334 at the concentration of 1 µM each were added to the reaction mixture with the volumes of 3, 2, 4, and 1 µl, respectively.

Thermocycling conditions for enterotoxin gene with a Gene Amp PCR System 9700 (AB Applied Biosystem, Stockholm, Sweden) were as follows: 95°C for 5 minutes, 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute, for 35 cycles, ending with a 5-min extension at 72°C. For the subtypes-PCR, the conditions were 95°C for 9 minutes, 95°C for 20 seconds, and 62°C for 2 minutes, for 35 cycles, ending with a 5-min extension at 74°C. PCR products (10 µl) were evaluated with a 1.5% (wt/vol) agarose gel (Gibco Life Technologies, Paisley, UK) at 120 mV for 30 min. A molecular marker (1 Kbp DNA Ladder; Gibco/BRL) was run concurrently. The DNA bands were visualized and photographed under UV light after staining with ethidium bromide.

3.4.5. Enzyme immunoassay for identification of rotavirus (Paper III)

Stool samples were analysed for Group A rotavirus using the IDEA^{TM} Rotavirus Kit for ELISA (DAKO Ltd., Cambridgeshire, UK) following the manufacturer's instructions. This test is a qualitative enzyme immunoassay for the detection of Group A rotavirus in human fecal samples. This test utilizes a polyclonal antibody in a solid-phase sandwich enzyme immunoassay to detect group specific antigen present in Group A rotavirus. Break-apart microwells are coated with a rotavirus specific polyclonal antibody. Fecal suspension is added to the microwells and incubated simultaneously with a rotavirus specific polyclonal antibody conjugated to horseradish peroxidase. If the antigen is present in the sample, this antigen is captured between antibody on the solid phase and the enzyme-conjugated antibody. The wells were read by spectrophotometer at the wavelength of 450 nm.

3.4.6. Antibiotic sensitivity test (Paper II)

Each minimum inhibitory concentration (MIC) was determined by agar dilution method following the recommendations from NCCLS [169, 170]. The following antibiotics were used for susceptibility testing: ampicillin (AMP; AstraZeneca, Stockholm, Sweden), chloramphenicol (CHL; Sigma-Aldrich, Stockholm, Sweden), trimethoprim/ sulfamethoxazole (SXT; Sigma), imipenem (IPM; Merck Sharp & Dohme B.V, Haarlem, Netherlands), cefuroxime (CXM; Sigma), cefotaxime (CTX; Sigma), nalidixic acid (NAL; Sigma), and ciprofloxacin (CIP; Bayer AG, Leverkusen, Germany).

3.4.7. Statistical analysis

The proportion difference was done by Chi-square test. In case the expected value for a cell was < 5, Fisher's exact test was used. P value < 0.05 was considered statistically significant.

Multiple comparisons of mean values of groups were done by Kruskal-Wallis H test; Mann-Whitney U test (for nonparametric data) was used for comparing two groups. P value < 0.05 was considered statistically significant.

For antibiotic sensitivity test, data were analyzed by WHONET 5 software.

3.4.8. Ethical approval

The project was approved by the Ethics Committees of the Karolinska Institutet, Stockholm, Sweden, and by Hanoi Medical University, Hanoi Vietnam.

4. RESULTS AND DISCUSSION

4.1. Identification and characterization of DEC (Paper I and II)

4.1.1. Development of multiplex PCR for identification of DEC from fecal samples

In this study, a multiplex PCR was developed by combining eight primer pairs specific for five main types of diarrheagenic *E. coli*. **Figure 3** shows the PCR products derived from pure cultures of reference strains: EAEC, EIEC, EPEC, EHEC (*vt*2, *eae*), EHEC (*vt*1, *eae*), and ETEC, from lane 2 to lane 7, respectively. However, the band seen in lane 4 (EPEC) contains two bands, 376 (*eae*) and 367 (*bfp*) so close together that they were impossible to be separated by agarose gel electrophoresis. Therefore, when the multiplex PCR was positive with EPEC from stool samples, it was necessary to run separately the specific PCRs with primers for *eae* and *bfp* to verify if this strain is typical or atypical EPEC.

The spiked samples with reference strains gave positive results for DEC and negative result for the negative control.

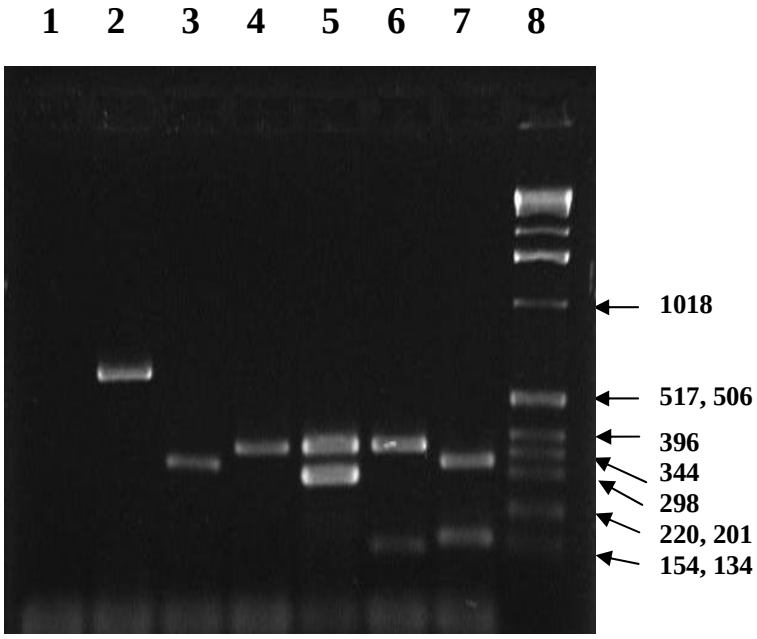


Figure 3. Multiplex PCR amplification of reference strains of diarrheagenic *E. coli* from pure cultures

Repeated experiments confirmed that the detection limit of DEC was approximately 10^3 cfu/ml of stool suspension. The multiplex PCR could detect two different DEC strains in a spiked stool sample. Several previous studies have shown mixed infections in stool samples from children with diarrhea, including bacterium with bacterium, bacterium with virus etc. [10, 14, 60, 62, 156, 200]. This showed the possibility to detect co-infections of DEC in stool samples. Due to the overlaps of some identical genes or close sizes of the amplicon, it was difficult to distinguish individual bands in some cases. In order to differentiate the band of *eae*A gene (376 bp) with *bfp*A gene (367 bp), and the band of *elt*B

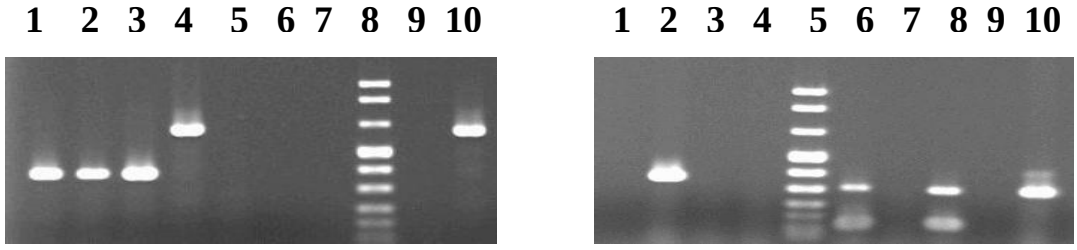
gene (322 bp) with *ial* gene (320 bp), it was necessary to run specific PCRs to verify these target genes of DEC.

