Karolinska Institutet

Thesis for doctoral degree (Ph.D.) 2009 Molecular and phenotypic characterization of diarrhoeagenic *Escherichia coli* from Nicaraguan children Bayardo Samuel Vilchez Rugama

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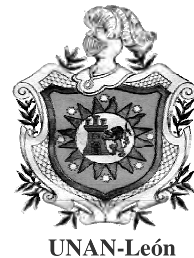

Stockholm 2009

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Published by Karolinska Institutet.
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ISBN 978-91-7409-718-4

Printed by

www.reproprint.se
Gårdsvägen 4, 169 70 Solna

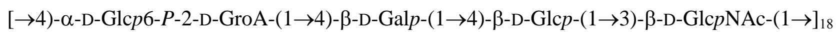
To Anielka, Fiorella and Mateo

ABSTRACT

Diarrhoeal diseases continue to be a foremost public health problem worldwide, with over 1.5 million deaths occurring each year, mostly in children in developing countries. In Nicaragua, the mortality due to diarrhoea in children less than 5 years of age is 11 per 10,000 inhabitants. Additionally, this group of children account for more than 120,000 thousands consultations due to diarrhoea per year, most of them occurring during the winter season. However, in Nicaragua, information about the prevalence of many of the enteric pathogens such as diarrhoeagenic *Escherichia coli* (DEC), is still lacking. Thus, the studies presented in this thesis focused on the identification, molecular and phenotypic characteristics of diarrhoeagenic *E. coli* pathotypes as enteric pathogens associated with diarrhoea in children from León, Nicaragua.

The results from studies I to III are: Five DEC types have been identified in 526 faecal samples from children less than 5 years of age, using PCR based methods. Additionally, the majority of the *E. coli* positives for a DEC type occurred alongside with the winter season in Nicaragua. The ETEC pathotype was identified with significantly higher prevalence in children with 78/381 (20.5%) compared to children without 12/145 (8.3%) diarrhoea. The distribution of the identified ETEC was as follow: *eltB* 72/90 (80%), followed by *eltB-estA* 14/90 (15.6%) and finally *estA* 4/90 (4.4%) positives. Using the biochemical fingerprinting method PhP-RE, ETEC positive samples seem to belong to defined biochemical phenotypes (BPTs). In addition, facts that a limited number of BPTs grouped *estA* positives, and *estA* ETEC samples were only isolated from children with diarrhoea suggest the spread of a marked pathogenic clonal group. On the other hand, atypical EPEC (only $eaeA^{+}$), was identified with similar frequencies in children with 61/381 (16%) and children without 30/145 (20.7%) diarrhoea. Additionally, clonal group analysis of atypical EPEC isolates showed no differences between children with and without diarrhoea. EIEC was identified at low, but similar frequency in both children with 3/381 (0.8%) and without 2/145 (1.4%) diarrhoea. Conversely, EIEC positive samples seem to be a clonal group, yet the number of samples was rather small. EAEC was the most prevalent pathotype of the identified *E. coli*. However, no differences were observed between the children with 106/382 (27.8%) and without 48/145 (33.1%) diarrhoea in terms of prevalence and phenotypic fingerprinting characteristics. Although, differences were appreciated between the herein tested EAEC isolates from children with 73/116 (62.9%) and without 28/68 (41.2%) diarrhoea, that showed aggregative adherence to Caco-2 cells. Additionally, a great variety of other putative virulence markers combination was detected, confirming the heterogeneity of this pathotype. EHEC was only identified in samples from children with 8/381 (2.1%) diarrhoea. The identified EHEC were distributed as follow: *vt2* 6/8 (75%), followed by *vt2-*

eaeA 1/8 (12.5%) and *vt1* 1/8 (12.5%) positives, at similar frequencies. In addition, the low diversity (Di=0.829) obtained from PhP-RE analysis of EHEC positive samples suggest the presence of pathogenic clonal groups. In addition, the majority of the group of children that required IRT 42/68 (61.8%) harboured EAEC, ETEC and EPEC either alone or in combination. Also, the diversity value (Di=0.937) of the isolates from these children suggest that these strains may represent virulent clones capable of causing a more severe case of diarrhoea. In summary, the ETEC pathotype play an important role in diarrhoea in children less than 5 years of age in León, Nicaragua. Nonetheless, EAEC, EPEC and EHEC pathotypes are to some extent important pathogens associated with diarrhoea in those children. In addition to all the above, the structure of the O-antigen polysaccharides (PS) from the EAEC strain 94/D4 and the international type strain from *Escherichia coli* O82 were determined in study IV. The O-antigen is composed of tetrasaccharide repeating units with the following structure:



[→4)-α-D-Glc*p*6-*P*-2-D-GroA-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1→]$_{18}$

LIST OF PUBLICATIONS

This thesis is based on the following papers:

I. Samuel Vilchez, Daniel Reyes, Margarita Paniagua, Filemon Bucardo, Roland Möllby and Andrej Weintraub. **Prevalence of diarrhoeagenic *Escherichia coli* in children from León, Nicaragua.** *J Med Microbiol* 2009; 58: 630-637.

II. Daniel Reyes, Samuel Vilchez, Margarita Paniagua, Patricia Colque, Andrej Weintraub, Roland Möllby and Inger Kühn. **Diversity of intestinal *Escherichia coli* populations in Nicaraguan children with and without diarrhoea.** *J Med Microbiol* 2009; 58: 1593-1600.

III. Samuel Vilchez, Margarita Paniagua and Andrej Weintraub. **Characterization of enteroaggregative *Escherichia coli* isolated from Nicaraguan children.** In manuscript.

IV. Samuel Vilchez, Magnus Lundborg, Felipe Urbina, Andrej Weintraub and Göran Widmalm. **Structural studies of the O-antigenic polysaccharides from the enteroaggregative *Escherichia coli* strain 94/D4 and the international type strain *Escherichia coli* O82.** *Carbohydr Res* 2009; doi:10.1016/j.carres.2009.09.033.

LIST OF ABBREVIATIONS

AA	Aggregative adherence
A/E	Attaching and effacing
ATCC	American Type Culture Collection
BPT	Biochemical phenotype
CFA	Colonization factor antigen
CFU	Colony-forming unit
CS	Coli surface antigen
CT	Cholera enterotoxin
CTFR	Cystic fibrosis transmembrane conductance regulator
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
DAEC	Diffusely adherent *Escherichia coli*
DEC	Diarrhoeagenic *Escherichia coli*
DNA	Deoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EAF	EPEC adherence factor
EHEC	Enterohaemorragic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
Gal	Galactose
Glc	Glucose
GlcNAc	N-Acetylglucosamine
GroA	Glyceric acid
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond coherence
HSQC	Heteronuclear single quantum coherence
HUS	Haemolytic uremic syndrome
ial	Invasion-associated locus
IL	Interleukin
IRT	Intravenous rehydration therapy
kb	Kilo base
kDa	Kilo Dalton
LEE	Locus of enterocyte effacement

LPS	Lipopolysaccharide
LT	Heat labile toxin
NAD	Nicotinamide adenine dinucleotide
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser effect spectroscopy
OM	Outer membrane
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PhP	PhenePlate system
PS	Polysaccharide
ST	Heat stable toxin
UTI	Urinary tract infection
VT	Verocytotoxin
VTEC	Verocytotoxin producing *Escherichia coli*

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1. INTRODUCTION

Diarrhoeal diseases continue to be a foremost public health problem worldwide, with over 1.5 million deaths occurring each year, mostly in children under 5 years of age in developing countries (16). In the Americas diarrhoea accounts for 13% of the deaths of these group of children. At the same time, in Nicaragua, the mortality rate in children beneath five is 11 per 10,000 inhabitants due to diarrhoea. Additionally, this group of children account for more than 120,000 thousands consultations due to diarrhoea per year, most of them occurring during the winter season (7, 65).

A broad range of recognized microorganisms such as viruses, parasites and bacteria are associated with diarrhoea in children. Amongst the bacteria, diarrhoeagenic *Escherichia coli* (DEC) are some of the most frequently detected pathogens worldwide (5, 30, 67). However, in Nicaragua, information about the prevalence of many of these enteric pathogens is still lacking. Nonetheless, several efforts have been done to have a clearer picture of the aetiology of the diarrhoea in Nicaragua. And good results have been achieved, followed by consequential intervention and immunization programs to reduce the burden of the disease, yet the puzzle is not completed and as mentioned earlier many cases still occur every year (64, 65). Thus, studies have been carried out in the country about the prevalence and association of several enteric pathogens with diarrhoea i.e., Rotavirus, Norovirus, enterotoxigenic *Escherichia coli* (ETEC), *Giardia lamblia, Entamoeba histolytica* and others (17, 18, 59, 76, 95). Although the study on the incidence of enterotoxigenic *E. coli* associated with diarrhoea in children less than 2 years of age gave valuable information about the ETEC pathotype (76), the importance of the other pathogenic *E. coli* pathotypes and their association with diarrhoea in children were not investigated.

1.1. *Escherichia coli* and diarrhoeagenic *Escherichia coli* (DEC)

Escherichia coli is a Gram-negative bacilli and a member of the family Enterobacteriaceae encountered as a normal inhabitant of the human and other mammals intestine. This microorganism colonizes the gastrointestinal tract of human infants within a few hours after birth. Thereafter, *E. coli* and its host coexist in good relation with mutual benefit for decades (47). However, the species *E. coli* comprises a versatile and extremely diverse group of organisms. Conversely, this harmless inhabitant of the gut can cause disease when the normal gastrointestinal barriers are breached or in immunocompromised hosts. Moreover, several well adapted *E. coli* clones have acquired specific virulence characteristics, which result on an augmented ability to adapt to new environments and allow them to cause a broad range of

diseases. Usually, the virulence features in *E. coli* clones are encoded on mobile genetic elements that can be inserted into different strains creating new combinations of virulence factors. Furthermore, these mobile genetic elements can be locked into the genome resulting in more stable clones. Only the most successful combinations of virulence factors have endured to become defined pathotypes of *E. coli* that are capable of causing disease in healthy individuals (Figure 1). Three clinical syndromes can result from an infection with *E. coli*: (i) enteric/diarrhoeal disease; (ii) urinary tract infections (UTIs); and (iii) septicaemia/meningitis. Among the enteric or diarrhoeagenic *E. coli* (DEC), there are six well described pathotypes: Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC) or Verocytotoxin producing *E. coli* (VTEC), Enteroinvasive *E. coli* (EIEC) and Diffusely adherent *E. coli* (DAEC) (47, 67). Consequently, the identification of DEC types requires differentiation from non-pathogenic members of the human normal intestinal flora. Molecular identification and classification of DEC is based on the presence of different chromosomal and/or plasmid-encoded virulence genes, which are absent in the commensal *E. coli*. In addition, the various pathotypes of *E. coli* tend to be clonal groups that are characterized by O, K, and H antigens, or by other phenotypic features (47).

