

2010

Thesis for doctoral degree (Ph.D.) 2010

ESCHERICHIA COLI FLORA AND DIARRHEA IN NICARAGUAN CHILDREN

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**Karolinska
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Stockholm, September 2010

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ISBN 978-91-7409-990-4

To all my family

Especially to

My dear wife María de la Concepción –Cony– and

My children: Norman, Josmary and Grethel

ABSTRACT

A combination of a phenotyping method (PhP typing) for the identification of clonal groups, and a genotyping method (PCR) for the identification of virulent strains of *E. coli* were used in order to obtain insight into diarrheal disease morbidity and epidemiology of diarrheagenic *E. coli* strains in children in León, Nicaragua. Fecal samples from 381 children aged 0-5 years suffering from diarrheal disease and 145 healthy children of the same age were analyzed. Besides, a cohort of 20 healthy infants aged 0-12 months were followed by repeated sampling (8 samples per infant). All samples were analyzed by a multiplex PCR (EAEC, ETEC; EPEC, EHEC, EIEC markers) on the primary streak and from the 282 positive samples 2,164 *E. coli* isolates were analyzed by single-colony PCR, and from all samples, eight *E. coli* isolates were phenotyped; totally 4,753 *E. coli* isolates were analyzed.

Our main results are:

- ◆ Diarrheagenic *E. coli* (DEC) positive samples were found at high rates in infants aged 3-60 months (Paper I), but were rare in infants below 3 months of age (Paper IV). Thus, colonization by DEC seems to start early in life and increase with age.
- ◆ The phenotypic diversities among all isolates from diarrheal cases and from healthy children were equal and high, thus giving indication that no large outbreak of DEC diarrhea occurred during the time period studied (Paper II)
- ◆ EAEC and EPEC could not be correlated to diarrhea, whereas ETEC and EHEC were significantly more common in diarrheal cases (Papers I, II, and III).
- ◆ ETEC *estA* and EHEC were only found in diarrheal cases, and isolates positive for these DEC types belonged to a few PhP types that could represent pathogenic clones (Paper III)
- ◆ DEC positive isolates seldom dominated the fecal *E. coli* flora, but for all DEC types, except for EAEC, a high proportion of DEC positive isolates in the fecal *E. coli* flora seemed to be correlated to diarrhea. Thus, a low proportion of DEC in the fecal *E. coli* flora may not be of significance for development of diarrhea, but may represent occasional carriage of virulence gene(s) by normal flora strains (Paper III).
- ◆ Newborns were found to rapidly exchange their *E. coli* strains during their first year of life. Colonization by single DEC positive isolates was rarely correlated to diarrheal disease (Paper IV).

Our findings suggest that there are certain stable types or clones of diarrheagenic *E. coli* that can circulate in a population, and have the possibility to colonize the intestine and to dominate the *E. coli* flora, thereby causing diarrhea. These are the true DECs. However, the virulence genes are promiscuous and may spread to the *E. coli* bacteria of the normal flora, whereupon these scarce gene markers are detected by the sensitive PCR test in samples from both healthy and diseased individuals, without necessarily having a pathogenic role.

Thus, the obtained information on the distribution of DEC pathotypes and their diversities may be used to optimize the use of today's diagnostic tools, which in turn might affect treatment approaches and vaccine development strategies.

LIST OF PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals

- I. Samuel Vilchez, **Daniel Reves**, Margarita Paniagua, Filemón Bucardo, Roland Möllby and Andrej Weintraub.
Prevalence of Diarrhoeagenic *Escherichia coli* in Children from León, Nicaragua
Journal of Medical Microbiology 2009; 58, 630–637
- II. **Daniel Reves**, Samuel Vilchez, Margarita Paniagua, Patricia Colque, Andrej Weintraub, Roland Möllby and Inger Kühn.
Diversity of intestinal *Escherichia coli* populations in Nicaraguan children with/without diarrhoea
Journal of Medical Microbiology 2009; 58, 1593–1600
- III. **Daniel Reves**, Samuel Vilchez, Margarita Paniagua, Patricia Colque, Andrej Weintraub, Roland Möllby and Inger Kühn.
Diarrheagenic *Escherichia coli* isolated from children in Nicaragua – Pathogens or occasional carriers of virulence genes?
Submitted for publication to Journal of Clinical Microbiology*
- IV. **Daniel Reves**, Margarita Paniagua, Erick Amaya, Patricia Colque, Roland Möllby and Inger Kühn.
Intestinal colonization patterns and phenotypic diversity of *Escherichia coli* flora in Nicaraguan infants from 0 to 12 months of age
Submitted for publication to Pediatric Research

* Now published as Ahead of print in a different version as
“Diarrheagenic *E. coli* markers and phenotypes among *Escherichia coli* in Nicaraguan infants”. *Journal of Clinical Microbiology* –DOI: 10.1128/JCM00228-10

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LIST OF ABBREVIATIONS

A/A	Aggregative adherence
A/E	Attaching and effacing
AID	Acute Infectious Diarrhea
BPT	Biochemical phenotype
DEC	Diarrheagenic <i>E. coli</i>
Di	Diversity index
DNA	Deoxyribonucleic acid
<i>E. coli</i>	<i>Escherichia coli</i>
EAEC	Enteroaggregative <i>E. coli</i>
ETEC	Enterotoxigenic <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
EHEC	Enterohemorrhagic <i>E. coli</i>
EIEC	Enteroinvasive <i>E. coli</i>
<i>ial</i>	Invasion-associated locus
LEE	Locus of enterocytes effacement
LT	Heat-labile toxin
PCR	Polymerase Chain Reaction
PhP system	Phene Plate system
Sp	Population similarity coefficient
ST	Heat-stable toxin
UPGMA	Unweighted pair group method using arithmetic averages

1 INTRODUCTION

1.1 *ESCHERICHIA COLI* – GENERALITIES

The scientific history of *Escherichia coli* (*E. coli*) started with its first description in 1885 by Theodor von Escherich (Escherich, 1988; Gross & Rowe, 1985), a pediatrician and scientist, who in a series of pioneering studies of the intestinal flora of infants discovered a normal microbial inhabitant of healthy individuals that he named *Bacterium coli commune*. Its actual name became used and officially accepted in 1958, in his honor (Kuhnert *et al.*, 2000).

The species *Escherichia coli* comprises Gram-negative, rod-shaped, non-spore forming, motile bacteria which are about 2 µm long and 0.6 µm in diameter, with a cell volume of 0.6-0.7 µm³ (Darnton *et al.*, 2007; Kubitschek, 1990). They are facultative anaerobes, oxidase-negative, glucose, lactose and sucrose fermenting, with an optimum growth pH of 6.0-7.0 and temperature of 37°C. However, some laboratory strains can multiply at temperatures up to 49°C (Fotadar *et al.*, 2005).

Taxonomically, *E. coli* belongs to the family *Enterobacteriaceae*, and it is a commensal bacterium residing as the most common and predominant inhabitant in the intestinal microflora of human and other mammals (Hill & Drasar, 1975; Nataro & Karper, 1998). However, the establishment of the intestinal *E. coli* flora, is rather a complex process influenced by microbial and host interactions, or by internal and external factors that can have a substantial influence on the prevalence and density of *E. coli*, e.g., delivery mode, and feeding habits, life-style, environment and immunological status (Adlerberth, 2008; Adlerberth & Wold, 2009; Penders *et al.*, 2006). Thus, this process should be considered during the study of *E. coli* transmission, colonization, establishment, and population structure.

The intestinal microflora comprises of about 10¹⁰–10¹¹ bacterial cells per gram of intestinal content, which is represented by more than 500 different species of bacteria (Penders *et al.*, 2006; Xu & Gordon, 2003). Although the anaerobic bacteria in the bowel outnumber *E. coli* by 100/1 to 10,000/1, *E. coli* is a first bacterial species, and predominant aerobic organism to colonize the intestinal tract during the infancy, reaching a very high density (higher than 10⁹ CFU per gram of feces), before the expansion of anaerobes (Penders *et al.*, 2006).

1.2 *ESCHERICHIA COLI* – ACQUISITION AND POPULATION STRUCTURE

Acquisition of *Escherichia coli*

The gastrointestinal tract of the newborn is considered sterile at birth. However, its colonization begins as soon as the newborn is exposed to a microflora, which occurs after the rupture of the fetal membranes. *E. coli* and other enterobacterial species are among the first colonizers of the newborn infant's intestine, within a few hours after birth (Adlerberth *et al.*, 1991; Fanaro *et al.*, 2003; Hooper & Gordon, 2001), and thereafter *E. coli* and the host derive mutual benefits for decades (Kaper *et al.*, 2004).

The initial *E. coli* strains colonizing the newborn intestine may have originated from the maternal fecal microflora during delivery (vertical transfer), or be transferred between infants via the nursing staff (horizontal transfer) (Adlerberth *et al.*, 1991; Bettelheim & Lennox-King, 1976; Fanaro *et al.*, 2003; Fryklund *et al.*, 1992). Another possible route of exposure is ingestion of contaminated food and water (Duriez *et al.*, 2001; Gorter *et al.*, 1998; Gorter *et al.*, 1991; Hammerum & Heuer, 2009).

Some studies in developed countries have shown that 42-49% of the newborn infants were colonized by *E. coli* strains, with a mean of 1.6–2.1 strains at day 3 in life (Kühn, 1986; Nowrouzian *et al.*, 2003). Interestingly, this flora was found to be stable and the corresponding carriage rate among infants was only 61–64% at 3-6 months of age, indicating an environment in the modern society in developed countries almost free of contaminating fecal bacteria. This should be compared to the rapid and high turnover of strains in developing countries, since 8.5 *E. coli* strains were found per child during a one year period in a comparable group of infants (Adlerberth *et al.*, 1998a).

Later in life, a newly introduced *E. coli* strain will not necessarily replace already existing strains in the intestines (Sears *et al.*, 1950; Sears *et al.*, 1956). In all humans there are some *E. coli* strains that will persist in the intestine of an individual for several weeks, months, and years in succession (resident strains). Whereas others disappear within a few days or weeks (transient strains), and they may be found in the microflora on a single occasion, or on a few occasions closely spaced in time (Sears *et al.*, 1950). Resident *E. coli* strains have an increased capacity to adhere to colonic epithelial cells by presenting different colonization characteristics, and are also more likely to belong to specific or particular “pathogenic” clonal groups (Adlerberth *et al.*, 1998b; Ishii *et al.*, 2007; Kaper *et al.*, 2004; Müller *et al.*, 2007; Pacheco *et al.*, 1997).

Population structure of *Escherichia coli*

The clonal structure of *E. coli* was first supported by serotyping analysis (Orskov *et al.*, 1976). As the relative frequency of many of the antigens varied with the isolate source but the O (somatic), K (capsular) and H (flagellar) antigens were non-randomly associated, and as some serotypes were distributed worldwide, it was postulated that the species *E. coli* consists of an array of stable lineages (called clones), among which few recombinations of chromosomal genes occurs. These recombinations have showed to be an important mechanism in the evolution of *E. coli*. Former (Ochman & Selander, 1984a; b) and recent (Duriez *et al.*, 2001; Hacker & Kaper, 2000; Hooper *et al.*, 2001; Robins-Browne, 2005) studies based on molecular analyses have shown that many *E. coli* clones have broad geographical and host distributions, and the observed genetic diversity of *E. coli* exhibits both host taxonomic and environmental components. This evolutionary relationship, nowadays, are more studied through the comparative studies of the entire *E. coli* genome (Dicksved *et al.*, 2007; Dobrindt *et al.*, 2003; Dobrindt *et al.*, 2002; Touchon *et al.*, 2009). However, although detailed, these studies have to be interpreted with caution as to their overall relevance of phylogeny, facing the huge diversity in *E. coli*. Thus, further characterizations of the commensal *E. coli* strains are necessary to understand how a helpful commensal can become a harmful pathogen.