When applied for verified DEC, our multiplex PCR had positive results for all of 99 DEC and negative results for 139 non-diarrheagenic *E. coli* demonstrating high sensitivity and specificity in detecting DEC.

PCR is a selective, sensitive, and specific assay. It becomes, however, cumbersome if being applied to numerous samples with various potential targets. The multiplex PCR has been used to identify and differentiate *E. coli* strains in a number of studies [40, 72, 181, 190, 192]. Many of previous studies on multiplex PCR focused on a single *E. coli* virotype, such as EAEC [41], EHEC [72], ETEC [238], STEC [190, 191]. Others target only a single serotype, for example, O157:H7 [80-82, 99, 203]. Other two studies target four types of DEC: EHEC, EIEC, ETEC, EAEC [189], and EHEC, EIEC, EPEC, ETEC [205]. The first one applied four different multiplex PCR reactions and the second one used three reactions. In this study, in order to identify five main types of *E. coli* (EAEC, EHEC, EIEC, EPEC, ETEC), it is necessary to perform several PCR reactions with different primers specific for the target genes. By combining eight primer pairs, we could identify these *E. coli* in a single reaction. It helped to minimize time and materials. It showed positive results with tested DEC and negative results with all non-DEC strains indicating high sensitivity and specificity.

4.1.2. Multiplex PCR for identification of DEC from fecal samples

A total of 162 DEC strains were isolated from 587 stool samples from children with diarrhea and from 249 samples of healthy group. **Figure 4** shows one of the results of multiplex PCR for DEC from stool samples in children with diarrhea.



Lane 1, 2, 3:	EPEC	Lane 2:	EPEC
Lane 4:	EAEC	Lane 6, 8:	ETEC
Lane 5, 6, 7:	Negative samples	Lane 1, 3, 4, 7, 9:	Negative samples
Lane 9:	Negative control	Lane 10:	EIEC
Lane 10:	Positive control	Lane 5:	Marker

Figure 4. Multiplex PCR results from DEC from stool samples

The prevalence of DEC in diarrhea group and in the control was 22.5% and 12%, respectively ($p < 0.001$) presenting the important role of DEC in causing diarrhea in children. The PCR assays detected 68 EAEC (11.6%), 12 EIEC (2%), 39 EPEC (6.6%), and 13 ETEC (2.2%) in the diarrhea group. In the healthy controls, the prevalence of

EAEC, EPEC, and ETEC were 18 (7.2%), 11 (4.4%), and 1 (0.4%), respectively. All detected EPEC were atypical. No EHEC strains were isolated from any of the groups. The lower prevalence of ETEC and EHEC in our study was the same as mentioned in other studies [3, 175, 179, 251]. In some studies, ETEC was found with a prevalence of 20.7% in patients [272], 28% patients and 16% in the controls [151]. No EHEC was found in other studies [3, 179, 251] or it was found at very low prevalence in children with diarrhea [34, 242]. In contrast, EAEC and EPEC were identified with higher prevalence in our study. In addition, in the diarrhea group, EAEC and EPEC were more frequently isolated in children less than 2 years of age (14.1% and 7.9%, respectively), whereas EIEC and ETEC were less frequently found (1.9% and 1%, respectively) (**Figure 5**). No significant differences were seen in isolation prevalence of DEC in the healthy group in terms of age group. Our study showed a causative role of EAEC and EPEC with diarrhea, especially, the children less than 2 years of age as mentioned in previous studies [140, 165, 168]. Our findings, however, were in contrast to the results of other investigators who have not found EAEC to be associated with diarrhea [98, 222].

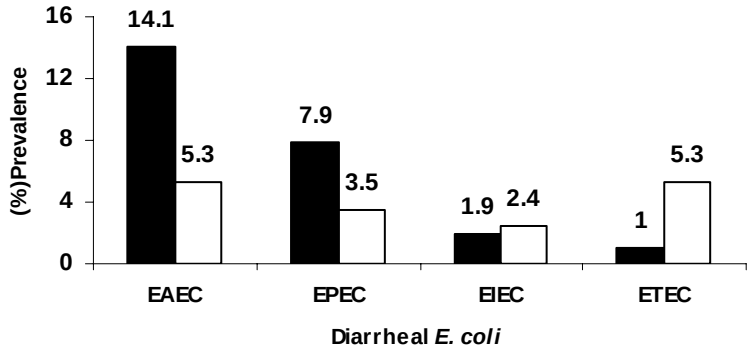


Figure 5. Prevalence of DEC in the diarrhea group vs. age group. There was a significant difference seen in EAEC ($p < 0.005$) and ETEC ($p < 0.005$). Closed bars: children 0-24 months; Open bars: children 25-60 months

One reason could be that the epidemiological characteristics of DEC (e.g., likely sources, reservoirs of infection, routes of transmission, seasonality…) are largely unknown in different geographical areas. In the present study, we did not isolate any *V. cholerae* or *Salmonella*. However, in the diarrhea group, 28 *Shigella* strains (4.7%) including 7 *S. flexneri*, 20 *S. sonnei*, and 1 *S. boydii* have been isolated. The isolation prevalence of *S. sonnei* in children less than 2 years of age and the older ones were 1.4% and 8.2%, respectively. The difference was statistically significant ($p < 0.0001$). Several studies from South East Asia and Vietnam showed the higher prevalence of *S. flexneri* than *S. sonnei*. It could be explained by the lower number of *Shigella* isolates in our study. Furthermore, over all, *S. flexneri* seemed more susceptible to antibiotics than *S. sonnei*, especially with the commonly used antibiotics (SXT, NAL, CIP) to treat shigellosis. This study increased our understanding of the role of *Shigella* and EIEC in children's diarrhea. Since *ial* is present in both *Shigella* and EIEC, we differentiated EIEC from *Shigella* by some biochemical tests and slide agglutination assay with anti sera specific for *Shigella* serotypes.

Table 4 shows age, the clinical symptoms of children with diarrhea in relation to DEC and *Shigella* infection alone. EAEC infection has tendency to be present in the younger

children compared to the infection of EIEC, ETEC, *S. flexneri*, and *S. sonnei*. The mean of age in month of children infected with EAEC was lower than that of those infected with EIEC, ETEC, *S. flexneri*, and *S. sonnei*. The difference was statistically significant. Children infected with EPEC had a significantly lower mean of age than those infected with *S. flexneri* and *S. sonnei*. Other comparisons do not show any significant difference. Our findings were similar to the studies by Echeverria et al., and Presterl et al. [62, 200]. Children having *Shigella* spp. in fecal samples seemed to have fever with a higher average of temperature than those who had DEC. We did not see any significant difference in terms of vomiting, dehydration, and episode/day of children infected with different categories of DEC and *Shigella* spp. Regarding stool properties, EAEC, EPEC, and ETEC were associated with watery diarrhea with a prevalence of 65, 79 and 100%, respectively. This characteristic is generally common for these pathogens, whereas mucous bloody stool and mucous stool were more prevalent in children infected with EIEC and *Shigella* spp. This showed the nature of EIEC and *Shigella* infection. Twenty-nine percent of children infected with EAEC also had mucous stools. This has been described in previous studies [100, 165]. In particular, five *S. flexneri*, three *S. sonnei*, and one *S. boydii* strains caused diarrhea with blood in stool. It has been shown that bloody, mucous stools are highly indicative of shigellosis [216].