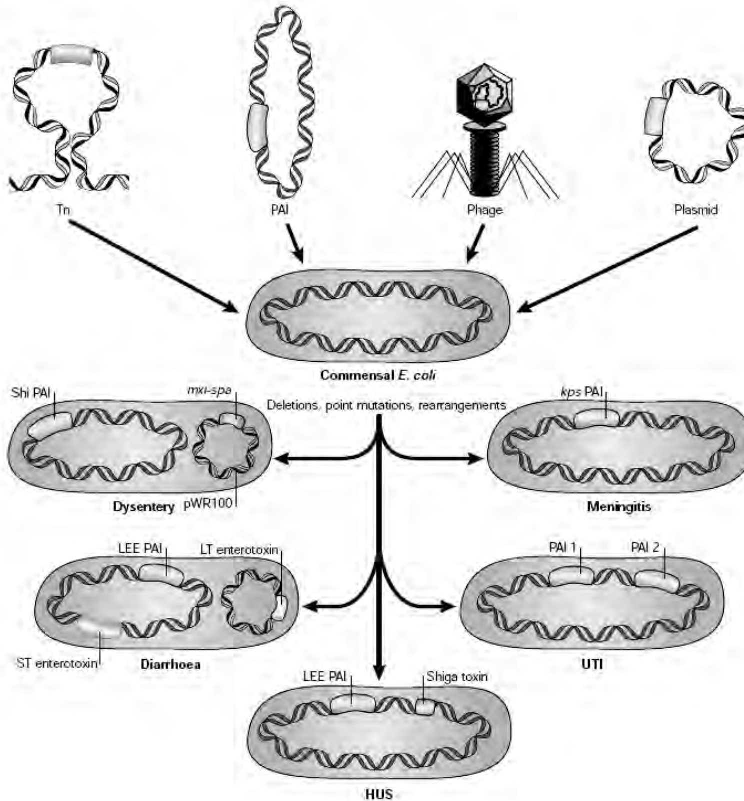


Figure 1. Schematic representation of the contribution of mobile genetic elements to the evolution of pathogenic *E. coli* [reproduced with the permission from (47)]. *E. coli* virulence factors can be encoded by several mobile genetic elements, including transposons (Tn) (for example, heat stable enterotoxin (ST) of ETEC), plasmids (for example, heat-labile enterotoxin (LT) of ETEC and invasion factors of EIEC), bacteriophage (for example, Shiga toxin of EHEC) and pathogenicity islands (PAIs) — for example, the locus of enterocyte effacement (LEE) of EPEC/EHEC and PAIs I and II of UPEC. Commensal *E. coli* can also undergo deletions resulting in 'black holes', point mutations or other DNA rearrangements that can contribute to virulence. These additions, deletions and other genetic changes can give rise to pathogenic *E. coli* forms capable of causing diarrhoea (ETEC, EPEC, EHEC, EAEC, DAEC), dysentery (EIEC), haemolytic uremic syndrome (EHEC), urinary tract infections (UPEC) and meningitis (MNEC).

1.2. Enterotoxigenic *Escherichia coli* (ETEC)

The enterotoxigenic *E. coli* (ETEC) is one of the most studied pathotypes of *E. coli,* which is defined by its ability to produce either the heat-labile toxins (LT), or the heat-stable toxins (ST), or both toxins (LT/ST). ETEC causes watery diarrhoea, which can vary from mild, self-limiting to a severe disease (47). Many studies have shown the association of ETEC with diarrhoea, particularly in children in the developing countries and travellers to developing countries (2, 62, 67, 71, 76). Additionally, ETEC belongs to a large number of serogroups, which the most commons associated with diarrhoea are: O6, O8, O11, O15, O20, O25, O27, O78, O128, O148, O149, O159 and O173 (91). ETEC is primarily spread through food or water contaminated with human waste, and the infectious dose is estimated to range from 10^6 to 10^{10} organisms. However, vulnerable populations such as children and elderly may be susceptible to infection at lower doses (33). Additionally, up to this date no licensed vaccine exists.

In order to cause diarrhoea, ETEC has to colonize the small intestinal mucosa first, by means of one or more colonization factors (CFs). The CFs are antigenically diverse, and thus usually are designated by CFA (colonization factor antigen), CS (coli surface antigen) or PCF (putative colonization factor) followed by a number. Even though more than 20 CFs have been described, yet epidemiological studies suggest that around 75% of human ETEC express either CFA/I, CFA/II or CFA/IV (47). Soon after, ETEC elaborates the LT and/or ST enterotoxins, which stimulate the small intestine epithelial cells. The LT toxins can be divided in two major groups LT-I and LT-II, which do not cross-react immunologically. The LT-I toxin is expressed by ETEC strains that are pathogenic to both humans and animals, while the LT-II is found mainly in ETEC strains of animal origin, but it has not been associated with the disease, neither in animals nor in humans. Thus, usually the term LT refers to the LT-I form. In addition, the LT toxin is functionally and structurally related to the cholera enterotoxin (CT), expressed by *Vibrio cholerae* serogroups O1 and O139 (87). Accordingly, the LT-I toxin has ~80% amino acid similarity with CT, consisting of an A subunit and five identical B subunits. The B subunits are responsible for the binding of the toxin to the cell surface gangliosides GM1 and GD1b, and the enzymatic activity of the toxin comes from the A subunit (33, 47, 67). As a whole, the LT toxin has ADP-ribosyl transferase activity, which transfers the ADP-ribosyl moiety from NAD to the α-subunit of the stimulatory G protein that regulates adenylate cyclase. The resulting activation of adenylate cyclase leads to increased levels of intracellular cAMP, and eventually activates the chloride channel and the CTFR regulator in the cells. The net consequence is increased chloride secretion from the cells leading to diarrhoea (47, 67). Independently of the toxicity of the LT toxin, it has been incorporated into many vaccine candidates due to its mucosal adjuvant properties (47, 93). The other mechanism by which the

ETEC pathotype causes diarrhoea is by means of the ST toxins. These toxins are small peptides that include two unrelated subclasses, the STa and STb. The STa class is associated with human disease. The mature STa is an 18- or 19-amino-acid peptide with a molecular mass of ca. 2 kDa. The main receptor for STa is a membrane-spanning enzyme called guanylate cyclase C, which belongs to a family of receptor cyclases. Binding of STa to guanylate cyclase stimulates guanylate cyclase activity, leading to an increased intracellular cGMP, which, in turn, activates cGMP-dependent and/or cAMP-dependent kinases, and finally increasing secretion. On the other hand, the STb is a 48 amino acid toxin associated with animal disease. The STb can elevate cytosolic Ca^{2+} concentrations, leading to the release of prostaglandin E_2 and serotonin, hence increasing ion secretion (47, 67).

1.3. Enteropathogenic *Escherichia coli* (EPEC)

The prototype pathogen that causes attaching and effacing (A/E) lesions is enteropathogenic *Escherichia coli* (EPEC), which adhere to epithelial cells in a characteristic pattern called localized adherence (LA). EPEC was the first pathotype of *E. coli* described, and it is associated with diarrhoea worldwide, representing a major health threat to children (31, 67). In addition, as with other diarrhoeagenic *E.coli* strains, transmission of EPEC is via the faecal-oral route, with contaminated hands, contaminated weaning foods, or contaminated fomites serving as vehicles. The infectious dose in naturally transmitted infections among infants is not known but is presumed to be much lower than 10^8 organisms. For decades, the mechanism by which EPEC caused diarrhoea was unknown and this pathotype could only be identified on the basis of O:H serotyping (47, 67). Some of the most prevalent serogroups of EPEC are: O18ac, O20, O25, O44, O55, O86, O91, O111, O114, O119, O125ac, O126, O127, O128, O142 and O158 (84). However, many advances in our understanding of the pathogenesis of EPEC diarrhoea and its epidemiology have been made. Nowadays, it is known that there are two variants of this pathotype typical EPEC and atypical EPEC, which differ in several characteristics. Typical EPEC is a cause of diarrhoea in developing countries, while atypical EPEC seems to be associated with diarrhoea in developed countries (3, 4, 47, 62, 96).

The central mechanism of EPEC strains is the characteristic A/E lesion, which involve microvilli destruction, intimate adherence of bacteria to the intestinal epithelium, pedestal formation, and aggregation of polarized actin and other elements of the cytoskeleton at sites of bacterial attachment (96). The genetic determinants for the production of A/E lesions are encoded by genes on a 35-kb pathogenicity island called the locus of enterocyte effacement (LEE). Additionally, the LEE encodes a 94 kDa outer membrane protein called intimin (encoded by *eaeA*), which mediates the intimate attachment of EPEC to epithelial cells (47, 96).

The LEE also contains open reading frames that encode a type III secretion system and the associated chaperones as well as effector proteins. One of these effector proteins is Tir (translocated intimin receptor), which is inserted into the host-cell membrane, functioning as a receptor for the intimin outer membrane protein. Additional EPEC virulence factors outside the LEE have also been described. One of these virulence factors is located in a 70-100 kb plasmid called EAF, a 14 gene-operon encoding a type IV pilus called the bundle-forming pilus (BFP). Subsets of genes also on the EAF plasmid are involved in the transcriptional regulation of other virulence factors, including *bfp*. Adding up, the LA phenotype characteristic of EPEC is dependent on the presence of the adherence EAF plasmid. Hence, the two variants of EPEC definition comes from the hybridization of EPEC strains with EAF probe: typical (bfp^{+} and $eaeA^{+}$) and atypical (bfp^{-} and $eaeA^{+}$) EPEC (23, 47, 96).

1.4. Enteroinvasive *Escherichia coli* (EIEC)

Enteroinvasive *E. coli* (EIEC) are biochemically, genetically and pathogenically closely related to *Shigella* spp. However, EIEC can be distinguished from *Shigella* by few minor biochemical tests, yet *Shigella* and EIEC share the essential virulence factors (47). These pathogens are responsible for different degrees of dysentery in humans. Also, EIEC may be responsible for invasive inflammatory colitis and in most cases for watery diarrhoea indistinguishable from that caused by the other pathotypes of *E. coli*. Epidemiologic studies of EIEC mostly describe outbreaks, usually food-borne or waterborne related. Even though the infectious dose is higher than that for *Shigella* spp., transmission person to person does occur (57, 67, 98). EIEC/*Shigella* causes diarrhoea by crossing the epithelial layer invading M cells. Entry into epithelial cells involves rearrangements of the cell cytoskeleton, leading to ruffling and engulfment of the bacterium within a vacuole. After, bacteria multiply intracellularly, moves through the cytoplasm and extent into adjacent epithelial cells without being exposed to the external surroundings. The characteristic ability to induce entry and dissemination of EIEC and *Shigella* spp. strains, from cell to cell spreading is present on a virulence plasmid of ~220 kb (47, 78). The virulence plasmid (VP) is composed of a mosaic of ~100 genes and numerous insertion sequences, which represent one-third of the VP. The VP contains a 30 kb region essential for entry into epithelial cells, also encodes a type III secretion system (TTSS) and a 120 kDa outer membrane protein call IcsA. The pathogenesis of EIEC/*Shigella* spp. strains seems to be the results of its plasmid-borne TTSS, which encodes several secreted proteins, such as IpaA, IpaB, IpaC, and IpgD, important for invasiveness. IpaB and IpaC proteins are important for pore formation. Additionally, IpaB can bind to the signalling protein CD44, resulting in cytoskeletal rearrangements and cell entry. Furthermore, IpaB can bind to the macrophage caspase 1, which results in apoptosis and release of IL-1 from macrophages. In the case of IpaC protein, it

induces actin polymerization, leading to the formation of cells extensions. Conversely, IpaA binds to vinculin and induces actin depolymerization, organizing the extensions. Finally, IpgD helps to reorganize host cell morphology (47, 67, 78). Although the TTSS of EIEC/*Shigella* spp. is important, additional virulence factors have been described, such as SigA, SepA and Pic, serine protease (12, 47). The most common serogroups of EIEC are: O28ac, O29, O112ac, O124, O136, O143, O144, O152, O159, O164 and O167 (91).