1.3 *ESCHERICHIA COLI* – IMPORTANT ROLES

Indicator for fecal contamination

Public and environmental health protection requires safe drinking water, which means that it must be free of pathogenic bacteria. Determining the source of fecal contamination in aquatic environments is essential for estimating the health risk associated with pollution, and for facilitating measures to remediate polluted waterways (Blanch *et al.*, 2006). *E. coli* constitute a part of the intestinal microflora of human and warm-blooded animals, and survive long enough in the different aquatic environments, and are easily isolated, enumerated and identified. It has been used as indicator of fecal contamination (McQuaig *et al.*, 2006; Whitlock *et al.*, 2002), and also to determine the quality and safety of water for consumption worldwide. This procedure - recommended by the U.S. Environmental Protection Agency (EPA) - is based on epidemiological studies that demonstrate a direct relationship between the density of *E. coli* organisms in water and the occurrence of swimming-associated gastroenteritis (WHO, 2003). Furthermore, this common organism may contribute to the dissemination of antibiotic resistant microorganisms between human and animal populations,

and it may also constitute the route by which resistance genes are introduced in environmental bacterial ecosystems (Kim & Aga, 2007; Kummerer, 2009).

Indicator for antibiotic resistance

Microbial resistance to antibiotics is an increasing public health problem worldwide; since administration of antimicrobial agents causes disturbances in the ecological balance between host and microorganisms (Pallecchi *et al.*, 2007; Paterson & Bonomo, 2005; Sullivan *et al.*, 2001), and may promote the emergence of antibiotic-resistant strains that increase in numbers, which may lead to more severe infections. *E. coli* and other members of the family *Enterobacteriaceae* are well known to develop or acquire resistance to a variety of antibiotics by different mechanisms. However, production of β -lactamases is the most common and clinically significant mechanism of resistance among this bacterial group (Suarez *et al.*, 2005; Torres *et al.*, 2007; Woodford *et al.*, 2007). Accordingly, the intestinal *E. coli* microflora may provide an important reservoir for antibiotic-resistant bacteria, and resistance genes, which may be transmitted further to potentially pathogenic bacteria (Pallecchi *et al.*, 2007). Thus, the ecological impact of different antimicrobial agents, as well as the development of antimicrobial resistance before it appears in pathogenic strains and in clinical infections, could be studied in the intestinal *E. coli* flora

Emerging pathogen with potential to spread virulence

Although *E. coli* strains are termed commensals and part of the normal intestinal microflora of human and warm-blooded animals, maintaining a healthy intestinal ecosystem, under certain circumstances they may cause diseases (Kaper *et al.*, 2004). Diseases caused by any *E. coli* strains are either a result of specific or non-specific infections. Unspecific infections may occur where the non-pathogenic, commensal *E. coli* strain become harmful, because of the fact that the host immune system is weak, e.g., in preterm-newborn infants, elderly, malnourished and immunocompromised individuals (Kaper *et al.*, 2004). Specific infections are caused by some subsets of *E. coli* strains that represent a versatile and diverse group of microorganisms with several highly adapted clones. These strains have acquired specific virulence factors, which confer them ability to adapt to new environments and make them capable to cause a broad range of infections in healthy individuals (Kaper *et al.*, 2004).

Interestingly, most of these virulent attributes that distinguish pathogenic *E. coli* from commensals are frequently encoded on mobile genetic elements such as plasmids, bacteriophages, and transposons that can be mobilized into different strains creating new and

successful combinations of virulence factors (Dobrindt *et al.*, 2003; Dobrindt *et al.*, 2002). Moreover, these novel combinations might be locked into the genome resulting in a more stable virulence clone to become specific pathotypes of *E. coli* capable to cause intestinal and extraintestinal disease (Dobrindt *et al.*, 2003; Dobrindt *et al.*, 2002; Kaper *et al.*, 2004). Major virulence factors associated to the pathogenicity of the different pathogenic group of *E. coli* are listed in Table 1, and pathogenic mechanisms associated with DEC infections are pictured in Figure 1.

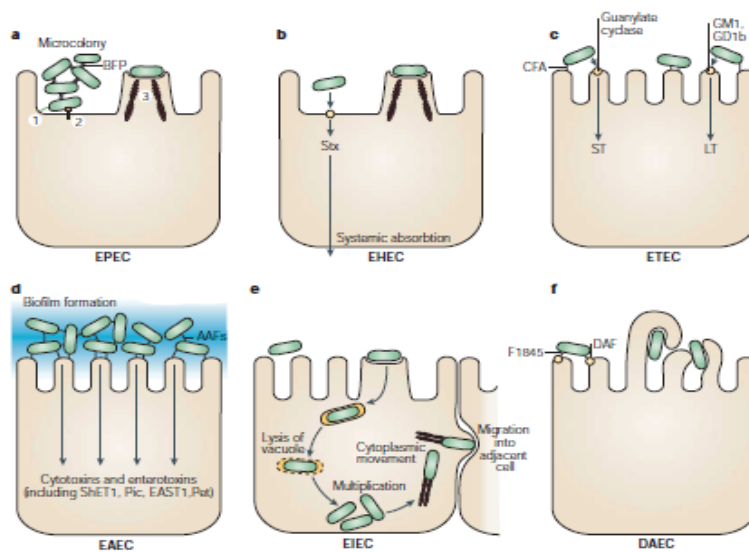


FIGURE 1. Pathogenic schema of diarrheagenic *E. coli*. The six recognized categories of diarrheagenic *E. coli* each have unique features in their interaction with eukaryotic cells. Here, the interaction of each category target cell is schematically represented. These descriptions are largely the results of *in vitro* studies and might not completely reflect the phenomena that occur in infected humans. **a)** EPEC adheres to small bowel enterocytes, but destroy the normal microvillar architecture, inducing the characteristic attaching and effacing lesion. Cytoskeletal derangements are accompanied by an inflammatory response and diarrhea. 1. Initial adhesion, 2. Protein translocation by type III secretion, 3. Pedestal formation. **b)** EHEC also induce the attaching and effacing lesion, but in the colon. The distinguishing feature of EHEC is the elaboration of Shiga toxin (Stx), systemic absorption of which leads to potentially life-threatening complications. **c)** Similarly, ETEC adheres to small bowel enterocytes and induce watery diarrhea by the secretion of heat-labile (LT) and/or heat-stable (ST) enterotoxins. **d)** EAEC adheres to small and large bowel epithelia in a thick biofilm and elaborates secretory enterotoxins and cytotoxins. **e)** EIEC invades the colonic epithelial cell, lyses the phagosome and moves through the cell by nucleating actin microfilaments. The bacteria might move laterally through the epithelium by direct cell-to-cell spread or might exit and re-enter the baso-lateral plasma membrane. **f)** DAEC elicits a characteristic signal transduction effect in small bowel enterocytes that manifests as the growth of long finger-like cellular projections, which wrap around the bacteria. AAF, aggregative adherence fimbriae; BFP, bundle-forming pilus; CFA, colonization factor antigen; DAF, decay-accelerating factor; EAST1, enteroaggregative *E. coli* ST1; LT, heat-labile enterotoxin; ShET1, *Shigella* enterotoxin 1; ST, heat-stable enterotoxin. [Reproduced with the permission from (Kaper *et al.*, 2004)]

TABLE 1. Intestinal and Extraintestinal Pathogenic *E. coli* (Kaper *et al.*, 2004; Nataro & Karper, 1998)

Pathotype	Main virulence factors	Structure/location	Clinical features
Intestinal <i>E. coli</i>			
Enterotoxigenic <i>E. coli</i> (ETEC)	Colonization factor antigens (CFA)/ oli surface antigens (CS) Heat-labile toxin (LT) Heat-stable toxin (STa, STb)	Fimbriae / Plasmid AB5 toxin / Plasmid Monomeric toxin / Plasmid	Watery diarrhea; vomiting Infantile diarrhea in developing countries and Traveler's diarrhea in all age
Enteropathogenic <i>E. coli</i> (EPEC)	<u>Pathogenicity island LEE:</u> Type III secretion system, intimin, Tir, EspA, EspB, EspC, EspD, EspF, EspH. <u>Adherence factor (EAF) plasmid:</u> Bundle-forming pili (bfp) Plasmid-encoded regulator (Per) No classic toxins produced, atypical EPEC lacks EAF plasmid.	LEE on chromosome EAF Plasmids	Watery to bloody diarrhea Classic type causing acute endemic and epidemic diarrhea in infants; occasionally associated to persistent diarrhea.
Enteraggregative <i>E. coli</i> (EAEC)	Aggregative adherence fimbriae (AAFs) Toxins (Pic, ShEt1, EAST, Pet, EspP). Dispersin, flagellin, aggR regulon.	Fimbriae / Plasmid Monomeric toxin / Plasmid	Watery mucoid diarrhea Classic type associated to persistent diarrhea. Second most important cause of traveler's diarrhea. Cause of AIDS-Associated diarrhea.
Enterohemorrhagic <i>E. coli</i> (EHEC)	<u>Pathogenicity island LEE:</u> Type III secretion system, intimin, Tir, EspA, EspB, EspD, EspF, EspH. Lymphocytes inhibits/Activation, adhesion (LifA/Efa) Verotoxins, <i>vt1</i> and <i>vt2</i> <u>Shiga toxin-producing <i>E. coli</i></u> <u>(O157) plasmid:</u> Enterohaemolysin (EHEC-Hly) Serine protease (EspP)	LEE on chromosome Excreted proteins / Plasmid AB5 toxin / Prophage / Chromosome Cytolsolin / Plasmid	Watery to bloody diarrhea Induce to Hemorrhagic colitis, hemolytic uremic syndrome, acute renal failure, and neurological disorders.
Enteroinvasive <i>E. coli</i> (EIEC)	Type III secretion system Invasion plasmid (pInv), IpaA, IpaB, IpaC, IpaH, IpgD; IscA Toxins (ShET1, ShET2) Serine Protease (SepA)	Excreted proteins / pInv Plasmid Excreted proteins / Plasmid	Watery to bloody diarrhea Induce to bacillary dysentery, invasive inflammatory colitis.
Diffusely adherent <i>E. coli</i> (DAEC)	Dr Adhesins family Fimbrial adhesin F1845	Monomeric toxin / Plasmid Fimbriae / Plasmid	Watery mucoid diarrhea Induces inflammatory bowel diseases.
Extraintestinal <i>E. coli</i>			
Uropathogenic <i>E. coli</i> (UPEC)	Adhesins (Type 1, FIC-fimbriae, Dr , P fimbriae (Pap), S fimbriae) Heam transport (Shu), autotransported protease (Sat) Cytotoxic necrotizing factor (CNF1) Haemolysin (HlyA)	Fimbriae / Plasmid Monomeric toxin / Plasmid	Cystitis and pyelonephritis (Colonizes periurethral area, ascends the urethra to urine bladder, attaches and invades epithelial cell)
Meningitis/sepsis-associated <i>E. coli</i> (MNEC)	Outer membrane proteins promotes invasion (OmpA, Iba, B, C; AsIA) Cytotoxic necrotizing factor (CNF1) K antigen capsules	Excreted proteins / Plasmid Glycoprotein / Plasmid	Neonatal meningitis / septicemia (Spreads hemathogeneously- translocates via blood to CNS)

1.4 DIARRHEAL DISEASES AND DIARRHEAGENIC *E. COLI*

1.4.1 Acute Infectious Diarrhea (AID) – A worldwide problem

Acute infectious diarrhea (AID) is a major cause of morbidity and mortality worldwide, and it remains a major public health challenge especially in developing countries where it is a leading cause of death. Every year nearly 1.4 billion episodes of AID occur in children less than 5 years of age in developing countries (Kosek *et al.*, 2003; Parashar *et al.*, 2003), of which 123.6 million episodes required outpatient medical care and 9 million episodes required hospitalization. It has been estimated that the mean number of episodes of diarrhea per year in children under 5 years of age from a developing region is 3.2, with the highest incidence (4.8 episodes), occurring during the first year of life, decreasing progressively to 1.4 episodes per year at 4 years of age. Furthermore, the highest age-mortality rate (8.5 children per 1000/year) occurred in children under 1 year of life (Kosek *et al.*, 2003). Improvements in sanitation, nutrition, education and early access to oral rehydration therapy among other measures have lowered the lethality of severe AID from 4.6 million in 1982 to an estimated 1.5–2.5 million in 2010 (Black *et al.*, 2010; O’Ryan *et al.*, 2010). However, it remains the second most common cause of death in children under 5 years of age worldwide according to the Global Burden Disease Report of the WHO (Boschi-Pinto *et al.*, 2008; Kosek *et al.*, 2003).