Table 4. Age, clinical symptoms of children with diarrhea vs. DEC and *Shigella* infection alone

Properties	EAEC (37)*	EIEC (10)	EPEC (19)	ETEC (8)	*S. flexneri* (5)	*S. sonnei* (16)	*S. boydii* (1)
Age in month	12.7	21.3	15.5	30.3	33	31.3	5
mean (range)	(2 – 47)	(10 – 50)	(6 – 49)	(4 – 59)	(18 – 46)	(2 – 55)	(5)
Fever ^{0}C	37.6	38.1	37.7	37.8	38.3	38.6	38
mean (range)	(36.7 – 39)	(37 – 40)	(37 – 40)	(37 – 39)	(37 – 39.5)	(37 – 40)	(38)
Vomiting (n, %)	18 (48.6)	5 (50)	6 (31.6)	6 (75)	2 (40)	7 (43.8)	0
Dehydration (n, %)	30 (81.1)	9 (90)	17 (89.5)	6 (75)	4 (80)	15 (93.8)	1 (100)
Stool properties (n, %)							
Watery	24 (65)	2 (20)	15 (79)	8 (100)	0	0	0
Bloody	0	0	0	0	1 (20)	0	0
Blood with stool	0	0	0	0	2 (40)	0	0
Mucus bloody stool	0	4 (40)	0	0	2 (40)	3 (19)	1 (100)
Mucous	11(29)	4 (40)	4 (21)	0	0	13 (81)	0
Others	2 (6)	0	0	0	0	0	0
Episodes/day	6.7	7.4	5.6	7.4	6.4	8.5	6
mean (range)	(3 – 21)	(3 – 16)	(3 – 11)	(4 – 13)	(3 – 10)	(4 – 17)	(6)

*: Number of isolates

4.1.3. Antibiotic susceptibility of isolated DEC and *Shigella* (Paper III)

Of the 162 DEC isolates, 86.4% were resistant to AMP, 77.2% to CHL, 29.6% to CXM, 24.1 % to CTX, 19.1% to NAL, 3.7% to CIP, 88.3% to SXT and all were sensitive to IPM.

Of the few *E. coli* strains resistant to CIP, five were EPEC and one ETEC. The traditional antibiotics, including AMP, CHL, SXT, showed low activity against the DEC strains ($MIC_{90} \geq 1{,}024$ mg/l for AMP and CHL, $MIC_{90} \geq 4/76$ mg/l for SXT) (**Table 5**). Most mild diarrhea cases are successfully managed with oral rehydration therapy. Only for more severe or persistent diarrhea cases should antimicrobial treatment be added. In the developing countries, ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole are widely used to treat diarrhea because of their low cost and ready availability. However, some previous studies have shown high prevalence of resistance to these antibiotics in enteric pathogens, especially DEC [104, 112, 199, 269]. The report of National Program for Surveillance in Antimicrobial Resistance of common pathogens in 2001 in Vietnam shows that more than 80% of *E. coli* are resistant to AMP, CHL, and SXT [267]. This raises the concerns on failure in treatment with these antibiotics against infection caused by *E. coli*. CXM, CTX and NAL showed moderate activity. They were still more active than the traditional antibiotics but less active than NAL with $MIC_{90} = 128$ mg/l. Although, the cephalosporins (cefuroxime and cefotaxime) and imipenem are not indicated to treat diarrhea, we have tested the DEC strains' susceptibility to these antibiotics since they could be empirically or incidentally used. According to the national household survey in 1997-1998, on average, about two thirds of those who are ill, treat themselves [44]. The differences of distribution of the resistance and the MICs were seen for individual antibiotics and each categories of *E. coli*. The multiple comparisons showed significant difference in resistance to CHL and CIP ($P = 0.004$ and 0.021). For SXT, the P value was borderlined significant ($P = 0.062$). When the comparisons of antibiotic resistance of two groups of *E. coli* were performed, EAEC were significantly more susceptible to CIP than EPEC and ETEC ($P = 0.003$ and $P = 0.013$). However, they were more resistant to CHL ($P = 0.002$ and $P = 0.006$). This group of *E. coli* also showed higher resistance to AMP and SXT ($P = 0.041$ and $P = 0.024$) as compared to ETEC. The other comparisons among *E. coli* types to antibiotics did not appear to be statistically different. Different resistance patterns were defined in the four categories of diarrheagenic *E. coli*. The most prevalent multiresistance pattern (resistance to at least 2 antibiotics) was AMP^r CHL^r CXM^s CTX^s NAL^s CIP^s IPM^s SXT^r in all types of *E. coli*; 34.8%, 16.6%, 28%, and 21.4% for EAEC, EIEC, EPEC, and ETEC, respectively. In EAEC strains, AMP^r CHL^r CXM^r CTX^r NAL^s CIP^s IPM^s SXT^r and in EPEC strains, AMP^r CHL^s CXM^s CTX^s NAL^s CIP^s IPM^s SXT^r were the second most prevalent multiresistance patterns with a prevalence of 17.4% and 18% of each category, respectively. Multi-antibiotic resistance was detected in 89.5% of all diarrheagenic *E. coli*, in 91.8% of EAEC, 100% of EIEC, 86% of EPEC, and 78.6% of ETEC. There was no significant difference in antibiotic resistance in DEC strains isolated from children with diarrhea compared to the healthy controls. Other investigators also described multiresistance problem among DEC [218, 269]. It is therefore reasonable to predict that this multiresistance showed by the different categories of DEC might emerge in other developing countries where the antibiotics are not appropriately used.

Twenty-eight *Shigella* strains were isolated from children with diarrhea only. Ampicillin and trimethoprim/sulfamethoxazole showed very low activity against *Shigella* strains with $MIC_{90} > 1024$ and $> 4/76$ mg/l, respectively. One strain was susceptible to all tested antibiotics. There were 22/28 (78.6%) multi-antibiotic resistant *Shigella* strains. One *S. boydii* strain was resistant to all tested antibiotics. The most common multiresistant pattern was AMP^r CHL^s CXM^s CTX^s NAL^s CIP^s IPM^s SXT^r with the prevalence of 35%. *Shigella* are becoming more resistant to commonly used antibiotic especially in developing countries [12, 43, 104, 215]. However, the prevalence of resistance to these antibiotics are lower in developed countries [199, 207]. The reason could be the more appropriate usage of antibiotics in developed compared to the developing countries.

According to our study, ampicillin, chloramphenicol, trimethoprim/sulfamethoxazole should not be used for treatment of diarrhea in children. Moreover, it should be taken into consideration that in spite of low bacterial resistant prevalence to quinolones, if they are widely used, a rapid emergence of quinolone resistance will likely occur.

Table 5. Antibiotic susceptibilities of diarrheagenic *E. coli* and *Shigella* strains

Organisms and agents	Number of isolates	% of resistant	MIC		
			MIC_{50}	MIC_{90}	Range
Diarrheagenic *E. coli*	162				
AMP		86.4	>1,024	>1,024	2->1,024
CHL		77.2	256	1,024	2-1,024
CXM		29.6	4	>1,024	0.032->1,024
CTX		24.1	0.064	>1,024	0.032->1,024
NAL		19.1	2	128	1->1024
CIP		3.7	0.016	0.25	0.008-128
IPM		0	0.125	0.125	0.064-1
SXT		88.3	>4/76	>4/76	0.032->4/76
Enteroaggregative *E. coli*	86				
AMP		90.7	>1,024	>1,024	2->1,024
CHL		87.2	512	1,024	2 - 1024
CXM		36	8	>1,024	0.032 - >1,024
CTX		26.7	0.064	>1,024	0.032 - >1,024
NAL		18.6	2	64	1->1,024
CIP		0	0.016	0.125	0.008 - 1
IPM		0	0.125	0.125	0.064 - 1
SXT		91.9	>4/76	>4/76	0.032 - 16
Enteroinvasive *E. coli*	12				
AMP		91.7	1,024	>1,024	8->1,024
CHL		83.3	256	512	2 - 1024
CXM		16.7	4	>1,024	0.064 - >1,024
CTX		25	0.064	>1,024	0.032 - >1,024
NAL		8.3	2	4	1-64
CIP		0	0.016	0.032	0.016 - 0.064
IPM		0	0.125	0.125	0.064 - 0.125
SXT		100	>4/76	>4/76	8-16
Enteropathogenic *E. coli*	50				
AMP		82	1,024	>1,024	2->1,024
CHL		64	256	1,024	4 - 1024
CXM		24	8	1024	0.064 - >1,024
CTX		20	0.064	64	0.032 - >1,024
NAL		24	2	128	2->1,024
CIP		10	0.016	1	0.016 - 128
IPM		0	0.125	0.25	0.064 - 1
SXT		84	>4/76	>4/76	0.032 – 16