1.5. Enteroaggregative *Escherichia coli* (EAEC)

Enteroaggregative *E. coli* (EAEC), which display a characteristic “stacked brick” like adhesion pattern when cultured with HEp-2 cells and other epithelial cell types, is an emerging pathogen associated with diarrhoea in children worldwide (25, 34, 79). The importance of EAEC as an agent of paediatric diarrhoea can be inferred from an increased number of reports of diarrhoeal cases in which EAEC are implicated. In a case-controlled study carried out in Vietnam by Nguyen *et al.* in 2005, EAEC were associated with diarrhoea in children less than 2 years of age (71). Furthermore, several other case-control studies suggest that EAEC is an important cause of diarrhoea in people of all ages in developing and developed countries (105). In addition, EAEC has been associated with diarrhoea in HIV-infected persons and with traveller’s diarrhoea from developed to developing areas (45). Several of the earliest epidemiological studies implicated EAEC as a cause of persistent diarrhoea (≥14 days) (11, 27, 35, 61). However, today it is known that EAEC cause both acute and persistent diarrhoea (34). EAEC is transmitted by the faecal-oral route, similar to ETEC. A high inoculum 10^{10} CFU size supports its role in food-borne related infections (35). Food-borne outbreaks like the one involving 2,697 Japanese school-children that became ill after consuming contaminated school lunches in 1993 was associated with an O-untypable EAEC strain (35, 41).

EAEC uses the strategy of causing diarrhoea, by colonizing the intestinal mucosa usually mediated by aggregative adherence fimbriae, formation of a mucoid biofilm, and secretion of enterotoxins and cytotoxins. Human and animal studies indicate that EAEC is able to bind to jejunal, ileal and colonic epithelium. Though, the major damage is in the colon, where EAEC elicit inflammatory mediators such as IL-8 and produces cytotoxic effects like microvillus vesiculation, enlarged crypt openings, and increased epithelial cell extrusion (34). Even though it is now clear that multiple factors contribute to EAEC-induced inflammation, the characterization of not yet described factors will facilitate the understanding of the mechanisms of this emerging pathogen. On the other hand, EAEC is a heterogeneous group, and not all strains are capable of causing the disease (45, 47). In a volunteer challenge study, four groups of five persons were fed with one of four different EAEC strains and only one of the strains

caused diarrhoea in one of the groups, while the other three strains were unsuccessful to cause diarrhoea (68).

EAEC is a group of *E. coli* which includes a considerable mosaic of serotypes that possess a variety of putative virulence factors and may express several different surface structures (67, 80), e.g. Huppertz *et al.* analysed EAEC from German children and found that of 14 typable isolates all belonged to different serotypes (37). However, serotyping of EAEC is a problem since many of the strains autoagglutinate due to their aggregative phenotype, and they are often described in the literature as non-typable or as O-rough (28). Some of the serogroups that have been identified within the EAEC group are O3, O7, O15, O21, O44, O77, O82, O86, O111, O126, O127 and O128 (6, 90, 91, 103). Additionally, it has been shown in a number of studies that many of the identified putative virulence factors are associated with diarrhoea. However, the association with diarrhoea of some of these factors has differed in others. Most EAEC strains harbour a 60-65 MDa virulence plasmid (pAA), from which a probe called CVD432 has been derived and used in many epidemiological studies (8, 44, 71, 104). Nonetheless, this probe does not discriminate between virulent and non virulent strains. Although, the pAA plasmid also encodes the following putative factors: (i) the aggregative (AA) fimbriae I, II and III (10, 21, 70); (ii) the transcriptional activator AggR (69); (iii) a cytotoxin designated Pet (26); (iv) the anti-aggregative protein (dispersin) encoded by the *aap* gene (86); and (v) a heat-stable toxin-1 (EAST-1) (82). Additionally, other toxins originally found in different enteric bacteria have been described in EAEC isolates such as SigA and SepA from *Shigella flexneri*, Sat from uropathogenic and diffusely adhering *E. coli* (DAEC), and Pic a mucinase encoded on the bacterial chromosome of *Shigella* and EAEC strains (12). In addition, many variations of clinical symptoms due to EAEC infections have also been associated with host genetic susceptibility factors, besides the heterogeneity of the EAEC strains (46). Polymorphisms in the promoter region of IL-8 located -251 bp upstream of the transcription start site have been identified. An AA or AT genotype at the -251 position in the promoter region of IL-8 results in greater chances of developing symptomatic EAEC infection compared with those of the TT genotype (46). Thus, there is still a lot to explore about this pathotype.

1.6. Enterohaemorragic *Escherichia coli* (EHEC)

Enterohaemorrhagic *E. coli* (EHEC) is a subset of Verocytotoxin-producing *E. coli* (VTEC), which is considered to be a human pathogen, specifically associated with diarrhoea. Numerous studies have documented EHEC as an important cause of diarrhoea (both bloody and non-bloody), and of haemolytic uremic syndrome (HUS) (15, 48, 50, 71). Although most of the cases of EHEC infection are uncomplicated non-bloody diarrhoea, in most cases that come to

medical attention the diarrhoea becomes bloody on the second or third day of illness, leading to a diarrhoea which persists for up to one week. Whereas most cases resolve at this stage, approximately 20% develop life-threatening complications, of which HUS is the most common. HUS is characterised by a lack of urine formation and acute kidney failure. Roughly half of all HUS patients entail renal dialysis. Moreover, HUS occurs most often in children under the age of 10 years (74, 75). Most of the studies on EHEC lead to ruminant animals, in particular cattle as the main reservoir for this pathotype. Beef has historically been mostly linked to infections, however a wide variety of other sources have been implicated, including unpasteurized milk and fruit juice. Waterborne transmission occurs through swimming in contaminated lakes, pools, or drinking untreated water. Direct contact with animal faecal material through recreational activities and person- to person- contact are also sources of infection (74, 75). The number of EHEC required to cause disease is very low and it has been reported to be as low as 10 colony forming units (CFU) (74).

The VTEC definition of EHEC strains is based upon the production of a family of toxins with cytotoxic activity against Vero cells *in vitro*. Additionally, as Verocytotoxins (VTs) are structurally and functionally similar to the Shiga toxin produced by *Shigella dysenteriae* type 1, a similar nomenclature system exists, the Shiga toxin producing *E. coli* (STEC) (48, 50). Though attempts to develop a consensus nomenclature have been made (84). The VTs consists of two main groups of closely related toxins, VT1 and VT2. Each of these holotoxins is composed of five glycolipid-binding B subunits, and one enzymatically active A subunit, which inhibit protein synthesis by cleaving ribosomal RNA (73). The major trait of EHEC is their ability to produce and release these VTs, which are also considered the major virulence factors. This makes the genes that encode the toxins ideal molecular targets for diagnostic EHEC infections. Though, beside the VT production, colonization of the host intestinal mucosa is a key factor for full virulence of EHEC, and several factors involved in the process have now been characterized. EHEC colonize the intestinal mucosa inducing a characteristic histopathologic lesion, defined as "attaching and effacing" (A/E), which is also a characteristic of EPEC. The A/E lesion is characterized by effacement of microvilli and intimate adherence between the bacteria and the epithelial cell membrane (47). In that sense, the intimin gene of EPEC strains is also used in molecular diagnostic of EHEC infections together with the toxins. Additionally, EHEC strains usually belong to different serotypes or serogroups, which are also useful for diagnostic and epidemiological studies. The most notorious serogroup is O157, which has been the cause of several outbreaks around the globe (13, 32, 66). Besides O157, some of the most important serogroups in terms of clinical infection are *E. coli* O26, O111, O103, and O145. Nonetheless, this list is not far-reaching and scores of other EHEC serogroups have also caused infections (75, 91).

1.7. Diffusely adherent *Escherichia coli* (DAEC)

The characteristic diffuse pattern of adherence to HEp-2 cells monolayers is what delineates the diffusely adherent *E. coli* (DAEC) pathotype. The first DAEC studies showing association with diarrhoea were controversial, in some but not all epidemiologic studies DAEC was associated with diarrhoea in children. In addition, the results of a challenge volunteer study showed that DAEC were unable to conclusively induce diarrhoea (94). However, it suggested that DAEC may cause disease in immunologically naive or malnourished children. The discrepancies among epidemiological studies are partly now explained by age-dependent association of DAEC with diarrhoea, particularly in children older than 1 year of age (47, 83, 89). DAEC strains induce a cytopathic effect that is characterized by the development of long cellular extensions, by means of a fimbrial adhesin called F1845. This adhesin belongs to the Dr family of adhesins, which use a cell surface glycosylphosphatidylinositol-anchored protein (DAF) receptor, that prevent cells from damage by the complement system. Dr fimbriae usually bind and cluster to the DAF receptor. All members of the Dr family elicit this effect. Binding of Dr adhesins is accompanied by the activation of signal transduction cascades. Additionally, it has been reported that infection of an intestinal cell line by strains of DAEC impairs the activities and reduces the abundance of brush-border-associated sucraseisomaltase and dipeptidylpeptidase IV (47). Altogether, this provides a possible mechanism for DAEC-induced enteric disease, and furthermore indicates the presence of other virulence factors in DAEC. Nevertheless, in general, little is known about the pathogenesis of diffusely adherent *Escherichia coli* strains.

1.8. Lipopolysaccharide (LPS)

Lipopolysacharide is not only the major constituent of the cell surface structure, the outer membrane (OM), but it is also a potent toxin (endotoxin) of Gram-negative bacteria. LPS covers approximately 75% of the OM, while the rest is mainly covered by porin proteins that form small channels through the OM. LPS is a virulence property of many Gram-negative bacteria, and it is usually important for colonization, i.e. EAEC 042 (42). In the prototype EAEC strain 042, the negatively charged LPS has been shown to interact with a cell surface positively charged protein nicknamed dispersin. This interaction prevents the positively charged aggregative fimbriae to collapse onto the bacterial surface. When dispersin was absent the bacteria increased aggregation and lost efficiency in colonization (101). LPS can be divided into two main domains (Figure 2): (i) lipid-A part, which is a phosphorylated and fatty acid-substituted glucosamine disaccharide glycolipid that anchor the LPS to the OM and it is also the toxic part of the molecule; and (ii) a polysaccharide part. The polysaccharide portion of the LPS

can be further divided in a core oligosaccharide proximal to the lipid part and an O-antigen polysaccharide, which gives the bacterial colonies a smooth appearance (97, 100, 106).