A diversity of recognized microorganisms such as bacteria, viruses and parasites can be associated with severe AID in children (Al-Gallas *et al.*, 2007b; Albert *et al.*, 1999; DuPont, 2009; O’Ryan *et al.*, 2005). However, etiological information that includes all the known agents of severe AID in children is scarce. Numerous studies performed in different countries have reported diarrheagenic *Escherichia coli* (DEC) pathotypes as being the most frequent and important among bacterial pathogens associated with AID in developing countries. However, the frequencies of these pathogens vary with geographic region and depend on the socioeconomic/sanitary conditions achieved (Black *et al.*, 2010; O’Ryan *et al.*, 2010). Altogether, they represent 30-40% of the cases (Albert *et al.*, 1999; O’Ryan *et al.*, 2005), and about 15-30% have required hospital care (Nataro & Karper, 1998; O’Ryan *et al.*, 2010).

In Nicaragua, like in other developing countries, the AID remains one of the most important health problems surpassed only by the respiratory diseases (MINSa-Nicaragua, 2008). Annually a main peak of AID has been reported (between May and August) when the winter season is started (Figure 2). According to Ministry of health in Nicaragua an estimates of morbidity rate in children aged less than five years of age account for more than 252.72 x

10,000 hab. (median calculated for week 33 in year 2008), corresponding to 70% of all national consultations. The mortality rate in this group is 1.34 x 100,000 hab. (median calculated for week 33 in year 2008) (MINSANicaragua, 2008).

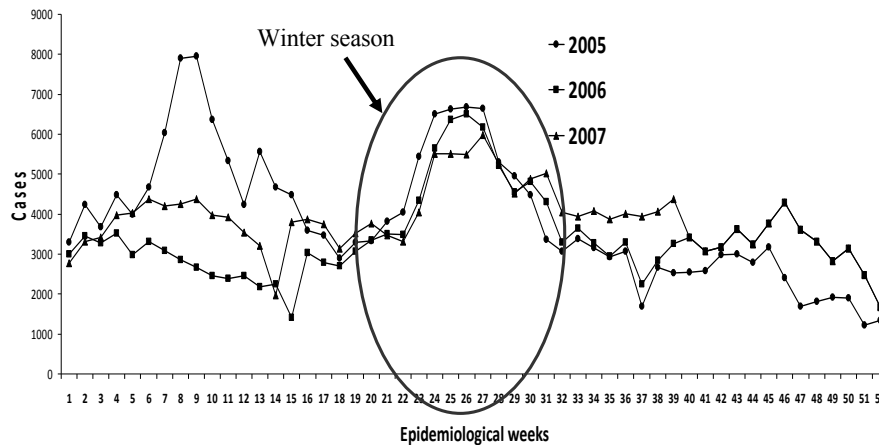


FIGURE 2. Frequency of acute infectious diarrheal disease (AID) in Nicaragua. Figure depicts the distribution of diarrheal cases during years 2005 to 2007. X-axis depicts number of the week. Adapted from SISNIVEN, MINSANicaragua (MINSANicaragua, 2008).

Over more than 10 years, a number of epidemiological studies on diarrheal disease have been carried out in infants and young children in Nicaragua. They have shown the importance of different pathogens associated with AID, like the diarrheagenic *E. coli*. Enterotoxigenic *E. coli* (ETEC), and enteropathogenic *E. coli* (EPEC) pathotypes were found to be the most frequent bacterial causes of diarrhea (Mayatepek *et al.*, 1993; Paniagua *et al.*, 1997). An important and valuable input was made in year 1997, when a large infant-cohort study was performed, that demonstrated the association between ETEC and infant diarrhea (Paniagua *et al.*, 1997). However, despite this valuable effort the complete picture of the epidemiology of the remaining diarrheagenic *E. coli* pathotypes and their association with diarrhea is still lacking.

1.4.2 Diarrheagenic *Escherichia coli* Pathotypes

The pathogenic *E. coli* species comprise a very versatile group with numerous virulence determinants (virulence factors) including adhesins, invasins, toxins and secretion systems that allow them act as causative agents in both human and veterinary medicine (Kaper *et al.*, 2004; Kuhnert *et al.*, 2000; Nataro & Karper, 1998). In humans, these pathogens are

responsible for three main types of clinical infections: (i) enteric or diarrheal diseases, (ii) urinary tract infections, and (iii) meningitis/septicemia. Based on their distinct virulence properties and clinical symptoms of the host, pathogenic *E. coli* strains are divided into numerous categories or pathotypes (See Table 1): Diarrheagenic *Escherichia coli* (DEC); Uropathogenic *Escherichia coli* (UPEC); Neonatal meningitis/sepsis associated *Escherichia coli* (MNEC) (Dawson et al., 1999; Kaper et al., 2004; Robins-Browne & Hartland, 2002; Stoll, 1997).

Diarrheagenic *Escherichia coli* (DEC) belong to one of the most frequent, interesting and widespread versatile pathogenic groups of bacteria that cause severe disease in humans (Campos et al., 2004; Evans & Evans, 1983; Hien et al., 2007; Müller et al., 2007; Nataro & Karper, 1998). DEC has been divided into six well-characterized subgroups or classes [enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), verocytotoxin producing *E. coli* (VTEC) or enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), diffusely adherent *E. coli* (DAEC)] based on clinical manifestations, phenotypic traits, specific virulence properties, and pathogenesis (Kaper et al., 2004; Nataro & Karper, 1998) (Figure 1, Table 1).

1.4.3 Enterotoxigenic *E. coli* (ETEC)

ETEC is one of the most studied pathotypes of DEC. Most studies have demonstrated the association of ETEC with diarrhea among infants less than five years of age (Aguero et al., 1985; Al-Gallas et al., 2007a; Ansaruzzaman et al., 2007; Hien et al., 2007; Paniagua et al., 1997; Peruski et al., 1999; Shaheen et al., 2009; Shaheen et al., 2004). Most of the illness, both in terms of numbers of cases and severity of symptoms, occur in infants after weaning. Moreover, epidemiological studies have implicated contaminated food and water as the most common vehicles of ETEC infection. Thus, sampling of both food and water sources from endemic areas have demonstrated the spread of ETEC bacteria in the community (Black et al., 1982; Long et al., 1994; Wood et al., 1983). A high infectious dose (ranging 10^6 to 10^{10} CFU) of ETEC has been demonstrated in human (Levine et al., 1979; Nataro & Karper, 1998), and a large number of serogroups of ETEC have been associated with diarrhea (Stenutz et al., 2006; Viboud et al., 1999).

ETEC strains cause cholera-like watery diarrhea through the elaboration and action of LT (heat-labile) and/or ST (heat-stable) enterotoxins or both. The ability of ETEC strains to produce diarrheal illness by either or both of these enterotoxins is what defines an ETEC.

However, to cause diarrhea, by LT and/or ST, the ETEC must adhere and colonize the intestinal mucosa. This is achieved by attaching with one or more colonization factor antigens (CFAs or CS), which are antigenically diverse and usually are encoded by plasmids (Nataro & Karper, 1998; Smith *et al.*, 1983). LTs of ETEC are oligomeric toxins that are functionally and structurally related to the cholera toxin (CT) expressed by *Vibrio cholerae*, serogroups O1 and O139 (Sixma *et al.*, 1993; Spangler, 1992).

There are two types of LTs, LT-I and LT-II, that are commonly found in human and animal isolates. However, the term LT refers to LT-I, which is associated with disease in both humans and animals, while LT-II is expressed only in animals, but it is rarely associated to disease (Nataro & Karper, 1998; Smith *et al.*, 1983). LT-I is constituted by ~80% amino acid identity with CT that consists of a single A subunit and five identical B subunits. The A subunit is responsible for the enzymatic activity, and the B subunits are responsible for the toxin binding to the cell surface ganglioside GM1. After endocytosis the A subunit stimulates a series of intracellular processes leading to increased level of cyclic adenosine monophosphate (cAMP), resulting in an increased phosphorylation of chloride channels, and hence a reduced absorption of NaCl. This increased extracellular ions content results in osmotic diarrhea. Besides, there is an increased secretion of ions in the villi crypts (Nataro & Karper, 1998).

STs are small peptides including two unrelated classes, STa and STb, which differ in structure and mechanism of action. Genes for both classes – *estA* and *estB* – have been found either on plasmids or on transposons. Only toxin of the STa class has been associated with human and animal diseases. It has been established that the STa receptor is located on the apical surface of enterocytes and that binding to the receptors leads to increased intracellular cyclic guanine monophosphate (cGMP) levels, which affects the electrolytic balance in a similar manner as LT (Kaper *et al.*, 2004; Nataro & Karper, 1998). In contrast, STb is primarily associated with diarrhea in piglets; however, some human ETEC isolates expressing STb have been reported (Nataro & Karper, 1998; Schulz *et al.*, 1990; Sears & Kaper, 1996). STb can elevate cytosolic Ca²⁺ concentrations, stimulating both the release of prostaglandin E₂ and serotonin, which lead to increased ion secretion (Kaper *et al.*, 2004; Nataro & Karper, 1998; Schulz *et al.*, 1990; Sears & Kaper, 1996).

The clinical features of ETEC diarrhea are constant with the pathogenic mechanisms of its enterotoxins, and characterized by watery diarrhea without blood, mucus or pus, fever and

vomiting (DuPont, 2009; DuPont *et al.*, 1971; Levine, 1987). The illness is typically abrupt, but can vary from mild, brief, and self-limiting to a severe disease similar to than seen in *Vibrio cholerae* infection (Levine *et al.*, 1977). Several studies have showed that the percentage of ETEC infection varies from 10 to 30% (Mangia *et al.*, 1993; Rivera *et al.*, 2010), and the clinical presentation, as with the other DEC:s, varies among different geographical areas.

1.4.4 Enteropathogenic *E. coli* (EPEC)

EPEC was the first pathotype of DEC described, and associated to the infant diarrhea diseases worldwide (Nataro & Karper, 1998). EPEC belongs to a number of specific O:H serotypes. Most of the serogroups of EPEC that have been frequently recognized so far are O18, O20, O25, O26, O28, O44, O55, O86, O91, O111, O112, O114, O119, O125, O126, O127, O128, O129, O142, and O158 (Nunes *et al.*, 2003; Rosa *et al.*, 1998; Trabulsi *et al.*, 2002). The characteristic intestinal histopathology – attaching and effacing (A/E) lesions - of EPEC are associated to striking cytoskeletal changes in the epithelial cell. This ability to induce attaching and effacing lesions is encoded by genes for the adherence factor intimin (*eae*), a type II secretion system (TTSS) that includes the *esc* genes and the translocated intimin receptor (Tir) (Nataro & Karper, 1998) (Table 1).

These genes are located on a 35-Kb pathogenicity island (PAI), called the locus of enterocyte effacement (LEE), which is present in all EPEC and EHEC (McDaniel *et al.*, 1995; Trabulsi *et al.*, 2002). The A/E mechanism induce microvilli destruction, intimate adherence of bacteria to the intestinal epithelium, pedestal formation, and aggregation of polarized actin and other elements of the cytoskeleton at the site of bacterial attachment (McDaniel *et al.*, 1995; Nataro & Karper, 1998). In addition, an EPEC virulence factor outside the LEE has also been described (Levine, 1987; Trabulsi *et al.*, 2002), which is located on a plasmid encoding a type IV pilus called bundle forming pilus (*bfp*). Besides, on another plasmid there is a plasmid encoded regulator gene (*per*) which regulates several chromosomal and plasmid genes necessary for the pathogenesis of A/E lesions (Levine *et al.*, 1985) (Figure 1). Beside the virulence factors described above, there are other potential virulence factors such as EAST1 (enteroaggregative *E. coli* heat-stable enterotoxin 1), originally indentified in Enteroaggregative *E. coli* strains, but its significance in pathogenesis is uncertain (Nataro & Karper, 1998) .