Table 5. *Continued*

Organisms and agents	Number of isolates	% of resistant	MIC		
			MIC_{50}	MIC_{90}	Range
Enterotoxigenic *E. coli*	14				
AMP		71.4	512	>1,024	2 - >1,024
CHL		57.1	128	1,024	4 - 1024
CXM		21.4	4	1,024	0.064 - >1,024
CTX		21.4	0.032	128	0.032 - 128
NAL		14.3	2	>1,024	2 - >1,024
CIP		7.1	0.016	1	0.008 - 32
IPM		0	0.125	0.125	0.064 – 0.25
SXT		71.4	>4/76	>4/76	0.064 - 16
Shigella	28				
AMP		75	256	>1,024	2->1,024
CHL		53.6	16	256	2 - 1024
CXM		14.3	2	128	0.032 - 1024
CTX		10.7	0.064	128	0.016 - 256
NAL		7.1	2	8	1-32
CIP		3.6	0.016	0.064	0.016 - 2
IPM		10.7	0.125	64	0.064 - 128
SXT		89.3	16	16	0.064 - 16
S. sonnei	20				
AMP		70	128	>1,024	2->1,024
CHL		40	4	256	2-1,024
CXM		15	0.064	128	0.032-1,024
CTX		10	0.064	.25	0.032-128
NAL		5	2	8	1-32
CIP		0	0.016	0.032	0.016-0.25
IPM		10	0.125	0.25	0.064-128
SXT		90	>4/76	>4/76	0.064->4/76
S. flexneri					
AMP		85.7	512	>1,024	4->1,024
CHL		85.7	128	256	8-128
CXM		0	0.125	2	0.064-4
CTX		0	0.064	0.25	0.016-0.25
NAL		0	2	2	1-2
CIP		0	0.016	0.016	0.016-0.016
IPM		0	0.125	0.25	0.064-0.25
SXT		85.7	>4/76	>4/76	1->4/76
S. boydii	1	Resistant to all tested antibiotics			

MIC_{50} and MIC_{90}: MICs required to inhibit the growth of 50 and 90% of tested strains, respectively.

4.2. Role of enterotoxigenic *Bacteroides fragilis* in diarrhea in children less than 5 years of age in Hanoi, Vietnam (Paper IV)

ETBF was detected in 7.3% (43/587) of fecal samples from children with diarrhea and in 2.4% (6/249) of samples from the controls ($P < 0.01$). The distribution of ETBF according to five groups of age is shown in **Table 6**.

Table 6. ETBF detection prevalence vs. groups of age

Group of children	Group of age (month)				
	0- ≤ 12	13 - ≤ 24	25 - ≤ 36	37 - ≤ 48	49 - ≤ 60
Diarrhea	12/240* (5%)	16/177 (9%)	5/95 (5.3%)	5/41 (12.2%)	5/34 (14.7%)
Control	0/48 (0%)	3/71 (4.2%)	1/48 (2.1%)	2/49 (4.1%)	0/33 (0%)

*: Denominators are total of children in each group of age

The rates of identification of ETBF were low in both groups of children at the ages from 0 to 12 months and from 25 to 36 months. In the diarrhea group, there was an increasing trend of ETBF detection over ages (Chi square for trend = 4.2, $P < 0.05$). When being analyzed according to whether the children were older than 1 year, the prevalence of ETBF isolation was 8.9% (31/347) in the diarrhea group, and 2.9% in the control showing the significant difference ($P < 0.01$). No difference was seen for children younger than 1 year in children with and without diarrhea.

IMS assay that has been modified as compared to the original one [278] was successfully used to detect ETBF from fecal samples. This is the first study on ETBF in Vietnamese children showing the role of ETBF in causing diarrhea. Our finding was the same as the other authors in the world who have found the significantly higher prevalence of ETBF in diarrhea children compared to the controls [213, 217]. However, our result showed a lower prevalence as compared to studies in American and Italian settings where 12 and 17% of ETBF, respectively, were identified in children with diarrhea [185, 214]. The higher prevalence of ETBF in children older than 1 year of age has been also confirmed by studies of Sack et al. [213, 214]. The hypothesis could be that the maternal antibodies protect children during the first 12 months of age [213].

Among the 43 fecal samples infected with ETBF from children with diarrhea, 19 samples contained ETBF as the only potential diarrheal pathogen. Twenty-four samples contained other pathogens as well, including 15 with rotavirus, 6 with DEC or *Shigella* spp., and 3 with rotavirus and DEC/*Shigella*. All the six ETBF positive samples in the control group were negative for other diarrheal pathogens.

Taking into account the ETBF infection, the clinical properties are shown in **Table 7**. Generally, the clinical symptoms such as watery diarrhea, vomiting, fever, and dehydration occurred in more than 40% of these subjects. There was no significant difference in terms of clinical symptoms in children infected with ETBF alone as compared to those with co-infections of ETBF. However, the children infected with more than one pathogen may suffer from the failure in treatment, especially with antibiotic-resistant bacteria.

Table 7. Clinical properties of diarrhea children infected with ETBF alone and with ETBF and other identified pathogens

Properties	ETBF alone (n = 19)	Co-infection (n = 24)
Age (month)		
0 - ≤ 12	7	5
13 - ≤ 24	5	11
25 - ≤ 36	1	4
37 - ≤ 48	3	2
49 - ≤ 60	3	2
Male/female	7/12	16/8
Stool properties		
Watery	10	15
Mucous	6	6
Mucous-bloody	1	1
Semi-solid	2	0
Vomiting	8	15
Fever	10	17
Dehydration	13	18
No. of episodes/day		
Range	2-9	3-14
Median	5	5
Mean	5.26	6.46

Based on the PCR results, three subtypes of the toxin were identified among the 43 ETBF isolates within the diarrhea group with the prevalence of 67.4% (29/43), 18.6% (8/43), and 16% (6/43) for *bft*-1, *bft*-2, and *bft*-3, respectively. In the controls, 5 *bft*-1 (83%) and 1 *bft*-2 (17%) among the six ETBF strains were identified. As mentioned in previous study [122], *bft*-1 is the most predominant subtype. It may be of interest to determine whether different subtypes will influence the pathogenecity of ETBF. Since the antibiotic resistance among *Bacteroides* isolates is still a concern in treatment of anaerobic infections [35, 253], the surveillance on antibiotic susceptibility of *Bacteroides* will be important in clinical aspect.