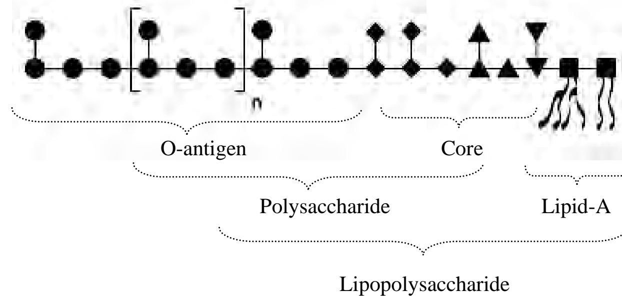


Figure 2. Schematic structure of a lipopolysaccharide molecule [reproduced and modified with the permission from (91)].

Additionally, the O-antigen specificity is used for serological classification of particular virulent strains of interest, resulting in a vast impact in the field of epidemiology; for the rapid tracking of strains related to infections e.g. outbreaks of disease caused by specific serogroups. Moreover, O-antigen diversity may contribute to bacterial evasion of host immune responses. Thus, the membrane attack complex (MAC) assembly can be affected by the chemistry, as well as the length and the relative amounts of long-chain O-antigens with different pattern of substitutions (51). Additionally, one specific example of the biological importance of the O-antigen can be presented as follows: the presence and length of O-antigens in *Shigella flexneri* have direct repercussion on the pathogenesis of this bacterium, by directing the invasion protein (IcsA) to its correct location at the cells to be invaded (51). This protein is essential for invasiveness and subsequent inter- and intracellular spread. Overall, it has been demonstrated that the roles of O-antigens in different bacteria are quite variable and the structural details of the O-antigens molecules can have a great influence on the biology of a given bacterium. Specifically, because of the monosaccharide that built up the O-antigen, the numerous possibilities of glycosidic linkages, the substitution patterns and their configuration can result in a great structure variation and uniqueness of the O-antigens. However, e.g. in *E. coli* more than 180 different serotypes have been described, but up till now less than half of them have been structurally elucidated (91).

1. AIMS OF THE STUDY

1. To determine the relative prevalence of five diarrhoeagenic *E. coli* pathotypes, and their association with diarrhoea in children from León, Nicaragua.

2. To investigate the phenotypic and molecular characteristics of the identified diarrhoeagenic *E. coli* pathotypes.

3. To elucidate the O-antigen structure of the enteroaggregative strain *E. coli* 94/D4, isolated from a Nicaraguan child with diarrhoea.

3. MATERIAL AND METHODS

A description of the methodology used in this thesis is described below, for more details see individual papers.

3.1. Study area: All clinical samples investigated in the present thesis came from subjects living in León, Nicaragua. Nicaragua is situated in the middle of the Central America isthmus that bridges the North and South America subcontinents, bordered by both the Caribbean Sea and the North Pacific Ocean, between Costa Rica and Honduras. The climate is tropical in the Pacific (22°C to 42°C) and the Atlantic (25.6°C to 26.4°C) parts, while cooler in the northern (5.5°C to 27.6°C) part of the country, with León being one of the warmest cities in the pacific (40). Additionally, it has two seasons summer (December to May) and winter (June to November). Nicaragua has an estimated population of ~ 5.1 million inhabitants and 11.8 % are children less than 5 years of age. The main health problems affecting these children are respiratory tract infections and diarrhoeal diseases (39, 64).

3.2. Papers I to III

3.2.1 Study subjects: A total of 526 stool samples from children younger than 60 months of age were investigated. This included 381 children with and 145 without diarrhoea attending four different health care centres and the main hospital in León. Information on some clinical and epidemiological features was obtained through questionnaires. Patients were enrolled in the study if they had diarrhoea, characterized by the occurrence of three or more loose, liquid or watery stools or at least one bloody loose stool in a 24-h period. Control subjects were healthy children with no history of diarrhoea for at least 1 month. Severity was defined as follows: 1, mild, when the episode lasted no longer than 3 days without fever and vomiting and with good toleration of oral rehydration therapy at home; 2, moderate, episode duration of more than 3 days with fever and/or vomiting and with toleration of oral rehydration at a health care centre; and 3, severe, episode with fever and vomiting, requiring intravenous rehydration and needing hospitalization (107) (Papers I, II & III).

3.2.2. Ethics: The studies described in this thesis were approved by the local bioethical committee of the Faculty of Medical Sciences, National Autonomous University of Nicaragua, León (Registration No. 62) (Papers I, II & III).

3.2.3. Clinical isolates: Stool samples collected in sterile plastic containers were cultured on MacConkey agar for the selection of *E. coli* isolates and on other media, such as Deoxycholate

citrate agar (for the selection of *Shigella* and *Salmonella*), with overnight incubation at 37°C. All samples were tested for *E. coli, Shigella* and *Salmonella* by colony morphology and conventional biochemical tests as described previously (29). In addition, samples were investigated for *Giardia lamblia, Entamoeba histolytica*, *Cryptosporidium*, *Trichuris trichiura,* Rotavirus and Norovirus, as described formerly (17, 18, 59, 95) (Papers I & II).

3.2.4. Polymerase chain reaction assays: Each culture plate was screened for the presence of different virulence markers detailed in Table 1, by boiling a bacterial suspension for 20 to 25 min, followed by centrifugation at 2,500 *g* for 10 min to pellet the cell debris. The supernatants were used for PCR. PCRs were performed in a 25 µl reaction mixture (RM) with a pureTaq Ready-To-Go PCR Bead (GE Healthcare UK). The bacterial lysate from negative, positive controls (Table 1) and the clinical isolates were used in the corresponding RM consisting of 10 mM Tris-HCl, (pH 9.0), 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM of each dNTPs 2.5 U of pureTaq DNA polymerase and each primer (from INTERACTIVA Biotechnologie GmbH, Germany). The primers and thermocycling conditions are listed in Table 2. PCR products (10 µl) were evaluated with a 1.5% (wt/vol) agarose gel (UltraPure™ Agarose, Invitrogen™ life technologies, USA) at 120 mV for 40 min. A molecular marker (TrackIt 100 bp DNA Ladder; Invitrogen™ life technologies, USA) was run concurrently. The DNA bands were visualized and photographed under UV light after the gel was stained with ethidium bromide (Papers I, II & III).

Table 1. Reference *E. coli* strains used in this thesis.

DEC type	Reference strain	Target gene(s)	Study
ETEC	ATCC 35401	*eltB*, *estA*	I & II
EHEC	ATCC 43890	*vt1*, *eaeA*	I & II
EHEC	ATCC 43889	*vt2*, *eaeA*	I & II
EPEC	ATCC 43887	*eaeA*, *bfpA*	I & II
EIEC	ATCC 43893	*ial*	I & II
EAEC	97R	*CVD432*	I & II
E. coli (negative control)	ATCC 11775	No virulence gene	I, II & III
EAEC	JM221	*sat, pic*, *aggA*	III
EAEC	042	*aap, aggR, pet, sigA, aafA*	III
EAEC	55989	*sat, sigA, pic, agg-3A*	III
EAEC	C1010-00	*sat, pic, sepA*, *agg-4A*	III

Table 2. List of primers and PCR conditions used in this thesis.

Target genes	Primer sequences (5' → 3')	Fragment size (bp)	PCR conditions (35 or 40 cycles)
eltB	TCT CTA TGT GCA TAC GGA GC CCA TAC TGA TTG CCG CAA T	322	30 sec 94°C, 30 sec 58°C, 1 min 72°C
estA	GTC AAA CCA GTA $^{G}_{A}$GG TCT TCA AAA CCC GGT ACA $^{G}_{A}$GG AGG ATT ACA ACA	147	
vt1	GAA GAG TCC GTG GGA TTA CG AGC GAT GCA GCT ATT AAT AA	130	
vt2	ACC GTT TTT CAG ATT TT$^{G}_{A}$ CAC ATA TAC ACA GGA GCA GTT TCA GAC AGT	298	
eaeA	CAC ACG AAT AAA CTG ACT AAA ATG AAA AAC GCT GAC CCG CAC CTA AAT	376	
bfpA	TTC TTG GTG CTT GCG TGT CTT TT TTT TGT TTG TTG TAT CTT TGT AA	367	
ial	CTG GTA GGT ATG GTG AGG CCA GGC CAA CAA TTA TTT CC	320	
CVD432	CTG GCG AAA GAC TGT ATC AT AAA TGT ATA GAA ATC CGC TGT T	630	
sat	TCAGAAGCTCAGCGAATCATTG CCATTATCACCAGTAAAACGCACC	930	30 sec 94ºC, 1 min 60 ºC, 1.5 min 72ºC
sigA	CCGACTTCTCACTTTCTCCCG CCATCCAGCTGCATAGTGTTTG	430	
pet	GGCACAGAATAAAGGGGTGTTT CCTCTTGTTTCCACGACATAC	302	
pic	ACTGGATCTTAAGGCTCAGGAT GACTTAATGTCACTGTTCAGCG	572	
sepA	GCAGTGGAAATATGATGCGGC TTGTTCAGATCGGAGAAGAACG	794	
Agg-4A	TCCATTATGTCAGGCTGCAA GGCGTTAACGTCTGATTTCC	411	
aggA	AAATATGAGAAGAAAGAA AAAAATTAATTCCGGTATGG	500	1 min 94ºC, 1 min 50ºC, 1.5 min 72ºC
aafA	CAGAATGTTTGCGATTGCTAC TTTGTCACAAGCTCAGCATT	468	
Agg-3A	GTATCATTGCGAGTCTGGTATTCAG GGGCTGTTATAGAGTAACTTCCAG	462	
aap	CTTGGGTATCAGCCTGAATG AACCCATTCGGTTAGAGCAC	310	45 sec 94ºC, 45 sec 55ºC, 45 sec 72ºC
aggR	CTAATTGTACAATCGATGTA AGAGTCCATCTCTTTGATAAG	457	

3.2.5. Biochemical fingerprinting assay: A total of 4009 *E. coli* colonies were typed using biochemical fingerprinting with the PhP-RE plates of the PhenePlate™ system (http://www.phplate.se) (55). In brief, each PhP-RE plate contains eight parallel sets of 11 dehydrated reagents, which provide a high level of discrimination among *E. coli* strains. A growth medium containing a pH indicator was added to the PhP plates, and eight *E. coli* colonies per plate were randomly picked and suspended into the first wells of each row of the plate, where after the homogenized bacterial suspensions were transferred to the remaining wells of the same row, containing the different dehydrated reagents. Test results were read after 16, 40 and 64 h incubation at 37°C, using a desktop scanner (HP Scanjet 7400c XPA) with a transparency adapter. The generated images were converted to numerical absorbance data by the PhPWIN software. After the last reading, the mean values from the readings of each well were calculated to give the biochemical fingerprint consisting of 11 numerical values of each tested isolate. The biochemical fingerprints of all isolates were compared pairwise to each other and the similarity between each pair was calculated as the correlation coefficient. The similarity matrix thus obtained was subjected to cluster analysis using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method (88). Isolates showing identical biochemical fingerprints were assigned to the same biochemical phenotype (BPT). When the PhPWIN software matched at least 1% of the 4009 analyzed isolates into the same BPT, a tentative common BPT (C-BPT) was defined. BPTs found in less than 1% isolates were in this study named as "others". The diversity was calculated using Simpson's index of diversity (36), which is a relative measure of the distribution of isolates into different types (Paper II).