Based on these properties, two variants of EPEC – typical and atypical EPEC – have been reported (Hien *et al.*, 2007; Nunes *et al.*, 2003; Trabulsi *et al.*, 2002) and their prevalence seems to be different between developing and developed countries. The typical EPEC is a cause of diarrhea in developing countries, while atypical EPEC seems to be associated with diarrhea in developed countries (Afset *et al.*, 2003; Afset *et al.*, 2004; Gomes *et al.*, 2004; Trabulsi *et al.*, 2002).

The most special feature of the epidemiology of disease due to EPEC infection is the remarkable age distribution. EPEC infection is primarily a disease of infants younger than 2 years of age (Nataro & Karper, 1998). EPEC infection, as with other DEC pathotypes, takes place via the faecal-oral route with contaminated food and water. The infectious dose among infants is not known, but is presumed to be much lower than 10^8 CFU/microorganism.

The clinical features of EPEC infection are characterized by watery diarrhea, vomiting and low-grade of fever. As compared to the developed countries, EPEC infection plays a more important role in developing countries, where it has been found as one of the foremost causes of diarrhea (Cravioto *et al.*, 1988; Cravioto *et al.*, 1990; Gomes *et al.*, 1991; Mayatepek *et al.*, 1993; Nguyen *et al.*, 2006). EPEC may also cause diarrhea under other circumstances, such as nosocomial outbreaks.

1.4.5 Enteroaggregative *E. coli* (EAEC)

EAEC was first described in 1985, recognized by its distinctive adherence to HEp-2 cells in an aggregative, “stacked brick-like” pattern (Nataro *et al.*, 1998; Pereira *et al.*, 2008). This adherence pattern, distinguishable from the adherence patterns manifested by EPEC and DAEC, was first significantly associated with diarrhea among Chilean children in 1987 (Nataro *et al.*, 1987). EAEC is defined as an *E. coli* pathotype that does not secrete heat-labile or heat-stable enterotoxins but adheres to HEp-2 cells and other epithelial cells in an aggregative adherence (A/A) pattern, the genes for which are encoded on 55–65 MDa plasmids (Nataro *et al.*, 1998).

Many epidemiological studies have used the A/A pattern and the plasmid-encoded probe “CVD432” or simply the A/A probe to identify EAEC (Baudry *et al.*, 1990; Germani *et al.*, 1998; Huppertz *et al.*, 1997; Okeke *et al.*, 2000; Okeke *et al.*, 2003). Moreover, a transcription activator known as “*AggR*”, the gene which regulates the A/A genes, has been described as the major EAEC virulence regulator (Nataro, 2005). Recently, some

epidemiological studies have suggested that CVD432-positive strains, which are predicted to carry the *AggR* regulators, are the true EAEC pathogens termed “typical EAEC” (Harrington *et al.*, 2006; Jenkins *et al.*, 2006a; Okeke *et al.*, 2003). However, A/A probe-negative isolates share virulence factors with A/A probe positive isolates, a finding which indicates that additional factors are involved in the A/A phenotype in these EAEC strains (Bouzari *et al.*, 2001) (Table 1, Figure 1).

The pathogenesis of EAEC is complex as the strains are relatively heterogeneous. However three major features of the EAEC infections have been suggested as basic strategy of EAEC: (i) abundant adherence to the intestinal mucosa (colonization), (ii) elaboration and secretion of enterotoxins and cytotoxins, and (iii) induction of mucosal inflammation (Harrington *et al.*, 2005; Nataro, 2005; Nataro *et al.*, 1998). Furthermore, several studies have reported the presence of fecal lactoferrin and proinflammatory cytokines, notably interleukin (IL)-8 (Greenberg *et al.*, 2002; Huang & Dupont, 2004; Jiang *et al.*, 2003; Kucharzik *et al.*, 2005; Steiner *et al.*, 1998), as well as the presence of a putative virulence factors, such as yersinia-bactin lectin (Basu *et al.*, 2004), in patients infected with EAEC.

Since its discovery, an increasing number of studies have associated EAEC with diarrhea in a variety of settings. These include endemic diarrhea of infants in both industrialized and developing countries (Elias *et al.*, 1999; Huang & Dupont, 2004; Huang *et al.*, 2006; Okeke & Nataro, 2001), persistent diarrhea among acquired immunodeficiency syndrome patients and traveler’s diarrhea (Jenkins *et al.*, 2006b; Knutton *et al.*, 2001; Rüttler *et al.*, 2002). The implication of EAEC in diarrheal outbreaks confirmed the fact that at least some strains exhibiting the A/A phenotype were true human pathogens, yet not in all studies were EAEC associated with diarrheal illness (Nataro *et al.*, 1995; Suzart *et al.*, 2001). Pathogenesis studies of EAEC experienced a step-forward in 1994, when the first EAEC volunteer study was published (Nataro *et al.*, 1995). In this report, it was showed that only one of four A/A probe positive EAEC strains elicited human diarrhea, confirming that not all EAEC strains were equally pathogenic.

Although not all EAEC strains have been associated to diarrheal disease, the most commonly reported symptoms associated with EAEC infection are watery diarrhea with or without blood and mucus, abdominal pain, nausea, vomiting, and low-grade fever (Adachi *et al.*, 2002). Besides, EAEC can cause both an acute and a chronic (>14 days) diarrheal illness in different parts of the world. A growing number of studies from both developing

and developed countries have supported the association of EAEC with persistent diarrhea in young children and adults (Araujo *et al.*, 2007; Bhan *et al.*, 1989; Fang *et al.*, 1995; Knutton *et al.*, 2001; Wilson *et al.*, 2001). It has also been reported that EAEC may be a leading cause of outbreaks of acute diarrheal disease affecting newborns and children (Cohen *et al.*, 2005; Itoh *et al.*, 1997; Knutton *et al.*, 2001; Koo *et al.*, 2008; Presterl *et al.*, 1999). EAEC have been reported as the second most common bacterial pathogen isolated in US adult travelers to developing countries and deployed US military personnel (Adachi *et al.*, 2001; Rüttler *et al.*, 2002).

In another study on adult US travelers to Mexico that evaluated the serologic response to the EAEC anti-aggregative protein dispersin, 48% of the travelers developed increases in antibody levels over time; the majority of patients, though, remained asymptomatic (Huang *et al.*, 2008). These findings may possibly suggest that EAEC infection shows a variety of clinical presentations and support the idea of a great heterogeneity of EAEC as a virulent pathogen (Nataro *et al.*, 1995; Suzart *et al.*, 2001).

1.4.6 Enterohemorrhagic *E. coli* (EHEC)

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 “Verocytotoxin-producing *E. coli*”) or STEC (“Shiga toxin-producing *E. coli*), formerly SLTEC (“Shiga-like toxin-producing *E. coli*”). EHEC colonize the intestinal mucosa inducing a characteristic attaching and effacing (A/E) lesion, which is also present in EPEC and EAEC. The classic intestinal histopathology characteristic of EHEC infection includes hemorrhage and edema in the lamina propria, which results in bloody diarrhea, hemorrhagic colitis, necrosis and intestinal perforation (Nataro & Karper, 1998). The major virulence factor, and a defining characteristic of EHEC, is the Shiga toxin (Stx), a potent cytotoxin that leads to cell death and will aggravate the symptoms in patients infected.

Most studies have reported EHEC strains as important pathogens causing diarrhea, with association to Hemorrhagic Colitis (HC) and Hemorrhagic Uremic Syndrome (HUS), which are due to the interaction of Shiga toxin (Stx) with endothelial cells (Brown *et al.*, 1989; Karmali, 2004). The term “enterohemorrhagic *E. coli*” (EHEC) was originally created to denote strains that cause HC and HUS, express Stx, cause A/E lesions on epithelial cells, and possess a ca. 60-MDa plasmid (Levine, 1987; Levine & Edelman, 1984). 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 pathogens (Levine, 1987; Levine & Edelman, 1984).

The Stx family contains two major, immunologically non-cross-reactive, groups called Stx1 and Stx2. A single EHEC strain may express Stx1 only, Stx2 only, or both toxins or even multiple forms of Stx2. Stx1 from EHEC is identical to Shiga toxin from *Shigella dysenteriae* type I. (Stx1 from some strains may differ from Stx in one residue) (Kaper *et al.*, 2004).

EHEC is an emerging pathogen that has stimulated worldwide interest in several large food-borne outbreaks. A wide variety of sources have been implicated in the EHEC transmission, including beef, unpasteurized milk, fruit juice, and contaminated drinking water (Kaper *et al.*, 2004).

EHEC belongs to different serotypes or serogroups, which are useful for diagnostic and epidemiological studies. The most notorious serogroups associated with EHEC is O157:H7, which has caused several large outbreaks of the disease, mainly in North America, Europe, and Japan (Ezawa *et al.*, 2004; Grimm *et al.*, 1995; Muto *et al.*, 2008; Ozeki *et al.*, 2003).

1.4.7 Enteroinvasive *E. coli* (EIEC)

Like most enteropathogens, these bacteria may also be important pathogens in developing countries where sanitation and hygiene levels have deteriorated. EIEC strains are biochemically, genetically, and pathogenically closely related to *Shigella* spp., but produce less severe diarrheal disease, compared to the dysentery caused by *Shigella dysenteriae* type 1 (Nataro & Karper, 1998). The precise pathogenetic scheme of EIEC has yet to be elucidated. However, pathogenesis studies of EIEC suggest that its pathogenic features are virtually those of *Shigella* spp. (Sansone, 1992). EIEC invades the colonic epithelial cell, thereby inducing an inflammation and mucosal ulceration, leading to the release of blood and mucus in the stool (Hart *et al.*, 1993), similar to bacillary dysentery.

Although EIEC is the prototype of invasive bacteria, that are unable to penetrate enterocytes via their luminal aspect. Instead, it passes through the 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 (Sansone, 1992). The ability of EIEC to penetrate, survive, and multiply within the colonic enterocytes is part of rearrangements of the cell

cytoskeleton, leading to disturbance and engulfment of the bacterium within a vacuole, a process which is encoded by a cluster of genes carried on a large plasmid (14-MDa). Afterwards, bacteria move through the cytoplasm and extend into adjacent epithelial cells without being exposed to the external surroundings. Infection results in a shigellosis-like syndrome in which patients exhibit abdominal pain, nausea, vomiting, fatigue, mucus, and bloody stools, but many cases show signs of watery diarrhea that is indistinguishable from that due to infection by other *E. coli* pathotypes (Nataro & Karper, 1998).

1.4.8 Diffusely adherent *E. coli* (DAEC)

DAEC is a category of DEC that produces the diffuse adherence on Hep-2 cells (Nataro *et al.*, 1998) similar to those EAEC strains. However, little is known about the pathogenesis of DAEC, but cells with a surface with fimbriae that mediate diffuse adherence have been cloned and characterized (Kaper *et al.*, 2004; Nataro *et al.*, 1998). Few epidemiological and clinical studies have been carried out to adequately describe the epidemiology and clinical aspect of diarrhea caused by DAEC. It was suggested that DAEC may cause disease in immunologically naïve or malnourished infants. Furthermore, DAEC has been associated to diarrhea in infants older than 1 year of age (Scaletsky *et al.*, 2002a; Scaletsky *et al.*, 2002b; Spano *et al.*, 2008).