4.3. Role of Group A rotavirus in diarrhea in children less than 5 years of age in Hanoi, Vietnam (Paper III)

4.3.1. Rotavirus infection

Among 587 fecal samples from children with diarrhea, 274 (46.7%) were positive for Group A rotavirus. The corresponding figure from the healthy controls was 9 (3.6%) samples showing the significant difference ($P < 0.0001$). Within the diarrhea group, the prevalence of detection in children less than 2 years of age was 51.1%, which was significantly different from that in the older one ($P < 0.001$). Rotavirus infection was the most prevalent in children in 13-24 month group. The second most common cases were seen in the group ≤ 12 and from 25-36 month group although cases were also seen in the older children. There was a significantly decreasing trend of rotavirus prevalence over age (Chi-square test for trend, 8.904; $P < 0.005$). The infection occurred year around but the prevalence trend was higher in September, October, November, and December. In the other months of the year, the number of infected cases decreased. February was the Tet (New Year) holiday in Vietnam resulting in a low number of diarrhea samples.

Our findings together with two previous studies in Vietnam [174, 176] have demonstrated the important role of Group A rotavirus in diarrhea in children less than 5 years of age. Many other studies have also shown the burden of rotavirus diarrhea not only in developed but also in developing countries [14, 15, 30, 70, 88, 128]. Our study indicated that rotavirus diarrhea was associated with children under 2 years of age and it was decreasing over age. The highest prevalence was seen in children from 13 to 24 months of age. Even, children under 3 months and under 6 months got rotavirus infection with a prevalence of 34.2 and 35%, respectively. It showed the early infection in a children's life. The tremendous prevalence of rotavirus infection emphasizes the urgent need for interventions, such as vaccines to prevent the morbidity and mortality, especially in developing countries.

4.3.2. Rotavirus infection in relation to clinical symptoms

For all the diarrhea children, the main clinical symptoms such as fever, vomiting, dehydration, type of stool, and number of episodes of diarrhea per day are shown in **Figure 6** and **Table 2 (Paper III)**. Fever, vomiting, and dehydration were common symptoms in rotavirus-infected children.

Dehydration occurred in 89% cases (243/274). There were significant differences in terms of vomiting, and dehydration in children positive with rotavirus compared to the negative ones ($P < 0.0001$, and $P < 0.001$, respectively). It has been shown that rotavirus diarrhea is more likely to be associated with vomiting, fever, and dehydration than diarrhea from other enteric pathogens [226]. These symptoms may occur with diarrhea alone or in combination resulting in hospitalization of children for treatment. In addition, watery stool was the suggestive symptom when it contributed to 81.1% of cases infected only with rotavirus. In our study, fever, vomiting, and dehydration were seen at prevalence of 59.1, 66.4, and 89%, respectively, in the children infected with rotavirus. Among 274 children infected with rotavirus, the most frequent combination of symptoms was fever-vomiting-dehydration (42%). The next most frequent combinations were vomiting-dehydration (20%), fever-dehydration (14%). Of the 49 children without fever, vomiting, and dehydration in 587 children, 13 were positive with rotavirus.

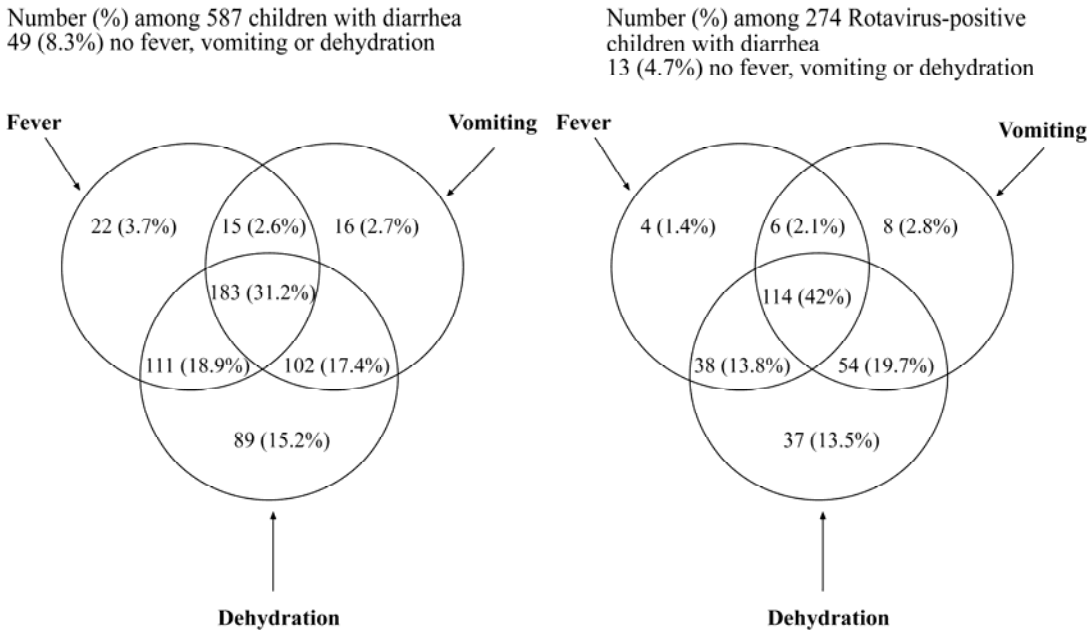


Figure 6. Relationships between rotavirus infection and clinical symptoms. Overlapping areas show the numbers and proportions of children with two or more symptoms.

We detected rotavirus at the highest rate among those with all three symptoms. Our study supported the conclusions from other studies that rotaviruses induce a clinical illness characterized by vomiting, diarrhea, fever, and dehydration (or some combination of these symptoms) [15, 30, 38, 212, 226, 237]. Mean of episodes of diarrhea per day in rotavirus-positive group significantly differed ($P < 0.001$) from that in the rotavirus-negative group.

4.3.3. Rotavirus and co-infections

In the present study, 233 bacterial pathogens were identified. The bacterial etiology consisted of 162 DEC, 28 *Shigella* spp., and 43 ETBF. Co-infections were 26.6% (73/274) in the diarrhea group and 22% (2/9) in the healthy one. The most common association was rotavirus and EAEC with a prevalence of 10.5% (29/274) followed by rotavirus and EPEC, 6.9% (19/274) and ETBF 5.4% (15/274). Albert et al. [1] reported that rotavirus infection was associated with ETEC, EPEC, and *Shigella* spp. at the prevalence of 17, 9.7, and 1.2%, respectively, in rotavirus-infected children. In a study carried out by Ming et al. [156] in China, only one child was shown to be infected with both rotavirus and ETEC. The co-infection could cause difficulties in treatment of diarrhea in clinical aspect, especially, when the bacterial pathogens are resistant to antibiotics.

The single or bacteria-associated rotavirus infection showed different prevalences in clinical symptoms. Overall, the clinical symptoms in the diarrhea group seemed to be more severe in children who were infected with either bacteria/rotavirus or both compared to those from whom no rotavirus, DEC, *Shigella*, and ETBF have been identified. In practice, the co-infection could cause difficulties in diagnosis, treatment, and prophylaxis of diarrhea in children. More studies would be necessary to further evaluate this aspect.