3.2.6. Adhesion assay: Monolayers of Caco-2 cells (ATCC HTB-37) were grown in a 24-well culture plate (Sterile Corning® CellBIND®) with α-MEM (Gibco) supplemented with 100 U penicillin ml^{-1}, 100 mg streptomycin ml^{-1} and 20% foetal bovine serum (HyClone Logan). The cells were maintained until confluent with approximately 4.0×10^5 cells per well. Additionally, bacterial isolates were grown in Luria broth at 37 °C overnight without shaking. Later, Caco-2 cells were infected in duplicates with EAEC isolates in antibiotic- and serum-free α-MEM containing 1% mannose and incubated in a humidified incubator at 37°C in an atmosphere containing 5% CO_2. After 3 h, supernatants were taken away and cells were washed to remove unbound bacteria, fixed with methanol for 5 min, stained with crystal violet and examined under phase-inverted light microscope at 10x. Each study included a well with no bacteria in the Caco-2-culture assay and a negative and positive control of non-pathogenic *E. coli* (Paper III).

3.3.7. Statistical analysis: Construction of databases and statistical analysis for papers I to III, were done using the software SPSS statistics version 17.

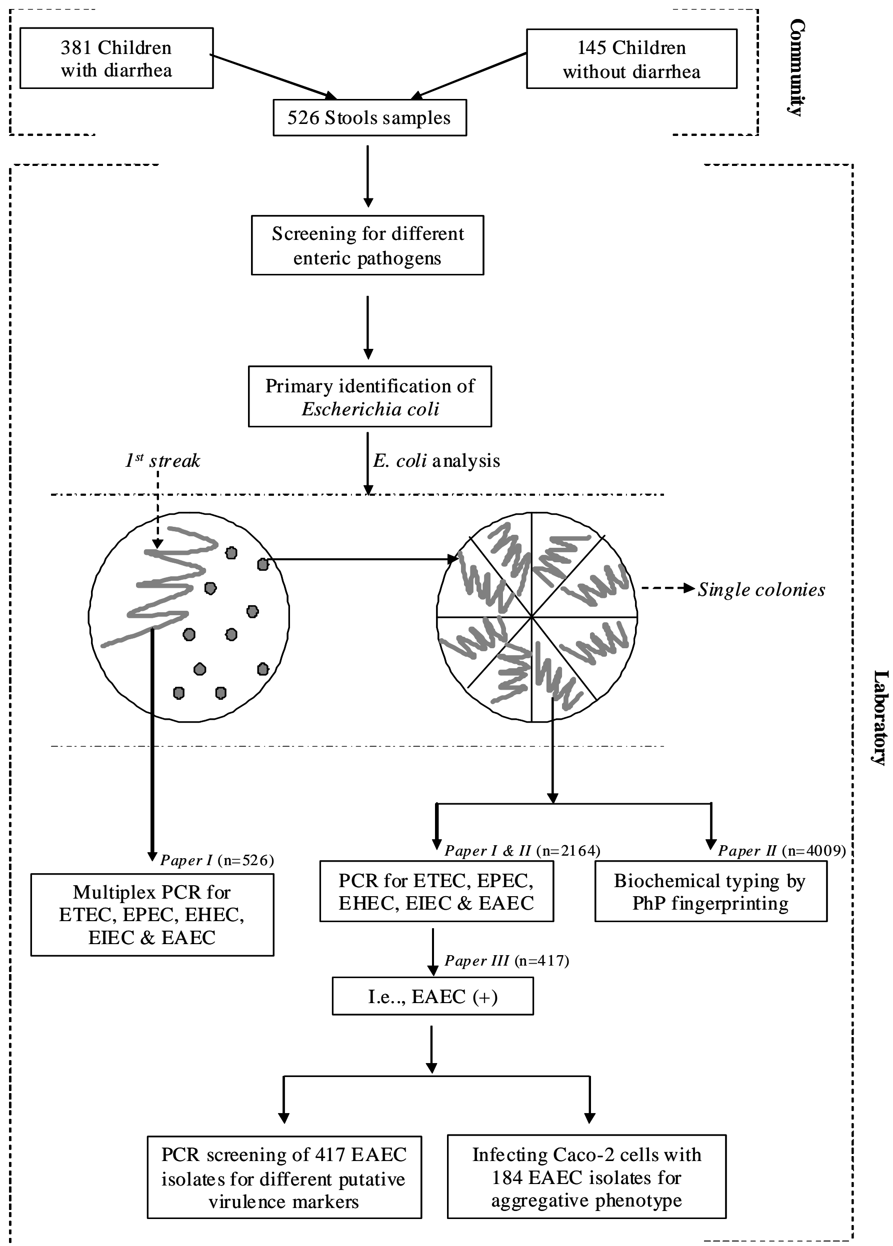


Figure 3. Schematic flowchart outlining the analysis for papers I, II and III.

3.3. Paper IV

3.3.1. Clinical strain: The investigated *Escherichia coli* strain 94/D4 was isolated in 1991 from the fourth episode of an infant aged 9 months, with acute diarrhoea in León, Nicaragua. The bacteria belong to the group of enteroaggregative *E. coli* and it was typed as O82:H8. The international type strain of *E. coli* O82 was obtained from the Culture Collection University of Gothenburg (CCUG 11384/O82:H-:K-). The bacteria were grown in a Luria-Bertani (LB) medium, using a 30-L fermentor (Belach AB) under constant aeration at 37ºC and pH 7.0. The bacteria were killed with 1% (v/v) formaldehyde (99).

3.3.2. Preparation of lipopolysaccharide and lipid A-free polysaccharide: The LPS were extracted and purified as previously reported (58). Lipid A-free polysaccharides from *E. coli* strain 94/D4 and O82 were prepared by treatment of the LPS with 0.1 M sodium acetate, pH 4.2, at 100ºC for 5 h and purified as previously described (53).

3.3.3. Immunochemical analyses: The rabbit anti-*E. coli* O82 specific antiserum was obtained from The International *Escherichia coli* Centre (WHO), Statens Serum Institute, Copenhagen, Denmark. The enzyme immunoassay (EIA) was performed as described previously (49, 99).

3.3.4. Component analyses: The LPS was hydrolyzed with 2 M triflouroacetic acid at 120ºC for 2 h. After reduction with $NaBH_4$ and acetylation, the sample was analyzed by Gas Liquid Chromatography (GLC). The absolute configuration of the sugars present in the O-antigen of *E. coli* strain 94/D4 and O82 were determined by derivation of the sugars as their acetylated (+)-2-butyl glycosides essentially as described (60).

3.3.5. Gas Liquid Chromatography analyses: Alditol acetates and acetylated 2-butyl glycosides were separated on an HP-5 column using a temperature program of 180ºC for 1 min, 3ºC min^{-1} to 210ºC, 10 min at 210ºC. The 2-butyl glyceric acid was separated on the same column as mentioned above with a temperature program of 130ºC for 3 min, 3ºC min^{-1} to 210ºC, 10 min at 210ºC . Additionally, the 2-butyl galactosides were separated on a DB-225 column with a temperature program of 180ºC for 1 min, 3ºC min^{-1} to 210ºC, 1 min at 210ºC, 4ºC min^{-1} to 230ºC, 10 min at 230ºC. Hydrogen was used as carrier gas. The columns were fitted to a Hewlett-Packard model 5890 series II gas chromatograph equipped with a flame ionization detector.

3.3.6. NMR spectroscopy: NMR spectra of the polysaccharide materials (5 mg) in D_2O solution (0.55 mL) were recorded at 60°C using Bruker AVANCE 500 MHz and AVANCE III

700 MHz spectrometers equipped with 5 mm PFG triple-resonance CryoProbes. Data processing was performed using vendor-supplied software. Chemical shifts are reported in ppm using internal sodium 3-trimethylsilyl-(2,2,3,3-$^{2}H_4$)-propanoate (TSP, δ_H 0.00), external 1,4-dioxane in D_2O (δ_C 67.40), and external 2% phosphoric acid in D_2O (δ_P 0.00) as references. One-dimensional ^{1}H, ^{13}C, and ^{31}P and 2D ^{1}H,^{13}C-HSQC NMR spectra were recorded for the PS from *E. coli* strain 94/D4 and *E. coli* O82. The assignments of the ^{1}H and ^{13}C resonances for the PS from *E. coli* strain 94/D4 were obtained by 2D NMR spectra using a multiplicity-edited ^{1}H,^{13}C-HSQC experiment (77), ^{1}H,^{1}HTOCSY experiments (14), a ^{1}H,^{13}C-HMBC experiment (72), and a band-selective constant-time ^{1}H,^{13}C-HMBC experiment (20). ^{1}H,^{31}P-hetero-TOCSY experiments (52) were also carried out. The inter-residue correlations were assigned using a ^{1}H,^{13}C-HMBC experiment (9, 108) and a ^{1}H,^{1}H-NOESY experiment (56). The chemical shifts were compared to those of the corresponding monosaccharides (43).

4. RESULTS AND DISCUSSIONS

In spite of many efforts, the puzzle of the aetiology of the diarrhoea in Nicaragua is not completed and many cases still occur every year (64, 65). On the other hand, results from studies like the one carried out by Paniagua *et al.* regarding "ETEC infant infections" gave valuable information for the planning of intervention studies using ETEC vaccines (76). However, it is clear from our present findings that the *E. coli* population structure is changing over time, which may be an important consideration for the development and evaluation of vaccines directed at protection against DEC infections. This can be appreciated as we compared the biochemical phenotypes (BPTs) of 797 *E. coli* isolates from a cohort study carried out in the 90's in same geographic area to those BPTs obtained in study II; only 4% of the isolates from the 90's were similar to any of the BPTs found in this study. In addition, the importance of the other pathogenic *E. coli* pathotypes and their association with diarrhoea in children has not been investigated earlier. Thus, we suspected that other pathotypes of *E. coli* were circulating in Nicaragua. In particular, we analysed isolates previously collected from children with diarrhoeal episodes from the 90's that were not classified as ETEC. Later, our results showed that these isolates were either enteropathogenic or enteroaggregative *E. coli* types (author's unpublished data). One of these isolates is *Escherichia coli* 94/D4, here investigated in study IV. Hence, studies aiming to determine the relative prevalence of diarrhoeagenic *E. coli* pathotypes, their association with diarrhoea, and the phenotypic and molecular characteristics of this pathotypes were needed. Accordingly, beside study IV, here we present the results of the investigations from three studies involving five different diarrhoeagenic *E. coli* pathotypes in relation to their importance and molecular and phenotypic characteristics as pathogens associated with diarrhoea in Nicaraguan children.

4.1. Prevalence of diarrhoeagenic *Escherichia coli*: molecular and phenotypic characteristics (Papers I, II and III)

The total *E. coli* flora, in all 526 studied stool samples, has been screened for diarrhoeagenic *E. coli* markers by multiplex PCR (Paper I). From the 526 samples, at least one diarrhoeagenic *E. coli* type was detected in 282 (53.6%) samples. In the diarrhoea group 205 (53.8%) and in the non diarrhoea group 77 (53.1%). However, a total of 348 diarrhoeagenic *E. coli* were identified from the 526 samples: 256 (67.2%) were identified in the samples of children with diarrhoea, and 92 (63.4%) in children without diarrhoea (Table 3). Forty-eight (12.6%) children from the diarrhoea group and fifteen (10.3%) from the non diarrhoea group harboured isolates positive for more than one diarrhoeagenic *E. coli* pathotype in study I. Additionally, as expected - in

Nicaragua most of the diarrhoeal episodes occurred during winter – the majority of the *E. coli* harbouring DEC virulence markers also occurred during the winter season (Figure 4).