2 AIMS OF THE PRESENT THESIS

The major aims considered in this thesis were:

- ◆ To determine the relative prevalence of five diarrheagenic *E. coli* (DEC) pathotypes in children younger than 5 years of age with and without diarrhea in León, Nicaragua
- ◆ To investigate the phenotypic diversity of the fecal *E. coli* flora in the society and in individual children
- ◆ To elucidate whether the DEC markers are confined to certain specific pathogenic clones, or whether they seem to be randomly distributed among various strains of *E. coli*
- ◆ To investigate the *E. coli* colonization patterns and strain persistence in a cohort of Nicaraguan infants aged 0 to 52 weeks
- ◆ To investigate the relation of DEC to diarrheal disease

3 MATERIALS AND METHODS

This section describes the general study design, samples collection, and the procedures that have been used for the laboratory work of this thesis. A more detailed description can be found in the *Materials & Methods* sections of the respective papers.

3.1 STUDY AREA

All clinical samples investigated in the present thesis came from children living in León, Nicaragua. The city of León is located in the northwest of the country, 90 km from the capital Managua. The city has an area of 820 km²; with an estimated population of 177,000 inhabitants. The climate is tropical with an average temperature of 25°C and two seasons a year, summer (December to May) and one rainy winter (June to November). Sanitary conditions are deficient in large parts of the city, mainly in the peri-urban and rural areas where poverty is prominent. Chlorinated tap water is available to less than 75% of the families and ~48% of the population has access to sewage system. The health system is organized on two levels: primary health-care, which is provided by health-care centers serving a population of about 30,000, and secondary-level care is provided mainly by the regional hospital (Peña *et al.*, 2008).

3.2 STUDY POPULATION

3.2.1 Epidemiological study (Papers I, II and III)

As a part of a passive-surveillance study conducted between March 2005 and September 2006 in León, Nicaragua, a total number of 526 stool samples (one from each subject) from 381 children with and 145 children without diarrhea, aged ≤60 months were collected. Five health-care centers, including the emergency pediatric ward of the main hospital, were involved in this study.

Clinical and epidemiological data from the children with diarrhea were recorded through questionnaires to evaluate the clinical symptoms.

Papers I, II, III: Children enrolled in the study were identified as diarrheal cases, characterized by the occurrence of three or more loose, liquid or watery stools, or at least one bloody, loose stool, in the preceding 24 h period (WHO, 2005). Control children were healthy children without a history of diarrhea and/or antibiotic treatment for at least 1

month previously to sampling, and who were attending a health-care program at health centers in the community. Severity of diarrhea was defined as: (i) mild, when the diarrhea episode lasted no longer than 3 days without fever, vomiting, and with a good tolerance to the oral rehydration therapy (ORT) at home; (ii) moderate, episode duration of more than 3 days with fever and/or vomiting, and with tolerance of ORT at a health care center; (iii) severe episode with fever and vomiting, requiring intravenous rehydration and hospitalization.

3.2.2 Cohort study (Paper IV)

During a one-year period – October 2006 through November 2007 – a longitudinal cohort infant study was performed in León, Nicaragua. The infants were recruited as newborns at the main hospital in León and afterwards monitored by home visits by a trained nurse.

All the infants included in this study (9 girls and 11 boys) were vaginally delivered. The infants' feeding patterns were recorded by interview of the mothers. Stool samples were collected by the mothers by spontaneous defecation of the children.

Predetermined sampling schedules were set for all children at 1, 2, 4, 12, 24, 36, and 52 weeks of age, irrespective of symptomatic sampling in connection with episodes of diarrheal disease. A total number of 160 regular stool samples (eight samples per infant) were available at the end of the study period. Besides, another 12 stool samples were collected in connection with diarrheal episodes.

3.2.3 Ethical Approval of the study (Papers I - IV)

Verbal and writing consent was obtained from the mothers or guardians of the children, and Ethics clearance was obtained from the Medical Bioethics Committee of the Faculty of Medical Sciences of the National Autonomous University of Nicaragua (UNAN) León, Nicaragua (registration no. 62).

3.3 LABORATORY PROCEDURES

3.3.1 Bacterial culture (Paper I, III, IV)

Stools samples collected in sterile screw-capped plastic containers without preservatives, were transported at 4°C to the Department of Microbiology at UNAN, León, Nicaragua, on the day of collection, where they were processed initially by standard culture and identification methods (Gillespie & Hawkey, 2006).

TABLE 2. Samples collected

Origin of samples	Number of collected samples (number of isolates typed)				
	Paper I	Paper II*	Paper III*	Paper IV	Total
Diarrheal children	381	381 (2,900)	205 (1,563)	-	381 (2,900)
Healthy children	145	145 (1,109)	77 (601)	-	145 (1,109)
Cohort study	-	-	-	160 (744)	160 (744)
Total	526	526 (4,009)	282 (2,164)	160 (744)	686 (4,753)

* Part of same samples and isolates as in earlier paper

Stools samples were plated on MacConkey agar (Oxoid Ltd, USA) for the selection of *E. coli* strains (Papers I-IV) and other media, such as deoxycholate citrate agar (Sigma-Aldrich, USA) for *Shigella* and *Salmonella* (Paper I), and the plates were incubated aerobically at 37°C overnight. From each plate showing suspected growth of *E. coli*, a full loop of *E. coli*-like colonies was stored at -70°C in Brain Heart Infusion broth (BD Diagnostics, Md, USA) containing 15% (v/v) glycerol, until they were transported to the Karolinska Institutet, Stockholm, Sweden for further studies. The presence of parasites (*Giardia lamblia*, *Entamoeba histolytica*, *Cryptosporidium*, and *Trichuris trichura*), and of Rotavirus and Norovirus was also investigated. However, in the present thesis we discussed and focused on the *E. coli* isolated. Figure 3 shows a scheme of the laboratory procedures employed for the characterization of *E. coli*.

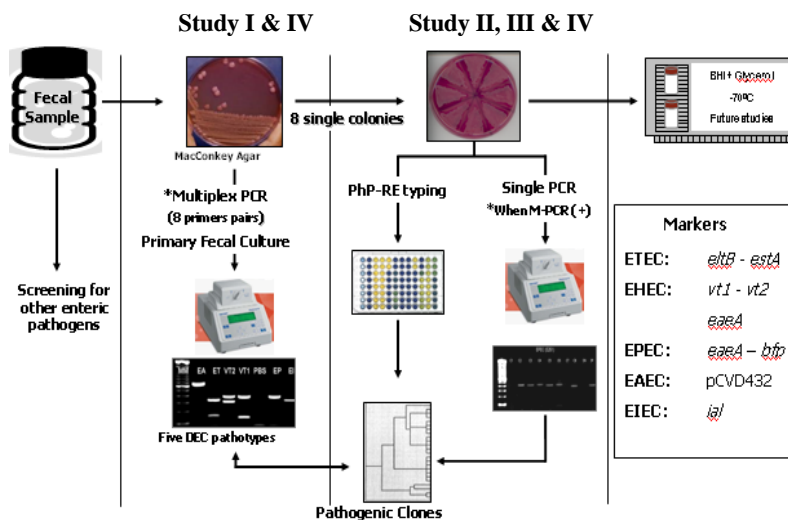


FIGURE 3. Flowchart for investigating *Escherichia coli* (isolates) for DEC pathotype markers and PhP-types.

3.3.2 Reference Strains (Papers I to IV)

Bacterial reference strains used for PCR assays of DEC (Papers I, III, IV), and Biochemical fingerprinting (PhP typing) (Papers II, III, IV) are listed in Table 3.

TABLE 3. Reference *E. coli* strains used in this thesis

Category	Reference Strain	Target marker(s)	Study
ETEC	ATCC 35401	<i>eltB</i> ⁺ , <i>estA</i> ⁺	I, II, III & IV
	NCTC 11601	<i>eltB</i> ⁺ , <i>estA</i> ⁻	II, III & IV
	NCTC 11063	<i>eltB</i> ⁻ , <i>estA</i> ⁺	II, III & IV
EPEC	ATCC 43887	<i>eaeA</i> ⁺ , <i>bfpA</i> ⁺	I, II, III & IV
EHEC	ATCC 43890	<i>vt1</i> ⁺ , <i>eaeA</i> ⁺	I, II, III & IV
	ATCC 43889	<i>vt2</i> ⁺ , <i>eaeA</i> ⁺	I, II, III & IV
EIEC	ATCC 43893	<i>ial</i>	I, II, III & IV
EAEC	97R*	pCVD432	I, II, III & IV
EC-negative	ATCC 11775	No virulence marker	I, II, III & IV

* The identity of this strain was verified by other methods, and was provided by a previous collaborative study between Nicaragua and Sweden.

3.3.3 Screening for DEC by PCR assays (Papers I, III, IV)

Papers I & IV: A one-step multiplex PCR assay using eight different specific primers as described (Paper I, Table 1) was performed to determine the prevalence of five different DEC pathotypes in the *E. coli* flora. The criteria for determining the different types of DEC by PCR were as follows: the presence of *eltB* and/or *estA* markers for ETEC; the presence of *vt1* and/or *vt2* for EHEC (the presence also of *eaeA* confirmed the presence of a typical EHEC); the presence of *bfpA* and *eaeA* for typical EPEC, (only *eaeA* for atypical EPEC); the presence of *ial* for EIEC/Shigella; and the presence of pCVD432 for EAEC (see also Figure 3).

A smear of each bacterial culture (the primary streak of *E. coli*-positive samples on the agar plates) was suspended in 1 ml PBS (phosphate buffered saline) to a density of four MacFarland standard (1×10^9 – 5×10^9 bacteria/ml), boiled for 20 minutes, followed by centrifugation at 2,500 g for 10 minutes to pellet the cell debris. Two μ l of DNA template was amplified in a final volume of 25 μ l reaction mixture using the pureTaq Ready-to-Go PCR Bead (GE Healthcare UK) and containing 200 μ M dNTPs, 10 mM Tris/HCl (pH 9), 50 mM KCl, 1.5 mM MgCl₂, 2.5 U pureTaq DNA polymerase, and 0.2 μ M of each primer mix (INTERACTIVA Biotechnologies GmbH, Germany). The PCR reactions were performed in a GeneAmp PCR system 9700 (Applied Biosystem) as follows: 1 cycle at

96°C for 4 min; 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min; ending with a 7 min extension at 72°C. The amplified DNA products (10 µl) were then electrophoresed on 1.5% agarose gel (UltraPure Agarose, Invitrogen Life Technologies) at 120 mV for 40 minutes and visualized under UV light after staining with ethidium bromide. Products were sized against a 100 bp DNA ladder (Invitrogen Life Technologies) used as a molecular weight marker. If the pooled DNA template result was negative following gel electrophoresis, the sample was considered as negative for DEC. If the bands were seen after gel electrophoresis, the band sizes on the gel were compared with the size marker bands in order to identify the suspected DEC in the stool samples. The residual cultured material from each PCR-positive sample was stored at -70°C for further characterizations.

Paper III: In this study, a total number of 282 samples positive for any of the DEC markers (Paper I) were further tested to confirm the presence of those DEC markers in eight randomly selected colonies per sample. All selected *E. coli* colonies (n=2,164) from the 282 samples were subcultured on fresh MacConkey agar plates and incubated at 37°C overnight. Afterward, each single colony was tested independently by a single-colony PCR assay with a primer pair specific for the DEC marker found in the multiplex PCR (Paper I) (Table 3), following the same procedures as described above. Minimum criteria for determination of DEC in the single-colony PCR were as defined above.

3.3.4 Biochemical Typing (Papers II, III, IV)

A total number of 4,009 (**Papers II & III**) and 744 (**Paper IV**) *E. coli* colonies were subjected to biochemical fingerprinting using the PhP-RE rapid screening microplates of the *PhenePlate* system (PhPlate-<http://www.phplate.se>) (PhP typing). This is a semi-automated and highly discriminatory typing system, based on measurements of the kinetics of a set of eleven biochemical reactions executed in 96-well microplates (Figure 4) (Möllby *et al.*, 1993).