4.4. Etiology and epidemiology of diarrhea in children less than 5 years of age in Hanoi, Vietnam (Paper V)

4.4.1. Etiology of diarrhea

In 587 children with diarrhea, Group A rotavirus was the most frequently identified enteric pathogen with a prevalence of 46.7%, showing the significant difference as compared to the control (3.6%). The second most common pathogen was the DEC belonging to four types: EAEC, EIEC, EPEC, and ETEC. The isolation prevalence was 22.5%, which was significantly different from that in the controls. EAEC was found with the highest prevalence among the isolated diarrheagenic *E. coli*. No EHEC, *Salmonella*, or *V. cholerae* were found showing that these pathogens did not play an important role in causing diarrhea in this poplulation. No children were colonized with two or more DEC. The prevalence of *Shigella* strains was 4.7% compared to 0% in the control ($P < 0.001$). The other bacterial pathogen identified in this study was ETBF. The detection prevalence was 7.3% in the diarrhea group and 2.4% in the control ($P < 0.01$). Three subtypes of ETBF were found with the prevalence of 67.4, 18.6, and 16%, respectively in children with diarrhea.

This is the first study in Vietnam looking at several kinds of possible enteric pathogens in diarrhea in children less than 5 years of age in relation to clinical and epidemiological aspects. It showed the roles of rotavirus, DEC, *Shigella*, and ETBF in causing diarrhea in children. It also presented the infection patterns of enteric pathogens in gastroenteritis in the northern of Vietnam. In the south, some national studies have shown certain prevalence of *Salmonella* and *V. cholerae*. However, these two pathogens have been rarely found in children with diarrhea.

In this study, we did not have possibilities to look for *Campylobacter* spp. However, this pathogen has been found to be associated with diarrhea in children in many studies [5, 73, 158, 227]. In Vietnam, a study in children less than 5 years of age carried out by Isenbarger et al. [111] showed that *Campylobacter* was found in 6.8% of cases. It is necessary to assess the role of *Campylobacter* in children's diarrhea in Vietnam.

Among the children with diarrhea, 79 (13.5%) had infection with two or more pathogens, and among the controls, 2 (0.8%) had mixed infection ($P < 0.00001$). Although we did not have the possibilities to look for other pathogens such as enteric viruses other than rotavirus, parasites, *Campylobater* spp., a potential pathogen was identified from 395 children with diarrhea (67.3%) and from 43 controls (17.3%) ($P < 0.00001$). It is a rather high prevalence as compared to other studies, even in adult patients [14, 31, 62, 241]. However, our prevalence was still lower than that in the study of Albert et al. [1] where 74.8% of samples have a potential enteric pathogen. Occurrence of single and mixed infections of enteric pathogens is shown in **Table 8**.

Regarding the seasonal pattern, although not many samples were collected during February due to the traditional Tet (New Year) holidays, a common characteristic has been found in the north of Vietnam, where there are four seasons in a year. Rotavirus infection occurred all year round but peaked during the fall and winter months, from September to December. This pattern was not observed in the south, where there are only two seasons per year, the rainy and the dry seasons. Rotavirus infections occur almost all year in the south, with less distinct seasonal differences [174, 176]. Our findings were similar to those of studies conducted in Korea, China, and Thailand but they differed from those of a Japanese study, in which rotavirus was rarely detected from September to December [71, 149, 226, 282]. The infections with bacterial pathogens peaked during the summer time

when there are suitable conditions such as humidity, high temperature, which facilitate the bacterial growth and transmission.

Table 8. Occurrence of single and mixed infections of enteric pathogens

Patterns of infection	No. (%) Group of children		Total (n = 836)
	Diarrhea (n = 587)	Control (n = 249)	
Group A rotavirus	201 (34.2)	7 (2.8)	208 (24.9)
EAEC	37 (6.3)	17 (6.8)	54 (6.5)
EIEC	10 (1.7)	0 (0)	10 (1.2)
EPEC	19 (3.2)	10 (4)	29 (3.5)
ETEC	8 (1.4)	1 (0.4)	9 (1.1)
S. flexneri	5 (0.9)	0 (0)	5 (0.6)
S. sonnei	16 (2.7)	0 (0)	16 (1.9)
S. boydii	1 (0.2)	0 (0)	1 (0.1)
ETBF	19 (3.2)	6 (2.4)	25 (3)
Group A rotavirus + EAEC	29 (4.9)	1 (0.4)	30 (3.6)
Group A rotavirus + EPEC	19 (3.2)	1 (0.4)	20 (2.4)
Group A rotavirus + ETEC	5 (0.9)	0 (0)	5 (0.6)
Group A rotavirus + *S. flexneri*	1 (0.2)	0 (0)	1 (0.1)
Group A rotavirus + *S. sonnei*	1 (0.2)	0 (0)	1 (0.1)
Group A rotavirus + ETBF	15 (2.6)	0 (0)	15 (1.8)
ETBF + EIEC	2 (0.3)	0 (0)	2 (0.2)
ETBF + *S. flexneri*	1 (0.2)	0 (0)	1 (0.1)
ETBF + *S. sonnei*	3 (0.5)	0 (0)	3 (0.4)
Group A rotavirus + EAEC + ETBF	2 (0.3)	0 (0)	2 (0.2)
Group A rotavirus + EPEC + ETBF	1 (0.2)	0 (0)	1 (0.1)
No pathogen	192 (32.7)	206 (82.7)	398 (47.6)

The reasons for children to attend to hospital for examination were: (i) diarrhea only (54.5%), (ii) diarrhea with vomiting (19.3%), (iii) diarrhea and fever (16.7%), and (iv) diarrhea together with vomiting and fever (9.5%). Taking the clinical symptoms into account, fever and vomiting occurred in 56.4 and 53.8% of children with diarrhea, respectively. Although 523/587 (89.1%) of children received oral rehydration fluid before hospitalization, 82.7% of them were still dehydrated. The most common risks with diarrheal illnesses are dehydration and, in developing countries, malnutrition. Thus, the critical initial treatment must include rehydration, which can be accomplished with an oral glucose or starch containing electrolyte solution in the vast majority of cases. Watery stools were predominant (66.4%), followed by mucous stools (21%). There was one bloody stool (0.2%). Other types of stool accounted for 12.4%. The mean number of episodes was 7, ranging from 2 to 23 episodes per day. When being analyzed as the single infections with identified pathogens, children infected with *Shigella* had fever with

temperature average significantly higher than that of those infected with rotavirus, DEC, and ETBF alone. Fever has been shown to be a common symptom in diarrhea caused by *Shigella* [11, 76]. In addition, the average age of these children was also significantly higher than that of children infected with other pathogens. Dehydration was the most common in children with rotavirus diarrhea. The relationship of clinical symptoms and single or multiple infections of identified enteric pathogens from 587 children with diarrhea is shown in **Table 9.**

Table 9. Clinical symptoms in relation to single and multiple infections in 587 children with diarrhea

Clinical symptoms	Pathogens found, n (%)			Total	Chi-square for trend, P value
	No (n = 192)	Single (n = 316)	Multiple (n=79)		
Fever	95 (49.5)	186 (58.9)	50 (63.3)	331	5.707 P = 0.016
Vomiting	78 (40.6)	190 (60.1)	48 (60.8)	316	15.319 P = 0.00009
Dehydration	144 (75)	278 (88)	63 (79.7)	485	4.265 P = 0.038
Watery stool	108 (56.3)	222 (70.3)	60 (75.9)	390	13.169 P = 0.00028

There was an increasing trend of prevalences of fever, vomiting, dehydration, and watery stool in children with diarrhea according to the increased numbers of pathogens found in fecal samples. Multiple infections could cause more damages to the epithelial cells or more changes in absorption and secretion functions of the intestines. In this study, 4.6% (27/587) children had persistent diarrhea. We did not see any associations between the infections of enteric pathogens with persistent diarrhea. EAEC that has been considered to be associated with this type of diarrhea only appeared in three cases, the second only to four cases of rotavirus infection. Fourteen persistent cases did not have any potential identified pathogens. The role of EAEC in causing persistent diarrhea should be more studied.