Table 3. DEC among children with and without diarrhoea in relation to total diversities of *E. coli* isolates from studies I and II.

DEC type	PCR Marker	No. (%) children with and without diarrhoea						Odds ratio (95% CI)*	*P* value**
		Diarrhoea group (n=381)	No. of isolates	Total Di	Non diarrhoea group (n=145)	No. of isolates	Total Di		
ETEC	All	78 (20.5)	-	-	12 (8.3)	-	-	2.4 (1.4-4.4)	0.001
	eltB	60 (15.7)	465	0.952	12 (8.3)	93	0.964	1.9 (1.1-3.4)	0.026
	estA	4 (1.0)	32	0.873	0 (0.0)	-	-	NA	0.216
	eltB-estA	14 (3.7)	105	0.912	0 (0.0)	-	-	NA	0.019
aEPEC	*eaeA*	61 (16.0)	457	0.938	30 (20.7)	236	0.946	0.7 (0.5-1.1)	0.205
EIEC	*ial*	3 (0.8)	20	0.821	2 (1.4)	13	0.833	NA	0.532
EAEC	CVD432	106 (27.8)	797	0.958	48 (33.1)	375	0.952	0.8 (0.6-1.1)	0.234
EHEC	All	8 (2.1)	57	0.829	0 (0.0)	-	-	NA	0.079
	vt1	1 (0.3)	8	-	0 (0.0)	-	-	NA	0.537
	vt2	6 (1.6)	41	-	0 (0.0)	-	-	NA	0.129
	vt2-eaeA	1 (0.3)	8	-	0 (0.0)	-	-	NA	0.537
Non-DEC	-	176 (46.2)	1337	0.959	68 (46.9)	508	0.953	NA	NA

*CI, Confidence interval; ***P value* for differences between children with and without diarrhoea; NA, not applicable; Di, Diversity index.

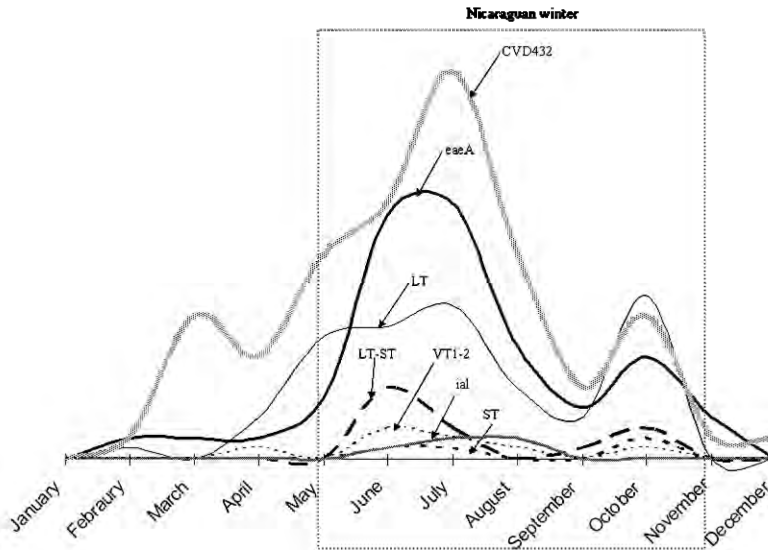


Figure 4. Diarrhoeagenic *E. coli* along with weather seasons in Nicaragua.

In study II, we also investigated whether the herein studied samples (n=526) may contain certain clonal groups of *E. coli*. Thus, the diversities among the *E. coli* isolates in these samples were examined. To explain a bit more, the total Di, as calculated in this study, was a simple shortcut to answer the question: Do we often find the same type (strain) in different samples in a given population, indicating an epidemic spread among the studied samples? The total Di of all 4009 (representing 526 samples) *E. coli* isolates was 0.97, which is similar to values that have been obtained in other studies on normal *E. coli* of faecal origin using PhP-RE plates (1, 102)**.** The Di values among isolates from various subpopulations were also calculated, and it was found that in some cases values lower than 0.97 were obtained, indicating that different infants were colonized by the same strains more often than in a normal population. This was observed for example in diarrhoeal children of 1 month (Di=0.859) and in hospitalized children with mucus stools (Di=0.890). Additionally, cluster analysis of data from all 4009 isolates revealed 24 common BPTs comprising 70% of the isolates, and 234 other BPTs found in only one or a few infants, comprising 30% of the isolates. The relative distribution of the common BPTs among the *E. coli* isolates from children with and without diarrhoea it shown in the above illustration (Figure 5). Most BPTs seemed to be distributed equally among the diarrhoeal and control children. However, some BPTs (e.g. 01, 03, 09, 18, 21 and 23) were more common among children with diarrhoea than in children without diarrhoea, whereas certain BPTs (e.g. 04 and 22) were more common among the control children. The most common type found in the present study was BPT06, comprising almost 387 isolates (9.7%). The biochemical fingerprint of this BPT could represent a clonal group of *E. coli* that is particularly common in the area of León.

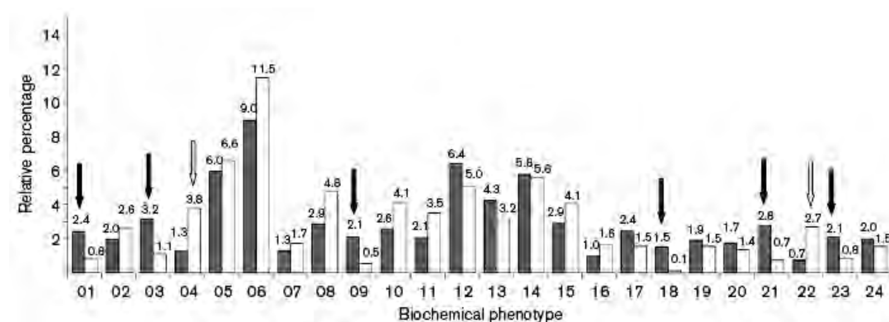


Figure 5. Relative distribution of the most common BPTs of *E. coli* found among the studied population, calculated as percentages of the total number of isolates within each group (i.e. diarrhoeal and control). Black arrows indicate BPTs that were found predominantly in children with diarrhoea, and white arrows indicate BPTs that were found predominantly in control children. These BPTs were significantly different between isolates originating from diarrhoeal patients and those of controls (P<0.01). Grey bars, diarrhoeal group (n=2900); white bars, control group (n=1109).

4.1.1. Enterotoxigenic *Escherichia coli* (ETEC)

The ETEC pathotype was identified significantly more often in children with diarrhoea (78/381; 20.5%) than in children without diarrhoea (12/145; 8.3%; P=0.001) in study I. Additionally, a higher prevalence was seen in children under 12 months of age, where also the significant difference (p<0.0001) in frequencies between children with (44/192; 22.9%) and without (5/88; 5.7%) diarrhoea was observed. Of the 78 children with diarrhoea harbouring ETEC isolates, 60 were *eltB*, 4 *estA* and 14 *eltB* - *estA*. In 12 of the non diarrhoea group children, ETEC was identified. However, these isolates were only positive for *eltB*, a finding which associates *estA* (p= 0.008) with diarrhoeal disease. Besides, isolates from samples positive for ETEC *eltB* showed somewhat decreased diversities in study II, whereas the low diversities among isolates from samples positive for ETEC *estA* was a clear indication that a limited number of BPTs were carriers of these virulence markers (Table 3). These BPTs could represent pathogenic clonal groups that have spread among the infants (Figure 6). Moreover, our results highlight the importance of ETEC as a cause of childhood diarrhoea in the studied region of Nicaragua. Unlike a study in Vietnam by Nguyen *et al.*, our study shows that the prevalence of ETEC in the diarrhoea group was significantly higher in children younger than 1 year of age as compared to older children; though, ETEC was detected in all age groups (71). Furthermore, our results support data from a previous prospective cohort study on the incidence of infection with ETEC in infants from Nicaragua, where it was shown that the highest incidence of ETEC diarrheal illness occurred during the first year of life (76). Thus, ETEC appears to cause secretory diarrhoea in the children population of León, early in life, well before 1 year of age, after which a high carriage rate is seen.

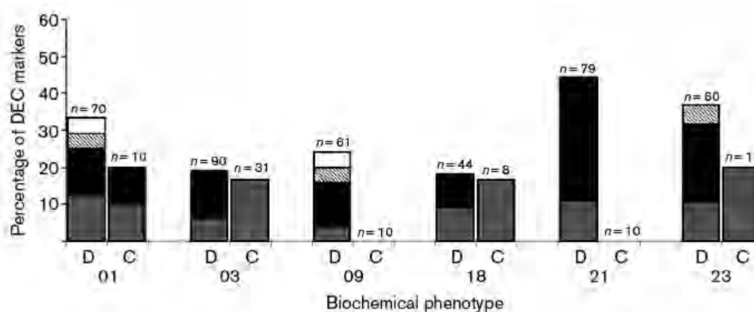


Figure 6. BPTs of *E. coli* found predominantly in children with diarrhoea and the proportion of samples presenting predominant DEC markers in these BPTs. The figure shows that *eltB+estA*-positive samples occurred mainly in BPTs 01 and 09 (*eltB*, heat-labile toxin gene; *estA*, heat-stable toxin gene). Furthermore, it can be seen that not only were these BPTs found more often in diarrhoeal children (Figure 5), but those from diarrhoeal samples were more often associated with virulence factors detected in the samples, especially types 09 and 21. D, Diarrhoeal group; C, control group. Grey bars, EPEC

(*eaeA* – attaching and effacing gene); black bars, ETEC (*eltB*); hatched bars, ETEC (*estA*); white bars, ETEC (*eltB*+*estA*).

4.1.2. Atypical enteropathogenic *Escherichia coli* (aEPEC)

EPEC is also a very important pathogen in children with diarrhoea. EPEC infection is primarily a disease of infants younger than 2 years of age. In study I, atypical EPEC (*bfp*$^{-}$ and *eaeA*$^{+}$) were detected in all age groups, but no statistically significant differences were seen in any of the groups. Additionally, isolates from children harbouring atypical EPEC showed similar diversities in the diarrhoea and the non diarrhoea groups in study II (Table 3). This finding has been seen in another study, where the EPEC pathotype was recovered more frequently from non diarrhoeal cases than from diarrhoeal (24). On the other hand, numerous case-control studies in many countries have found that EPEC is more frequently isolated from children with diarrhoea than from healthy controls (47, 67). In a study carried out in a northern city of Nicaragua, EPEC was found to be significantly more frequent in diarrhoeal (16%) than in healthy children (4%) (62). Conversely, it has also been shown that typical EPEC (*bfp*$^{+}$ and *eaeA*$^{+}$) is a cause of diarrhoea in developing countries, while atypical EPEC seems to be associated with diarrhoea in developed countries (3, 4, 47, 62, 96). In our study the identification rate of atypical EPEC from children with (61/381; 16%) diarrhoea was slightly lower than that from children without diarrhoea (30/145; 20.7%) diarrhoea. Our findings enlighten the variation in prevalence of one DEC type (EPEC), as causative agent of diarrhoea within the same country. Additionally, the role of atypical EPEC in diarrhoea in the present study was not established decisively.