In order to obtain a representative collection of *E. coli* isolates from each sample, eight *E. coli* colonies per sample were selected for PhP typing. Each PhP microplate was inoculated with the eight isolated colonies into the first column (one of each row) containing a growth medium (bromothymol blue as pH indicator) only; thereafter the homogenized *E. coli* bacterial suspensions were transferred to the other wells containing eight parallel sets of eleven different dehydrated reagents (Figure 4). The PhP microplate were incubated at 37°C, and were read after 16, 40 and 64 h incubation, using a desktop scanner with a transparency

adapter (HP Scanjet 7400c XPA scanner). The plate images generated by the scanner were then converted to numerical absorbance data using the PhP-WIN software, resulting in a set of eleven mean values of the reactions for each tested isolated (biochemical fingerprints; Figure 4).

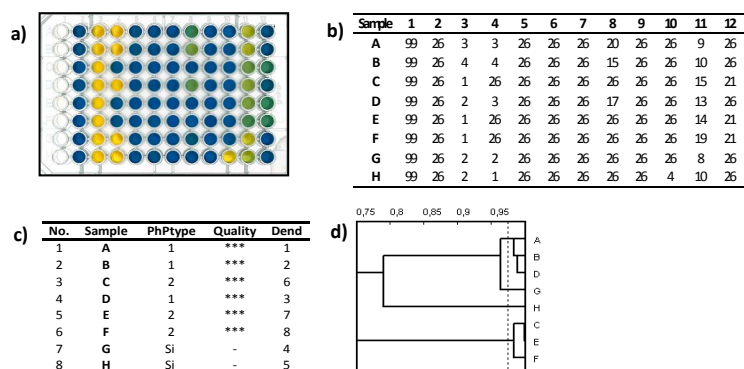


FIGURE 4. PhP typing; from plate image to analysis. **a)** Reactions of eight isolates on eleven reagents in PhP-RE plate; **b)** Quantification of the reactions (1-12) leading to biochemical fingerprints (A-H). Content of wells 1-12: Inoculation's well, Cellobiose, Lactose, Rhamnose, Deoxyribose, Sucrose, Sorbose, Tagatose, D-Arabitol, Raffinose, Gal-Lacton, Ornithine; **c)** List of PhP types obtained; **d)** Dendrogram of samples A-H.

Data generated by PhP typing were used to calculate pairwise similarities between the isolates. The similarities, expressed as correlation coefficients, were clustered using the unweighted pair-group method with arithmetic average (UPGMA) (Sneath & Sokal, 1973) and illustrated in dendrograms (Figure 4). The similarities between different samples (i.e. *E. coli* populations) were calculated as population similarity (Sp) coefficients (Kühn *et al.*, 1991), which measures the proportion of isolates that belong to the same type in the two populations that are compared with each other (Paper IV). The phenotypic diversities for *E. coli* in different populations, e.g., individual samples, DEC pathotypes, all isolates, etc., were calculated using Simpson's index of diversity (Di) (Hunter & Gaston, 1988). Di is a relative measure of the distribution of isolates into different types. A high value (maximum 1) indicates an evenly distribution of the isolates into many different types, whereas a low value (minimum 0) indicates a few dominating types in the population.

3.4 ANALYSIS OF DATA

3.4.1 Analysis of the PhP-Data (Papers II, III, IV)

All data handling, including conversion of plate images to numerical data, calculation of similarities between *E. coli* isolates, diversity within samples and phenotypic similarities between populations, as well as construction of dendrograms were done with the PhPWIN-Software version 6 (PhPlate - <http://www.phplate.se>).

3.4.2 Statistical Analysis (Papers I, II, III, IV)

A non-parametric Mann–Whitney U-test was used for pairwise comparisons, and Fisher’s two-tail exact test, a χ^2 test and Kruskal–Wallis test, were used for contingency analyses, where applicable.

Statistical analyses for Paper I and II were performed using the SPSS Software version 17.

Statistical analyses for Paper III and IV were performed using the GraphPadPrim Software.

4 RESULTS AND DISCUSSION

4.1 OCCURRENCE AND PHENOTYPIC DIVERSITY OF DEC TYPES

The first study included in this thesis aimed at the identification and determination of the relative prevalence of virulence markers of five different diarrheagenic *E. coli* (DEC) types in the fecal flora by using a multiplex-PCR. A total of 282 DEC positive samples were detected from 381 stool samples from children with diarrhea and from 145 without diarrhea. Figure 5 shows one of the results of multiplex PCR for DEC on reference strains and stool samples from children with diarrhea.

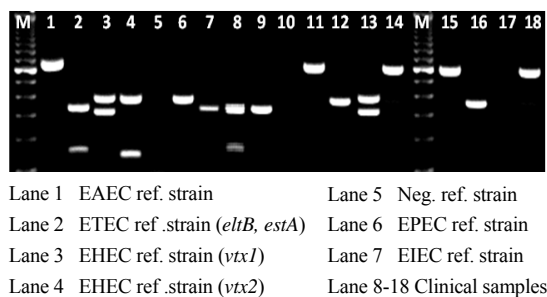


FIGURE 5. Multiplex PCR results for DEC types (see Table 2)

It was found that as many as 54% (n=205) of the diarrheal group and 53% (n=77) of the non-diarrheal group were positive for at least one DEC marker. Besides, several samples from diarrheal (12.6%) and from non-diarrheal group (10%) harbored samples positive for more than one DEC marker. Thus, overall, 348 DEC types were isolated from the 526 samples; 256 (67.2%) were from children with diarrhea and 92 (63.4%) in children without diarrhea (Paper I, Table 2).

Enteroaggregative *E. coli* (EAEC) was the most prevalent DEC type identified, but no significant difference was observed between diarrheal and non-diarrheal group (Paper I, Table 2). In addition, *E. coli* isolates from EAEC-positive samples showed as high a phenotypic diversity as isolates from the non-DEC *E. coli* samples (Paper II, Table 3). These findings reflect the heterogeneous nature of this group of DEC as reported previously (Czeczulin *et al.*, 1999; Jenkins *et al.*, 2007; Nataro *et al.*, 1995; Okeke & Nataro, 2001; Suzart *et al.*, 2001) and is in accordance with the theory that only a subset of EAEC strains are capable to cause disease in humans (Nataro *et al.*, 1995; Wilson *et al.*, 2001).

Such EAEC strains did not seem to be common in this study. The lack of association with diarrhea could also be due to a high level of asymptomatic carriers, with (or without) previous episodes of enteritis.

However, the high prevalence of CVD432 positive EAEC strains in this study, as well as in earlier reports, could indicate that such bacteria are endemic in many parts of the world and that its appearance in the intestinal fecal flora only represents a repeated exposure with this pathogen without any clinical relevance. Our results are in contrast to other investigators who have found a causative role of EAEC with diarrhea (Jenkins *et al.*, 2007; Levine & Edelman, 1984; Nguyen *et al.*, 2005; Regua-Mangia *et al.*, 2009). However, most studies have reported similar results as our findings, where EAEC was isolated at higher frequency rates from controls than from children with diarrhea (Spano *et al.*, 2008; Vernacchio *et al.*, 2006).

Enteropathogenic *E. coli* (EPEC) has been reported as an important causative agent of diarrhea in infants younger than two years of age in many countries (Levine & Edelman, 1984; Nataro & Kaper, 1998; Nguyen *et al.*, 2005). There are two subtypes of EPEC: typical EPEC (*bfp*⁺ & *eaeA*⁺), as a cause of diarrhea in developing countries, and atypical EPEC (*bfp*⁻ & *eaeA*⁺) that seems to be more associated with diarrhea in developed countries (Afset *et al.*, 2003; Afset *et al.*, 2004; Kaper *et al.*, 2004; Trabulsi *et al.*, 2002). Controversially, in our study, we could only identify atypical EPEC. However, the identification rate of atypical EPEC was slightly higher in children without diarrhea than in children with diarrhea (Paper I, Table 2). This finding was obtained in another study, where EPEC was recovered more frequently among non-diarrheal than from diarrheal cases (Echeverria *et al.*, 1989). Atypical EPEC comprise a complex group among DEC types, displaying a diversity of serotypes and genetic virulence markers, which makes it difficult to determine which strain is truly pathogenic (Hernandes *et al.*, 2009; Trabulsi *et al.*, 2002). As for other DEC types, a higher prevalence among diarrheal samples together with a low diversity is a good indication on the prevalence of virulent clonal groups that are circulating in the community. However, the diversity among *E. coli* isolates of EPEC-positive samples from children with diarrhea was similar to those from children without diarrhea, and only slightly lower than among normal *E. coli* flora (non-DEC samples) (Paper II, Table 3). Thus, the role of atypical EPEC in diarrhea in this study could not be fully established, and these EPEC strains do not seem to belong to virulent clones that have spread in the population. Moreover, compared with a previous study carried out in a northern city of Nicaragua, our study highlighted the variation in prevalence

of this DEC pathotype as a causative agent of diarrhea within the same country (Hien *et al.*, 2008; Hien *et al.*, 2007; Mayatepek *et al.*, 1993).

Enterotoxigenic *E. coli* (ETEC) was isolated significantly more often in children with than without diarrhea (20.5% vs. 8.3%, $P < 0.001$) (Paper I, Table 2). ETEC was detected in all age groups, but an even more striking difference ($P < 0.0001$) in frequencies between children with (22.9%) and without (5.7%) diarrhea was observed in children under twelve months of age (Paper I, Table 3). This result supports data from a previous study carried out in a cohort of infants with ETEC infection in the same area, where the highest incidence of ETEC diarrheal illness was observed during the first year of age (Paniagua *et al.*, 1997). ETEC isolates carrying *eltB* showed somewhat decreased diversities, and were detected in both diarrheal and non-diarrheal groups, whereas the much lower diversities among samples positive for ETEC carrying *estA* only clearly indicated that a limited number of clones were carriers of these virulence markers, and were correlated to diarrhea (Paper II, Table 3). Besides, *estA* carrying isolates were never detected in healthy children. Thus, this phenotype represents stable pathogenic clonal groups that spread among the infant population.

Enterohemorrhagic (EHEC) and Enteroinvasive (EIEC) *E. coli* were identified at low frequencies. A finding, in general, in agreement with other studies in developing countries (Gomes *et al.*, 1991; Hien *et al.*, 2008; Hien *et al.*, 2007; Nguyen *et al.*, 2005). However, EHEC was detected in eight samples from diarrheal children but in no healthy child, and isolates from those samples showed lower diversities (Paper II, Table 3), suggesting the presence of clonal groups. Albeit, these two phenotypes could not be considered an important cause of diarrhea in the studied population.

4.2 CLINICAL FEATURES AND PHENOTYPIC DIVERSITY

In general, clinical symptoms among children suffering from diarrhea were similar regardless of the isolated DEC pathotype (Paper I, Table 5). Furthermore, when any of the individual DEC-type positive children were analyzed, the severity of the diarrheal cases ranged from mild to severe dehydration. Moreover, the diversity in this group was slightly lower than in the non-diarrheal group (Paper II, Tables 1, 2). EAEC was the most frequent pathotype (32.4%) associated with severe dehydration, followed by ETEC (27.9%), and EPEC (20.6%) (Paper I). Additionally, a low total diversity ($D_i=0.937$), as well as intra-diversity (intra- $D_i=0.410\pm 0.334$) of the *E. coli* isolates from this group of children was observed (Paper II, Table 2), whereas children with mild/moderate dehydration (receiving oral rehydration)

showed similar Di as children without diarrhea (Paper II, Table 2). These findings suggest that virulent *E. coli* clones could have colonized children subjected to hospitalization care and had caused diarrhea with a high degree of dehydration.