4.4.2. Epidemiology of diarrhea

The information of demographic, epidemiological data and clinical symptoms is shown in **Table 10 and 11**. Among 836 children, the male/female ratio in children with diarrhea was 1.64 and 1.18 for the control showing a significant difference. The higher prevalence of male with diarrhea is also seen in the national census in 2002 in Vietnam [171]. The ratios of male versus female in terms of single infections of DEC and rotavirus, which had the relatively high numbers (74 and 201 children, respectively), were 1.6 and 1.9, respectively. Other studies on rotavirus diarrhea in children have also indicated the same higher ratios of male compared to female [70, 176, 202, 212, 237]. No reasonable explanations have been given for this distribution. There could be some factor related to genetics that may contribute to the higher risk of male to get diarrhea. This remains an open question.

Of those with diarrhea, 40.9% were less than 1 year of age, and 71.1% were less than 2 years of age. This probably showed an association between diarrhea and children less than 2 years of age as described in other studies [29, 103, 177, 184, 243]. Children without diarrhea had the average of current weight significantly higher than those with diarrhea in both groups of age. This could be explained by decreased nutrient absorption capacity in children with diarrhea. Patients ate less during diarrhea. Nutrient requirements were increased as a result of infection. Diarrhea is an important cause of malnutrition. Each episode of diarrhea can cause weight loss and growth faltering. Moreover, if diarrhea occurs frequently, there may be too little time to "catch up" on growth between episodes. Children who experience frequent episodes of acute diarrhea, or have persistent diarrhea, are more likely to become malnourished than children who experience fewer or shorter episodes of diarrhea. Of patients less than 6 months of age, 22.3% were fully breastfed as compared to 36.4% for the controls. There was no significant difference. Looking at children up to 3 months of age, these prevalences were 36.8 and 71.4% showing a significant difference (P = 0.026). Breast milk is the best and safest food for young babies. The value of breast milk as a source of nutrition and as a preventive measure to protect children from infections especially from diarrhea, as well as its psychological benefits, have been reported in several studies [8, 54]. The finding of our study clearly showed that breastfeeding could partially play an important role in preventing children diarrhea. The breastfeeding promotion campaign in Vietnam recommends that children be exclusively breastfed during the first 4 months of life and no solid food given before 6 months of age [171]. In the group of children up to 4 months of age, the prevalences of the fully breastfed ones were 40 and 60% in children with diarrhea and the controls, respectively, showing no significant difference. The data in the census in Vietnam shows that 36% of children at this age group are fully breastfed and this drops to 19% for children less than 6 months of age [171]. The difference between our study and the census is due to the fact that we only investigated children living in Hanoi, whereas, the national census has the data from children all over the country.

Table 10. Distribution of children regarding age group

Age group (month)	No. (%) of children		Total
	Diarrhea	Control	
0 - 12	240 (40.9)	48 (19.3)	288 (34.4)
13 - 24	177 (30.2)	71 (28.5)	248 (29.7)
25 - 36	95 (16.2)	48 (19.3)	143 (17.1)
37 - 48	41 (7)	49 (19.7)	90 (10.8)
49 - 60	34 (5.8)	33 (13.3)	67 (8)

Right before attending the hospital, 162/587 (27.6%) of children had been treated with antibiotics. They included 35 children with acute respiratory infections. Of them, 17 children had pneumonia, 11 had bronchitis, 1 had otitis media, and 6 had sore throat. The other 30 children had other kinds of diseases such as wounds, anemia, renal failure, etc. Ninety-seven of 162 antibiotic-treated children did not suffer from any diseases other than diarrhea that could be detected at the examination by the pediatricians or were reported by their parents. There was no significant difference in identification prevalence of enteric bacteria in children with diarrhea, who received and did not receive antibiotics before

attending the hospital. In previous 30 days before being enrolled in the study, 21.8% of children with diarrhea and 23.6% of the controls used antibiotics. Among the antibiotics used for children with diarrhea right before they attended hospitals, trimethoprim/ sulfamethoxazole, amoxicillin, and ampicillin were the most prevalent (36.4, 11.7, and 8.6%, respectively). According to the report from the parents of 162 children, who had been treated with antibiotics before attending hospital, only 89 (54.9%) had advice from the healthcare staffs (including pharmacist). Antibiotic therapy is not appropriate in the management of simple gastroenteritis even when a bacterial cause is suspected because most cases of acute diarrhea are self-limited. Despite this, a recent multicenter study from Europe showed that 44% of physicians would use antibiotics [53]. A community study in Vietnam [137] showed that 75% of the children had been treated with antibiotics during the month preceding the study; of these, 80% had been purchased from private drug outlets. According to the census in Vietnam [171], 23% of children less than 3 years of age with diarrhea had been treated with antibiotics.

As mentioned above, 89.1% of children with diarrhea had been given oral dehydration fluid, including ORS, and other fluids, before hospitalization. About 63 percent of children had received ORS. This knowledge is very important since fluid and nutritional support remains the mainstay of therapy for both viral and bacterial gastroenteritis. ORS designed to replace the acute electrolyte and fluid losses in acute diarrhea are widely available. Unfortunately, both in developed and developing countries despite the clear recommendations for ORT's use, the most recent WHO assessment is that ORT is being used in less than 50% of the world's episodes of acute diarrhea [53]. In an investigation in Vietnam [171], 40% of children with diarrhea were given a solution prepared from ORS packets. More than 60% of the mothers know about ORS. However, the awareness is different from different age groups, lowest is at the group of less than 25 years of age. Furthermore, young mothers tend to know less about the drinking and eating patterns in case their children have diarrhea. The education also plays an important part to knowledge of the mothers. It has been shown in the investigation that the more education they have, the more they know about the way to take care of their children with diarrhea, and the lower prevalence of diarrhea their children may get. In our study, more mothers younger than 25 years old and less mothers with higher education were seen in children with diarrhea compared to the controls (**Table 11**).

In the last 6 months preceding the study, 21.6% of children in the diarrhea group had had at least one episode of diarrhea. The corresponding figure was 15.3% in the control group ($P = 0.034$). It has been said that the previous infections such as diarrhea, respiratory tract infections etc. may give an increased risk to the children to get diarrhea.

Among children from 9 to 24 months of age, who were supposed to have vaccination against measles, 87% in diarrhea group and 96.5% in the controls were vaccinated ($P < 0.05$). In Vietnam, about 83% of children at the age of 12-23 months are vaccinated against measles [171]. Measles may be ultimately responsible for child deaths because of complications from pneumonia, diarrhea and malnutrition. Measles is also the major cause of preventable blindness in the world, affecting the same disadvantaged populations. The mechanisms by which measles predisposes to diarrhea are not clear but may include: (i) a direct effect of measles virus on the bowel epithelium, and (ii) virus induced immunosuppression, which can last for several months after an episode of measles and reduces the child's defenses against a variety of pathogenic bacteria and protozoa [273]. The full vaccination coverage given at the proper age could prevent measles and its complications as well.