4.1.3. Enteroinvasive *Escherichia coli* (EIEC)

EIEC isolates were identified at low frequencies in both the diarrhoea and the non diarrhoea groups in the first study. On the other hand, in study II isolates from samples positives for EIEC showed low diversities indicating the presence of a pathogenic clone (Table 3). Then again, the number of samples positive for EIEC was rather small. The low frequency of the EIEC pathotype in the studied population is, in general, in agreement with other studies performed in different parts of the world (30, 71).

4.1.4. Enteroaggregative *Escherichia coli* (EAEC)

EAEC was the most prevalent pathotype of *E. coli* identified in study I. However, no differences were seen between diarrhoeal (106/381; 27.8 %) and non-diarrheal children

(48/145; 33.1%). Moreover, the *E. coli* isolates from EAEC positive samples in study II showed the same diversity as isolates from non-DEC samples (Table 3). It is well known that this pathotype of *E. coli* display a characteristic "stacked brick"-like adhesion pattern when cultured with HEp-2 cells, and is an emerging pathogen associated with diarrhoea in children worldwide (34, 47). It has also been shown that EAEC is a heterogeneous group of *E. coli* (22, 44) and not all strains are capable of causing diarrhoea. Furthermore, the DNA probe (CVD432) used in study I to detect EAEC is a derivate from the pAA plasmid. This probe was first described by Baudry *et al.* in 1990, showing 89% sensitivity and 99% specificity (8). Since then, the CVD432 EAEC probe has remained as the most popular target in molecular assays for the detection of EAEC strains (19, 45, 85). However, this probe does not discriminate between the virulent and non-virulent EAEC strains. Hence, study III was initiated and 417 of 468 CVD432 originally positive isolates were subjected to a further characterization by detecting 11 genes encoding for putative virulence factors. Additionally, further characterization was done on 184 selected isolates by an adherence assay using Caco-2 cells in order to investigate their aggregative phenotype and to confirm their EAEC status. Thus, study III showed that, of the 184 isolates positive with the CVD432 probe, only 101 showed aggregative adherences (AA) to Caco-2 cells in a stacked-brick pattern. This ability of enteroaggregative *E. coli* strains to adhere in aggregative pattern in cell lines has been associated with the diarrhoeal outcome in different studies (2, 81). On the other hand, our results indicate that the CVD432 probe PCR do not correlate well with the adherence assay, as only 54.9 % of the originally CVD432 positive isolates were confirmed to have the phenotypic characteristic of enteroaggregative *E. coli*. Nevertheless, using the adherence assay as reference, a significant difference could be observed between EAEC positive isolates from children with (73/116; 62.9%) and without (28/68; 41.2%) diarrhoea ($P<0.05$). These differences were not observed with the CVD432 probe alone before. The most frequently detected putative virulence genes among all isolates tested for virulence markers were *aap* (78.7%), *aggR* (66.2%), *sat* (51.8%) and *sigA* (41.2%). Statistical analysis did not show any significant association of the herein identified genes to isolates from children with or without diarrhoea. Even though, other studies have shown a clear association of some of these markers with the diarrhoeal outcome (44, 45, 71, 80, 81). These findings corroborated the fact that isolates that belong to the EAEC pathotype are heterogeneous, and not all strains are capable of causing diarrhoea (47, 68). In addition, many variations of clinical symptoms due to EAEC infections have also been associated with host genetic susceptibility factors, besides the heterogeneity of the EAEC strains (46).

4.1.5. Enterohemorragic *Escherichia coli* (EHEC)

EPEC and EHEC share *eaeA* (the intimin structural gene), but the major virulence factors defining the characteristics of EHEC are the verocytotoxins. In study I, we identified a few stool samples positive for the EHEC pathotype from the children with diarrhoea (8/381; 2.1%), but none from the children without. In addition, isolates from samples positive for EHEC showed low diversities, an additional indication of the possible presence of pathogenic clones (Table 3). An example of an EHEC pathogenic clone is shown next (Figure 7). However, in general our results agree with the low prevalence of EHEC infection in developing countries (15, 92).

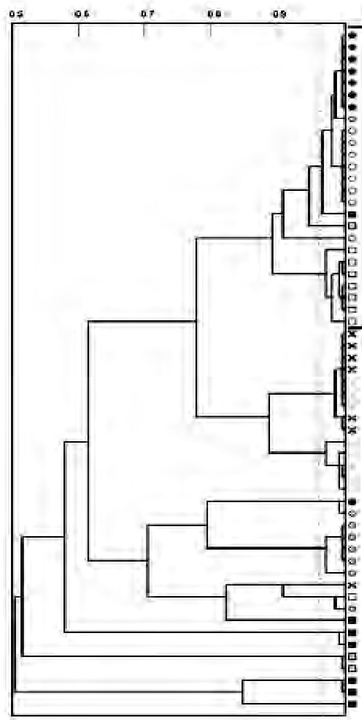


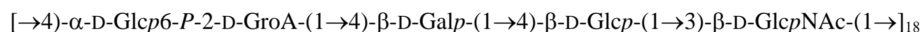
Figure 7. Dendrogram depicting 57 *E. coli* isolates from eight infant stool samples that were EHEC positive by multiplex PCR screening. The eight different symbols indicate isolates from the eight samples. The total diversity among the isolates was 0.829. The figure shows a cluster of identical or similar isolates (vertical solid line) from six different infants. These isolates could represent a virulent clonal group.

4.1.6. Diarrhoeagenic *Escherichia coli* and the severity of the diarrhoeal outcome

Clinical symptoms from the children with diarrhoea such as fever, vomiting, nausea, loss of appetite, abdominal pain, stool properties and the number of episodes per day, were recorded. In general the frequencies of symptoms were similar regardless of the identified DEC pathotype (study I). This suggests that, based on these clinical findings, an infection with e.g. ETEC could not be distinguished from an infection with atypical EPEC, EIEC, EAEC or EHEC. Nevertheless, when any of the DEC types was identified, the severity of the diarrhoeal cases were as follow: 139 cases (67.8%) were mild, 24 (11.7%) moderate and 42 (20.5%) severe. In the severe case group EAEC was identified in 22 children (32.4%), followed by ETEC in 19 (27.9%) and atypical EPEC in 14 (20.6%), either alone or in combination. This is of particular notice as the majority (42/68; 61.8%) of the children requiring intravenous rehydration therapy (IRT) due to severe dehydration harboured a DEC type. Additionally, a low diversity value (Di: 0.937) of the isolates coming from these children was obtained. Whereas the diversity values of the isolates from mild/moderate cases (oral rehydration therapy) and control children were 0.972 and 0.966, respectively (study II). Altogether, these findings suggest that the hospitalized children requiring IRT were colonized with virulent *E. coli* clones that were capable of causing a high degree of dehydration. Another example is presented in study III, where EAEC isolates showed marked differences between the groups of isolates from children with severe diarrhoea (10/12; 90%) and control children (28/68; 41.2%; *P= 0.007*), with a positive AA pattern.

4.2. The structure of the O-antigen polysaccharide from the enteroaggregative *E. coli* strain 94/D4 (Paper IV)

In study IV, the investigated *Escherichia coli* strain 94/D4 was isolated in 1991 from the fourth episode of an infant aged 9 months with acute diarrhoea in León, Nicaragua. The bacteria belong to the group of enteroaggregative *E. coli* (EAEC) and it was typed as O82:H8. Thus, the structure of the O-antigen polysaccharides (PS) from the EAEC strain 94/D4 and the international type strain from *Escherichia coli* O82 were determined. Component analysis and ^{1}H, ^{13}C and ^{31}P NMR spectroscopy experiments were employed to elucidate the structure. The O-antigen is composed of tetrasaccharide repeating units with the following structure:



[→4)-α-D-Glc*p*6-*P*-2-D-GroA-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→3)-β-D-Glc*p*NAc-(1→]$_{18}$

The first interesting feature about the O-antigen structure from the LPS of enteroaggregative *E. coli* 94/D4 here investigated is the chemical and immunochemical relationships to an *E. coli* strain belonging to serogroup O82 as confirmed by enzyme immunoassay using specific anti-*E. coli* O82 rabbit sera. The assay showed identical reactivity to the LPS of the two strains (Figure

8), in agreement with the structural analysis of their O-antigen polysaccharides. The serogroup O82 has been associated with Verocytotoxin-producing *E. coli* (VTEC) strains causing diarrhoea in humans (38).

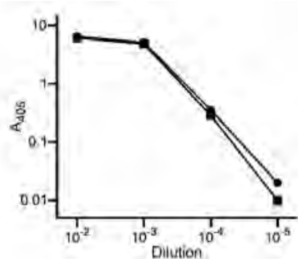


Figure 8. Enzyme immunoassay with rabbit anti-*E. coli* O82 sera titrated against LPS isolated from *E. coli* strain 94/D4 (■) and *E. coli* strain O82 (●).

Second, the phosphate glyceric acid moiety in the O-antigen polysaccharide from *E. coli* strain 94/D4, is similar to that present in a S-layer glycoprotein from *Bacillus alvei* CCM 2051 (Figure 9) (63). This similarity reflects on the suggested hypothesis that the architecture of the S-layer glycans of the *B. alvei* resembles that of polysaccharides of Gram-negative bacteria (54, 63).

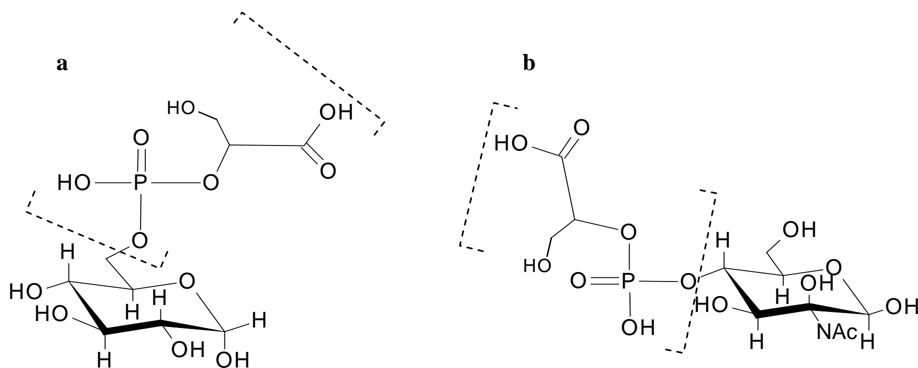


Figure 9. (a) α-D-Glc*p*6-*P*-2-D-GroA present in the O-antigen from *E. coli* 94/D4; (b) β-D-Man*p*NAc4-*P*-2-D-GroA present in the repeating unit of a glycan from the S-layer glycoprotein of *B. alvei* CCM 2051.

Third, besides having the unusual phosphodiester-linked substituent, the herein studied *E. coli* O-antigen contains the disaccharide lactose as a structural element. This substituent has been previously reported in an *E. coli* for the O21 O-antigen polysaccharide and the EAEC strain 105 (Figure 10). The EAEC 105 strain was also isolated in 1991 from a Nicaraguan child with diarrhoea (90). Thus, here we have two different EAEC strains circulating in the same geographic area at the same time, expressing heterogeneity in their surface O-antigens. However, the O-antigen structures contain a probable common epitope.