TABLE 4. Occurrence of samples presenting at least one DEC positive isolate among eight tested isolates and the proportion of DEC positive isolates according to single colony PCR assay. Data were derived from *E. coli* in 282 fecal cultures that were positive on the primary PCR screening

	No. of positive/No. tested (% positive)		
	Diarrheal [†]	Controls [†]	Total
Samples	168/205 (82%)	48/77 (62%)	216/282 (77%)
Isolates	632/1563 (40%)	196/601 (33%)	828/2164 (38%)

[†]Fisher's exact test was used for calculation, P-value < 0.05

Hence, to elucidate whether DEC markers were confined to certain pathogenic clones that could be related to diarrhea, 2,164 *E. coli* isolates from the originally DEC-positive samples were subjected to further analysis for virulence markers, using a single-colony PCR assay for the same DEC markers (Paper III, Table 1). Overall, 828 (38%) DEC-positive isolates were identified in these samples (Table 4). Only in a few cases all eight isolates analyzed from a DEC positive stool sample were DEC positive. For EAEC, a high proportion of positive isolates in a sample seemed as common in non-diarrheal samples as in diarrheal samples (Figure 6a), whereas for ETEC and EPEC, only samples from diarrhea were dominated by DEC positive isolates (Figure 6b, 6c). Furthermore, among ETEC- and EPEC-positive control samples, more than half of the samples presented no DEC positive colony at all (Figure 6b, 6c). It thus seems that at least for ETEC and EPEC a high percentage of DEC positive isolates in the intestinal flora is more correlated to diarrhea than only presence of DEC *per se* as detected in the primary streak. These findings might also be interpreted that a low concentration of DEC in the fecal flora may be found in early stages of a diarrheal episode (Iijima *et al.*, 2007). Thus, we did not find any correlation of the proportion of DEC-positive isolates to diarrhea for EAEC, but for ETEC isolates carrying *eltB* and *estA* markers either alone or in combination and somewhat for EPEC.

The multiplex streak-PCR has previously been shown to be a very sensitive method that can detect low amounts of DEC in a fecal sample (Iijima *et al.*, 2007; Nguyen *et al.*, 2005), and it is valuable for the purpose of excluding DEC negative *E. coli* samples (Müller *et al.*, 2007;

Nguyen *et al.*, 2005; Schierack *et al.*, 2006). In fact, 23% of the samples positive in the primary streak analysis were missed upon analyzing 8 single colonies per sample (Paper III). However, this indicates very low mean numbers of positive bacterial *E. coli* cells compared to the negative cells in the fecal sample. Such samples were also more common in controls than in diarrheal samples. Thus, it could be argued that a finding of DEC markers by PCR on the primary streak only probably has a high risk of being false positive.

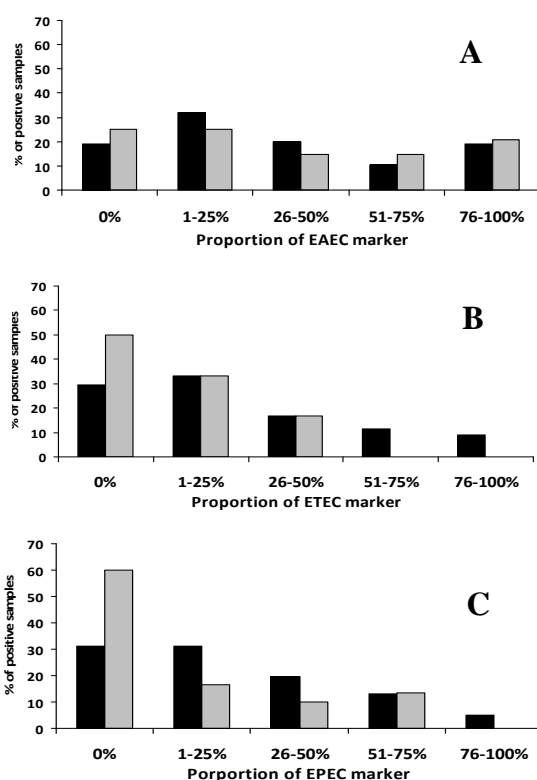


FIGURE 6. Proportion of DEC positive isolates among eight tested *E. coli* isolates from samples that had been DEC positive on multiplex PCR analysis of the fecal *E. coli* culture.

Black bars, diarrheal samples;

Grey bars, control samples.

4.3 COMMON PHENOTYPES

Cluster analysis of standardized biochemical fingerprinting data from all 4,009 isolates revealed 24 common biochemical phenotypes (BPTs) comprising 70% of the isolates, and 234 less frequent BPTs, including those only detected once. Most common BPTs seemed to be distributed equally among the diarrheal and non-diarrheal children. However, some BPTs (e.g. 01, 03, 09, 18, 21 and 23) were significantly more common among children with diarrhea than in children without diarrhea, whereas certain BPTs (e.g. 04 and 22) were more

common among the control children (Paper II, Figure 2). The most common type found in the present study was BPT 06, comprising almost 387 isolates (9.7 %).

Further comparisons of the distribution of DEC-positive isolates among these 24 BPTs were performed (Paper III, Table 2). Some BPTs were equally common among DEC positive and DEC negative isolates, e.g. 05, 06; whereas other BPTs were predominant among certain DEC groups, e.g. 01, 03 09, and 23 (ETEC, EPEC), 02, 04 and 22 (EAEC), 08 and 13 (EAEC, EPEC), 18 and 21 (ETEC).

The diversity indices (Di) among *E. coli* isolates from different DEC subpopulations were calculated separately. In general, Di values among DEC positive isolates were rather high, but slightly lower than among non-DEC isolates. Exceptions were ETEC-positive isolates carrying *estA* and EHEC positive isolates carrying *vt2*, which both showed low Di values, indicating a dominance of clonal groups among these DEC types (Paper II, Table 3).

4.4 DOMINATING PHENOTYPES

A more detailed PCR analysis on single isolates from DEC-positive samples (Paper III) was useful in order to elucidate whether isolates carrying virulence markers are confined to specific pathogenic clones associated with diarrhea.

Thus, our combined data from single-colony PCR and PhP typing indicated that diarrhea was caused by certain DEC clones that dominated the fecal flora (Schierack *et al.*, 2006). This could be anticipated from the higher proportion of DEC types, such as ETEC and EPEC (33% and 25%, respectively) in diarrheal than in control samples. Data from these combined analyses were used to define five different PCR/PhP patterns (A, B, C, D and E; Paper III, Figure 2) for EAEC, ETEC and EPEC positive samples (for EHEC and EIEC the number of positive samples was too small to merit an analysis of such patterns). The patterns were defined according to the phenotype distribution and according to the presence or absence of DEC markers among individual phenotypes obtained from the eight isolates tested (Paper III, Figure 2). In Pattern A all or almost all DEC positive isolates belonged to a single dominating phenotype and this pattern was found only in children with diarrhea for ETEC positive isolates, a finding which indicated a dominating pathogenic clone. The PhP data for those isolates correlated with the PhP data for ETEC reference strains ATCC35401 belonging to serogroup O78:H11 and NCTC11603 belonging to serogroup O159:H34, which were mentioned more than 30 years ago (Paper III, Figure 3).

These findings suggest that certain stable clones of DEC can circulate in a population, which can colonize the intestine and dominate the *E. coli* flora, thereby causing diarrhea. However, the virulence genes of these pathogenic clones may spread easily to the *E. coli* bacteria of the normal flora or environmental flora, and be detected by sensitive PCR tests without having a pathogenic role (Müller *et al.*, 2007; Schierack *et al.*, 2006).

It should also be mentioned that a similar trend was seen for EPEC with pattern B, but for EAEC the situation was almost the reverse with dominating patterns more common in control samples. The latter finding again points at the low relevance of the sole detection of EAEC pathotype marker pCVD432 for diagnostic purposes.

4.5 E. COLI COLONIZATION IN HEALTHY INFANTS

In the cohort study of twenty newborn infants carried out in León, Nicaragua (Paper IV), it was observed that *E. coli* was the most common enterobacterial species that colonized during the first two weeks of life (65%), and thereafter other *Enterobacteriaceae* were more frequently found (Paper IV, Table 1).

A pattern of successive changes of *E. coli* PhP-types was observed in all infants. PhP-types that dominated the flora in one sample were often completely replaced by another type on the next sampling occasion. Based upon the distribution of PhP-types in each sample, the individual samples from each infant were compared and the similarities between the *E. coli* populations were calculated as population similarity (S_p) coefficients. Among the eight samples investigated per infant, an average of 9.4 different PhP-types (range 5 to 25) were found during the first 12 months of life of the infants (Paper IV, Table 1). Normally, the fecal samples were dominated by one or two *E. coli* PhP-types and some of these were found again in later samples. For residential strains the time of residence varied between 2.5-51 weeks (median value 29.5 weeks) (Paper IV, Figure 1). A similar finding was reported in previous studies on infants from developing countries (Adlerberth *et al.*, 1998a; Matta & Urrutia, 1971), but the proportion here was higher than that reported from developed countries (Kühn, 1986; Nowrouzian *et al.*, 2003). Since the colonization of infants could be influenced by their environmental conditions, including the feeding habits (Adlerberth *et al.*, 1998a; Magne *et al.*, 2006), this suggests that infants in developing countries are exposed to a greater variety of external bacterial floras.

Furthermore, the total diversity (i.e. the diversity among all studied isolates) and the intra-diversity (i.e. the diversity among *E. coli* in an individual sample) indices were calculated for different age groups and compared to values obtained from a similar cohort study performed on Swedish infants using the same methods in year 2000 (Kühn *et al*, manuscript in preparation). It was found that in Nicaraguan infants, the total diversity seemed not to change with age (Paper IV, Table 2), and in overall, it was similar to that of Swedish infants, i.e. 0.955. However, among infants from both countries, the average intra-diversity of the *E. coli* flora increased with age (Paper IV, Figure 3), and for all age groups the intra-diversity was higher among Nicaraguan than among Swedish infants. Totally, the intra-diversity was significantly higher among samples from Nicaraguan infants ($P=0.008$).

The 24 different PhP-types that were particularly common among 4,009 *E. coli* isolates from 526 Nicaraguan infants with and without diarrhea (Paper II), could also be identified to a part in the present cohort study (Figure 7). It was observed that the same PhP-type (BPT 06), is the most prevalent in both studies. In contrast, 01, 13 and 17 are overrepresented in the cohort material. This finding suggests that these phenotypes are prevalent and very endemic in this part of Nicaragua, and could represent stable clones that circulate in the infant population.

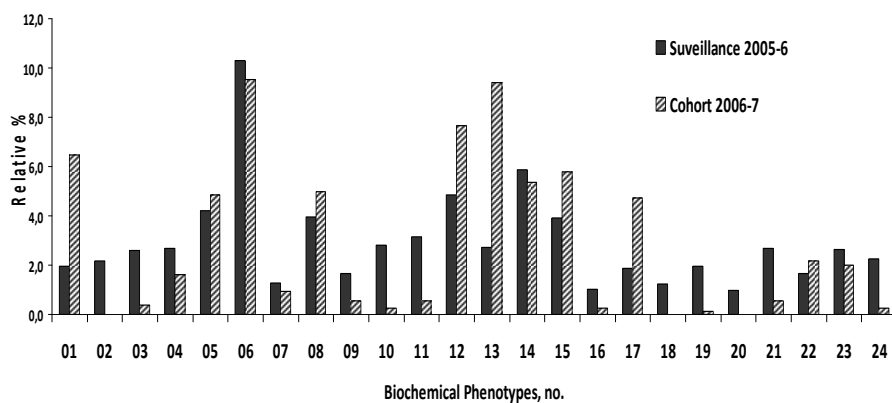


FIGURE 7. Relative distribution of PhP-types among *E. coli* isolates from Nicaraguan infants aged 0 to 12 months. Surveillance year 2005-6 (Paper II) consisted of 2,131 isolates (up to eight colonies from one sample) from each of 280 infants with/without diarrhea. The cohort samples (2006-7) resulted in 744 isolates (eight colonies from each of 93 samples) from 20 infants. Besides the 24 phenotypes depicted in the figure, about 20-35% of the isolates belonged to “other” PhP types.