Table 11. Clinical aspects and epidemiological factors related to the risk for diarrhea

Characteristic	Group of children		P value
	Diarrhea (n = 587)	Control (n = 249)	
Child's characteristics			
Gender: Male/Female	365/222	135/114	< 0.05
Weight: Mean of weight at birth (kg):			
Children ≤ 2 years old	3.06	3.16	< 0.05
Children > 2 years old	3.10	3.16	> 0.05
Mean of current weight (kg):			
Children ≤ 2 years old	8.50	9.61	< 0.01
Children > 2 years old	13.66	14.92	< 0.01
Fully breastfed (children ≤ 6 months)	23 (22.3%)*	12 (36.4%)	> 0.05
Clinical symptoms (diarrhea only)			
Fever	256 (43.6%)		
Vomiting	316 (53.8%)		
Dehydration	485 (82.6%)		
Kinds of stool:			
Watery	390 (66.4%)		
Bloody	1 (0.2%)		
Mucous	123 (21%)		
Others	73 (12.4%)		
Episodes per day** (mean)	7		
Mother's characteristics			
Age < 25	102 (17.4)	14 (5.6)	< 0.05
High education	206 (35.1)	128 (51.4)	< 0.0001
Hygienic conditions			
Often hand washing***	250 (42.6)	211 (84.7)	< 0.001
Water resource (pipe water)	408 (69.5)	231 (92.8)	< 0.0001
Convenience (hygienic toilet)	469 (79.9)	247 (99.2)	< 0.0001
Living standard			
Very poor	18 (3.1)	1 (0.4)	Chi square for trend = 3.403 P = 0.065
Poor	152 (25.9)	58 (23.3)	
Middle	204 (34.7)	90 (36.1)	
Fair	121 (20.6)	53 (21.3)	
Rich	92 (15.7)	47 (18.9)	
Information on health and sanitation			
Daily, weekly	112 (19.1)	65 (26.1)	< 0.05

*: Percentage in the defined group

** Episodes per day: When children were examined

***: Mother's hand washing before feeding children

Regarding the living standard, there was a decreasing trend of children acquiring diarrhea in terms of increasing level of income of their families (P = 0.065). In general, the prevalence of children with diarrhea was significantly higher in the families where the hygienic water and toilet were lacking, mothers less often washed their hands before feeding children, mothers and fathers had lower education, and the information on health and sanitation less often reached the families.

Diarrheal diseases are water-, hygiene-, food-, and sanitation-related and have multiple oral-fecal transmission routes [52, 65, 110]. In principal, enteric pathogens in feces infect humans via fluids, foods, and personal contact. Appropriate practices that stop these contaminations are very important. These include: (i) the disposal of stools in such a way that they are isolated from all future human contact, (ii) washing hands before preparing food, eating, storing food, and feeding the children. Hand washing can interrupt some transmission routes of enteric pathogens to the host. In this study, we have only asked about the mothers' hand washing routines before they gave food to their children. Some other details of hand washing are also necessary to ask the mothers such as washing hand after using the toilet, after changing a diaper, before preparing food, before eating, before touching the cooking or before drinking water. There are a number of epidemiological studies on hand washing which claim substantial reduction in diarrheal morbidity [107, 126, 196, 270]. However, water availability is likely to have an impact on the frequency of hand washing.

In terms of hygienic water supply, it is not easy for every family to fully get it for domestic uses. It depends on the geographical areas, infrastructure supply, and the supply capacity of water plant. In Hanoi, Vietnam, people in some areas still do not have access to sufficient pipe water. During the summer, when the water consumption rises dramatically, some families have shortage of hygienic water. They may have to find other additional sources or to try to store water in different ways. These facilitate the microbial contamination resulting in diarrhea especially in children. This together with suitable conditions for bacterial growth (warm temperature in summer time, for example) could partially explain for the high prevalence of diarrhea in children during this time. Improved water supplies can result from government-sponsored programs, in which families and communities may play an important role, or from other community or family efforts, such as collecting and storing rainwater.

As mentioned above, there is an association between stool disposal and child diarrhea. We have seen that having hygienic toilet in the households might prevent about 20% of children from getting diarrhea. In fact, not all the households in Hanoi have their own hygienic toilets. In some cases, they have to share with others. Sometimes, these types of conveniences are not hygienic enough. People, in some areas, can use indigenous composting latrine or defecation pit. The problem of safe stool disposal has also been investigated in many other studies [93, 155, 163, 252]. Safe stool disposal is one of the key barriers to the transmission of enteric pathogens.

Diarrhea prevention is one of the important issues of the public health. It is necessary to propagandize the knowledge, skills, and appropriate activities to the public. However, the frequency of how often this type of information reaches the households plays an important role in anti-diarrhea campaign. In our study, to the question: "How often does your family receive information about health and sanitation?", 26.1% of households in healthy group claimed that they receive the information daily or weekly, compared to 19.1% in the diarrhea group, showing a significant difference. That showed a fact that, although, many diarrheal diseases can be preventable by following simple rules of personal hygiene and safe food preparation, the public healthcare workers should pay more attention to

effectively inform the population about health and sanitation through the mass media to the households.

In conclusion, many aspects of diarrhea need to be more investigated in order to decrease the morbidity and mortality of diarrhea in Vietnamese children less than 5 years of age.

5. CONCLUSIONS

The study focused on identification of some potential enteric pathogens causing diarrhea in relation to clinical and epidemiological aspects in children less than 5 years of age in Hanoi, Vietnam. The results can be summarized as follows:

1. Detection and characterization of DEC and other enteric pathogens from children less than 5 years of age in Hanoi, Vietnam

- A multiplex PCR has been successfully set up and applied to identify five main categories of DEC from fecal samples.

- Four DEC types have been identified with significantly higher prevalence in children with diarrhea compared to the control. No EHEC, *Salmonella*, and *V. cholerae* were found.

- EAEC, EPEC were more frequent in children less than 2 years of age, whereas ETEC and EIEC were more predominant in the older ones.

2. Antibiotic resistance in diarrheagenic *Escherichia coli* and *Shigella* strains isolated from children in Hanoi, Vietnam

- The isolated DEC and *Shigella* were resistant with high prevalence to ampicillin, chloramphenicol and trimethoprim/sulfamethoxazole.

- About 90% of all DEC and 79% percent of *Shigella* were multiresistant.

3. Diarrhea caused by rotavirus in children less than 5 years of age in Hanoi, Vietnam

- Group A rotavirus was found with significantly higher prevalence in diarrhea children than in the control. It was associated with children less than 2 years of age.

- Rotavirus infection occurred year around and picked during wintertime.

- Watery diarrhea, vomiting, dehydration were common in rotavirus diarrhea.

4. Diarrhea caused by enterotoxigenic *Bacteroides fragilis* in children less than 5 years of age in Hanoi, Vietnam

- Enterotoxigenic *B. fragilis* were identified with higher prevalence in diarrhea children as compared to the control showing a significant difference. ETBF infection was associated with children older than 1 year of age.

- Three subtypes of ETBF have been identified.

5. Etiology, epidemiological factors of diarrhea in children less than 5 years of age in Hanoi, Vietnam

- Group A rotavirus, diarrheagenic *E. coli*, *Shigella*, and enterotoxigenic *B. fragilis* play an important role in causing diarrhea in children less than 5 years of age in Hanoi, Vietnam.

- More than 70% of children with diarrhea were less than 2 years of age.

- The epidemiological factors such as lack of fresh water supply, unhygienic toilet, low family income, infrequent getting information of health, and low education of parents could give rise to the morbidity of diarrhea in children.

Diarrhea is still a health problem in children not only in Hanoi, but also nationwide in Vietnam. Some enteric pathogens such as rotavirus, DEC, *Shigella*, ETBF are the major causes of diarrhea. This study has contributed to the knowledge of enteric pathogens that cause diarrhea in children in Vietnam. The implications of the techniques such as PCR, ELISA will improve the quality of detection of the pathogens. This will make the clinical diagnosis better and help the pediatricians in treatment of children with diarrhea. The findings from the study will also help the policy makers to improve the health care program to provide better services to children.

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