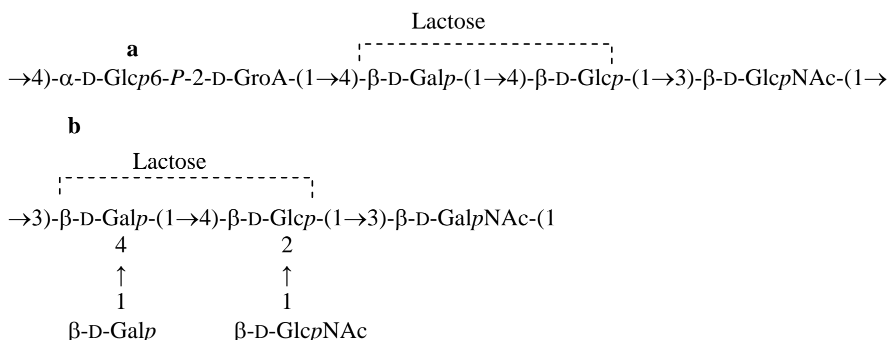


Figure 10. (a) The lactose moiety present in O-antigen EAEC 94/D4; (b) and O-antigen EAEC 105.

Fourth, together with the heterogeneity of the biological repeating unit, the O-antigen has a 3-substituted *N*-acetyl-D-glucosamine residue at its reducing end. This suggests that the biosynthesis of the O-antigen in this bacterium took place by the polymerase-dependent pathway typical for heteropolysaccharides (91). This pathway is one of the three that have been reported for the biosynthesis of the O-chain part of LPS in Gram-negative bacteria. Finally, the O-antigen from EAEC 94/D4 has ~18 repeating units on average. The consequences of having such a long polymer are not easy to explain. However, it can perhaps be associated with a better ability of these strains to evade the immune system of the host. This could be as a result of surface thickness in the bacterium outer cell wall conferred by the length of O-antigen.

5. CONCLUSIONS

The studies presented in this thesis focused on the identification, molecular and phenotypic characteristics of diarrhoeagenic *E. coli* pathotypes as enteric pathogens associated with diarrhoea in children from León, Nicaragua.

The results from study I to III can be summarized as follows:

1. Five DEC types have been identified in 526 faecal samples from children less than 5 years of age. Additionally, the majority of the *E. coli* positive for a DEC type occurred alongside with the winter season in Nicaragua, where most of the diarrhoeal cases also occur.

2. The ETEC pathotype was identified with significantly higher prevalence in children with compared to children without diarrhoea. Additionally, a higher prevalence was seen in children less than 12 months of age. The identified ETEC were distributed as follow: *eltB* positive were the more prevalent, follow by *eltB-estA* and finally *estA* positive. In terms of phenotypic characteristics, isolates from ETEC positive samples seem to be pathogenic clonal groups as their low diversities indicate. Also, there was a clear differentiation of those BPTs that predominate in children with to that in children without diarrhoea. Furthermore, a limited number of BPTs grouping *estA* positives ETEC and the fact that *estA* ETEC samples only happen in children with diarrhoea suggest the spread of a marked pathogenic clonal group.

3. Atypical EPEC (only *eaeA* positive) was identified with similar frequencies in children with and without diarrhoea. Additionally, clonal group analysis of atypical EPEC positive samples showed no differences either between children with and without diarrhoea.

4. EIEC was identified at low, but similar frequency in both children with and without diarrhoea. Although EIEC positive samples showed low diversities suggesting a pathogenic clonal group, the number of samples positives for EIEC was rather small.

5. EAEC was the most prevalent pathotype of *E. coli* identified. However, no differences were observed between children with and without diarrhoea in terms of prevalence and phenotypic fingerprinting characteristics. Although, a difference was appreciated between EAEC isolates showing aggregative adherence to Caco-2 cells among EAEC isolates from children with and without diarrhoea. On the other hand, beside the CVD432 marker

original used to identify EAEC strains, a great variety of putative virulence markers combination was observed, but no differences were again appreciated between children with and without diarrhoea.

6. EHEC was only identified in samples from children with diarrhoea. The herein identified EHEC were distributed as follow: *vt2* positive were more prevalent, followed by *vt2-eaeA* and *vt1* positive, at similar frequencies. In addition, the low diversity of EHEC positive samples suggests the presence of pathogenic clonal groups, though the general prevalence of this pathotype was rather small.

7. Even though no statistical differences were appreciated for EAEC and atypical EPEC, a finding is that the majority of the group of children that required IRT harboured EAEC, ETEC and EPEC either alone or in combination, but with no other enteric pathogen. Additionally, the low diversity value of the isolates from these children suggest that these strains may represent a virulent clone capable of causing a more severe case of diarrhoea.

8. In summary, the ETEC pathotype play an important role in diarrhoea in children less than 5 years of age in León, Nicaragua. Nonetheless, EAEC, EPEC and EHEC pathotypes are to some extent important pathogens associated with diarrhoea of those children.

The main result from study IV is:

1. The structure of the O-antigen polysaccharides (PS) from the EAEC strain 94/D4 and the international type strain *Escherichia coli* O82 were determined. The O-antigen is composed of tetrasaccharide repeating units with the following structure:

$[\rightarrow 4)\text{-}\alpha\text{-}\textsc{d}\text{-Glc}p6\text{-}P\text{-}2\text{-}\textsc{d}\text{-GroA-}(1\rightarrow 4)\text{-}\beta\text{-}\textsc{d}\text{-Gal}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-}\textsc{d}\text{-Glc}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-}\textsc{d}\text{-Glc}p\text{NAc-}(1\rightarrow]_{18}$

6. ACKNOWLEDGEMENTS

I would like to thanks the people who directly or indirectly contributed to the realization of this thesis:

Professor Andrej Weintraub*, my main supervisor and the boss, for being wholehearted with our projects, scientific minded, encouraging, patient, for being not just my supervisor, for being a friend. Thanks for sharing your knowledge with me through many fruitful discussions about science and everything.It has been a privilege to be your student.* ***"You are the boss".***

Dr. Felipe Urbina, *my co-supervisor for introducing me into the field of biochemistry and for giving me the chance to work in the* ***Dr. Ernesto Medina*** *(former Rector UNAN-León) and yours joined project on bacterial antigens. Also, thanks to* ***Dr. Edmundo Torres*** *for giving me the opportunity to work with you on laboratory quality assurance, at the Vice-rectory office of research and postgraduate studies (VIP)****.*** *Thanks to the three of you for believing in me since the very beginning. Many thanks to* ***Dr. Carlos González*** *(for sharing your knowledge and experience),* ***Mercedes Corea****, and* ***Yami,*** *for the nice moments at the VIP office.*

Professor Göran Widmalm*, thanks for nice collaboration and for always answering my questions about carbohydrate chemistry and their possible biological implications.*

Professor Carl Erik Nord, *former head of the Division of Clinical Bacteriology, for allowing me to work in your lab, your support and good inputs in our projects.*

Professor Anders Vahlne*, head of the Division of Clinical Microbiology,* ***Professor Bengt Wretlind, Professor Gunnar Sandström****,* ***Professor Charlotta Edlund*** *and* ***Professor Matti Sällberg*** *for good comments during seminars.*

Present and former colleagues from former Bacteriology, for making such a nice lab-environment, ***Sonja, Hanna G., Axana*** *(thanks for the caco-2 cells)****, Cristina, Hanna B., Haihui, Benjamin, Amir, Oonagh, Tara, Anna R., Anna F., Sohel, Mamun.*** *The newly joined Virology,* ***Sarene, Malin, Sepideh, Antony and Halime*** *(the fun office)****, Lars, Erwin, Alenka, Annica, Masoud, Maria, Gustaf, Samir, Piotr, Babi, Eva, Anna and Alberto.***

Monica Sörensson, *I have no words to thank you. Thanks for all the lab and life tricks you have taught me over the years. Thanks for being my adopted Swedish mom. Also, thanks to you and* ***Roffe*** *for such nice times I spent at your house.*

Ann-Chatrin Palmgren, Kerstin Bergman and Åsa Sullivan *(the PhD students' moms), thanks for such care and concern for all PhD students from Bacteriology.*

Gudru Rafi, *thanks for all the help with paper work when I needed and for the good language comments of the thesis. Also, thanks to* ***Karin*** *for the handling of disposal material and keeping our stock of glassware always clean.*

Thanks to ***Nora****, Prof. Andrej's wife for all the meals you have prepared for us. For always welcoming me into your Stockholm and Summer homes. Thanks for such of nice spent times.*

Pierre*, the little brother. Life is too short…just keep on being yourself. Thanks for the labelling of those hundreds eppendorf tubes.*

Professor Roland Möllby, Inger Kühn and Patricia Colque *(for your honest concern about Nicaraguans)****,*** *thanks to all of you for your help, support and fruitful collaboration all these years.*

My Nicaraguan friends with whom I have the pleasure to share this experience of pursuing the dream of becoming a PhD, so far from home and families, to ***Erick, Daniel, Filemón and Fernando.*** *For all what we have aprendido, vivido, sufrido and celebrado through the years.*

Thanks to ***Dr. Rigoberto Sampsom*** *former Rector of UNAN-León (R.I.P);* ***Dr. Rodolfo Peña****, Dean of the Faculty of Medical Sciences;* ***Dra. Mercedes Cáceres****, Vice-dean of the faculty, for your support through the years. Members of the Microbiology and Parasitology Department at UNAN-León,* ***Orlando****,* ***Byron, Teresa, Alleyda, Kenia, Eugenia, Silda, William, Rosario, Isabel, Edelma and Elizabeth,*** *for your support. Also, thanks to* ***Patricia, Soledad*** *and Doña* ***Silvia****.*

Margarita and Felix*, with you I had the first notion about the importance of children diarrhoeal disease in Nicaragua. Thanks for sharing your more than 15 years of experience on the matter.*

To my parents in law *for all your support and helping with my baby girl, my sister in law* ***Martha*** *for being a second mom to Fiorella.*

Thanks to ***my family****, my Papá* ***Bayardo,*** *for always reminding me the importance of education and being there when I need you. My mom* ***Mercedes,*** *for your love and help with Fiorella when I was and was not around. My Grandma* ***Candida****, my aunts* ***Olivia*** *and* ***Lola****, my brothers* ***Freddy*** *and* ***Uriel,*** *and my sister* ***Isabel*** *for all your love and support. Also to the rest of the family I've not mentioned here.*

Finally, but not least to ***Anielka,*** *for all your love and support through the years. For your patience and for being such a strong woman. When we got married I was starting the PhD studies (some 4 years ago). Soon, we got* ***Fiorella*** *(our amazing built-up character little girl). Since she was born I've been on and off with you guys, most of the time in Sweden. Now, we have* ***Mateo****, and I was not there when he joined us. But, I promise, I will do the impossible to be with you guys from now on. I am not goin' to forget about science, thou!*

May the Lord accomplish his purpose in all of the above people mentioned.

This work was supported by UNAN-León, grants from the Swedish Agency for Research Cooperation with Developing Countries (Grant 2004-0671), Swedish Research Council (VR) and The Knut and Alice Wallenberg Foundation**.**

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