In contrast to the results of paper I only 18% of the samples from the present cohort study were DEC positive (Paper IV, Table 1). This finding could be related to the fact that very few samples from the first three months of age of the infants were DEC positive (only 4%),

whereas at higher ages this figure rose to 42%. This supports the assumption that Nicaraguan infants are colonized with DEC pathotypes to an increasing extent during the first year of life, starting already during the first months. Furthermore, it is quite clear by the results that a positive finding of a DEC by PCR does not have to be related to diarrheal disease (Paper IV, Table 1). In total, a relatively low number of diarrheal episodes were reported for this cohort, as compared to previous studies (Paniagua *et al.*, 1997; Shaheen *et al.*, 2009; Shaheen *et al.*, 2004). The reason for this is unknown, but might be due to underreporting or to the social status of the cohort, but it is also well known that the frequencies of diarrheal episodes vary with time in Nicaragua (MINSAs-Nicaragua, 2004; 2008).

4.6 E. COLI PHENOTYPES IN THE ENVIRONMENT (UNPUBLISHED DATA)

A total of 1,145 *E. coli* isolated colonies from wells and sewage water were subjected to PhP typing. The PhP patterns of those isolates were compared with the 24 common BPTs earlier identified in the children (Paper II). It was evident that the same phenotypes were prevalent in both infants, wells and sewage (Figure 8), although BPT 06 was clearly overrepresented in the wells. However, in none of the isolates from environmental water DEC pathotype markers were identified. This preliminary finding indicates that the same phenotypes circulate in clean and contaminated water as in both healthy and diarrheal children. However, the “truly pathogenic clones” discussed in section 4.4 may thus predominantly be found in the intestines, alternatively at a very low frequency in the environment, and upon colonization of the child they disseminate the virulence genes to the normal *E. coli* flora strains.

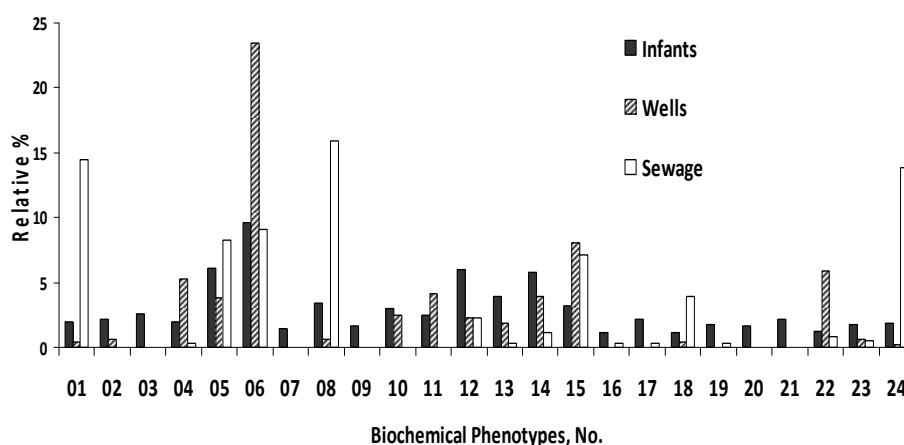


FIGURE 8. Relative distribution of PhP types in wells and sewage water compared to those of children investigated in Paper II. Besides the 24 phenotypes depicted in the figure, about 20-35% of the isolates belonged to “other” PhP types.

5 CONCLUSIONS

In this thesis we have used a combination of a phenotyping method (PhP typing) for the identification of clonal groups, and a genotyping method (PCR typing on whole fecal *E. coli* flora and on eight individual isolates per sample) for the identification of virulent strains in order to obtain insight into diarrheal disease morbidity and epidemiology of diarrheagenic *E. coli* in an understudied area of the world.

Our main findings were:

- ◆ DEC positive samples were found at high rates in infants aged 3 - 60 months (paper I), but were rare in infants below 3 months of age (Paper IV). Thus, colonization by DEC seems to start early in life and increase with age

- ◆ The phenotypic diversities among all isolates from diarrheal cases and from controls were equal and high, thus giving indication that no large outbreak of DEC diarrhea occurred during the time period studied (Paper II)

- ◆ EAEC and EPEC could not be correlated to diarrheal disease, whereas ETEC and EHEC were significantly more common in diarrheal cases (Papers I, II, and III).

- ◆ ETEC *estA* and EHEC were only found in diarrheal cases, and isolates positive for these DEC types belonged to a few PhP types that could represent pathogenic clones (Paper III)

- ◆ DEC positive isolates seldom dominated the fecal *E. coli* flora, but for all DEC types, except for EAEC, a high proportion of DEC positive isolates in the fecal *E. coli* flora seemed to be correlated to diarrhea. Thus, a low proportion of DEC in the fecal *E. coli* flora may not be of significance for development of diarrhea, but may represent occasional carriage of virulence gene(s) by normal flora strains (Paper III).

- ◆ Newborns were found to rapidly exchange their *E. coli* strains during their first year of life. Colonization by single DEC positive isolates was rarely correlated to diarrheal disease (Paper IV).

Our findings suggest that there are certain stable types or clones of diarrheagenic *E. coli* that can circulate in a population, and have the possibility to colonize the intestine and to dominate the *E. coli* flora, thereby causing diarrhea. These are the true DEC. However, the virulence genes are promiscuous and may spread to the *E. coli* bacteria of the normal flora, whereupon these scarce gene markers are detected by the sensitive PCR test in samples from both healthy and diseased individuals, without necessarily having a pathogenic role.

Thus, the obtained information on distribution of DEC pathotypes and their diversities may be used to optimize the use of available diagnostic tools, which in turn will affect treatment approaches and vaccine development strategies.

6 ACKNOWLEDGEMENTS

A lot of people supported me during my PhD studies and made my life in Stockholm an unforgettable time. Now, it is time to finish this task and what a best way to acknowledge to all these people. I would like to express my deep gratitude to all who contributed directly or indirectly in making this work. In particular, I would like to thank:

Professor Roland Möllby, my main supervisor, for giving me the opportunity to visit his laboratory aimed at developing skills in the world of research and science. Thanks for giving me the privilege to be your student during this time in KI, Sweden and for share your knowledge with me through many interesting and fruitful discussions into my manuscripts. For your excellent scientific guidance, and support during all these years. I wish you and your family all the best of this world. *“Tack så mycket för all!”*

Associate professor Inger Kühn, my co-supervisor, I am very grateful with you for all great ideas and for your scientific input within the manuscripts. Thanks for your never ending enthusiasm and all support during my project. You have been such an inspiration to me, which I have learnt a lot working with you. I am also grateful with you for introducing me into your nicer and very practical system “the *PhP-system*”, which it was a very important tool to characterize my “thousands” *E. coli* isolates. Thanks for sharing me very good and great moments during our lunchtime. *“Jag är innerligt Tacksam!”*

Professor Andrej Weintraub, for being my mentor and for your important contribution in the design and positive input in all concerning to our projects. Thanks for your sincere friendship and for your support during these years. Thanks to **Nora**, your wife, for all delicious meals that she prepared during I visited your home. *“Tack så mycket”*

Professor Hans Hallander, former coordinator of the Swedish-Nicaraguan collaboration. Thanks for your positivity feedback in planning the studies design for the grant applications at the beginning. **Professor Lennart Svensson**, from Linköping University, for invited me to participate at the gastroenteritis meetings and for contributing in the positive feedback in our project. **Professor Tore Midtvedt**, **Ass.Professor Elisabeth –Lisa– Norin**, **Professor Jan-Ingmar Flock**, **Margareta Flock**, and **Anna Karin Pettersson**, for your friendship and very good moments in our lunch and coffee times.

Actual and former members from the Möllby's group: **Keun-Young Kwak (Dae Ho)**, my lunch-mate, thank you for your sincere friendship and for being so nice person. Sincerely my best "foreign" friend; **Sara Ashá, Lena Guldevall, Mats (Dr. Pling), Jenny Gabrielson, Aina Iversen**, and **Mokhlarsur Rahman (Muhki)**. All of you guys for making the group a nice place to come in, and for providing an excellent work environment. **Erika Lindbom**, the newly joined member of the group, thanks for the labelling of those "hundreds" eppendorf tubes containing the *E. coli* isolates. **Maj Ringman**, thanks for your sincere friendship and for your kindly care with me about the Swedish life during my first visit in Stockholm.

Patricia Colque-Navarro (Dra. Colque), for being my friend, colleague, and for always encouraging me through all the ups and downs during the last four years I have been here in Stockholm, and for all your care and love to all us, Nicaraguan students in Sweden. Also, I would like to thank your husband **Jorge "chochi" Montii**, for his very kindly friendship and for his very good dinners to me and my Nicaraguan friends.

Esperanza Gutierrez – Señora Hope – for being a special person and for giving me your hospitality, and for allowing me to feel your home like my own. Thanks to **Ivette**, your daughter, for her kindly friendship.

In Nicaragua, I would like to express my gratitude to:

MSc. Margarita Paniagua, former coordinator of the "Diarrhea Disease Project", with her I learnt the early methods of bacterial culturing and who invited me to start in the young researcher program, which it leads me to get this PhD

Dr. Félix Espinoza, my Nicaraguan co-supervisor, who introduced me in the PCR assays for investigating Diarrheagenic *E. coli*. Thanks for introducing me into the fieldwork activities for the samples collection when I began my first typing analyses, and for sharing your knowledge and enthusiasm in this interesting research area.

Dr. Ernesto Medina, former rector of the UNAN, León, who supported the idea of the formation of young researcher and for your support at the beginning of the project of PhD studies. **MSc. Teresa Rivera** (R.I.P) from who I learnt on the bacteriology in my early days in our Laboratory. **Dr. Edmundo Torres**, former vice-rector of Research and Postgraduate studies of UNAN, León for being a kind person and always been open to listen and support

me where it available. *Dra. Mercedes Cáceres*, former vice-dean of our faculty and former coordinator of the “Infectious Disease Program” between Nicaragua-Sweden, for your support in these years. *Dr. Leonardo Mendoza*, former vice-rector of Research and Postgraduate studies of UNAN, León for being a very open persons and supported me where it available. *Mercedes Corea*, secretary of the vice-rectory of Research and Postgraduate studies of UNAN, León, for your nicer attention when I needed your help.

My Nicaraguan friends and colleagues, *Filemón, Samuel, Erick* and *Fernando*, with whom I have shared this experience and the pleasure, so far away of our home and our families, to become a PhD.

Former and Actual members of the Microbiology Department of UNAN, León: *Ana Esther, Elizabeth, William, Orlando, Byron, Edelma, Silda, Eugenia, Aleyda, Isabel, Rosario, Teresa, Kenia*, for being my work mates and for your support. *Cristian, Erick, Alisset, Alba Estela, Martha*, for their secretary assistances. All the technicians of the Department: *Patricia, Soledad, Brenda, Edwin*, for your effort and support in the laboratory assistances and preliminary analysis in our lab; *Jayintzina, Oscar, Reymundo, Antonia, Mercedes M.*, and *Candida*, for their friendship and support any time in our lab; *Silvia A., Maria Mercedes D.*, for their valuable activities during the field-work and sample collection.

My friend *Calixto*, for helped me to get the epidemiological data from the Ministry of Health

Finally, but no least, I would like to thank to **all my family**, especially to my wife *Maria de la Concepción – Cony* – for your love, and for being a great support and for taking care of our home during all this time. Thank you for always being there for me; and to my children *Norman, Josmary* and *Grethel*, whom I have lost to attend when I have been abroad. To my mother *Danelia*, my Sisters *Mélida, Lizeth*, and my brother *Sergio*; to my mother in law *Norma*, and my sister in law *Norma Jessenia*, for their support and for taking care of *Norman* and *Josmary*.

I am grateful for the financial support provide by the Swedish Agency for Research and Economic Cooperation (SAREC) (grants 2004-0671 – 75007292 and 2008-20 002992) and the National Autonomous University of Nicaragua (UNAN), León, Nicaragua.

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