VIRULENCE FACTORS AND CLONAL RELATEDNESS OF ENTEROTOXIGENIC *ESCHERICHIA COLI* (ETEC) ISOLATED FROM CHILDREN WITH DIARRHOEA IN BOLIVIA

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To my lovely parents
Justo and Virginia

My son, keep your father's commands
and do not forsake your mother's teaching.

Bind them upon your heart forever;
fasten them around your neck.

When you walk, they will guide you;
when you sleep, they will watch over you;
when you awake, they will speak to you.

For these commands are a lamp,
this teaching is a light,
and the corrections of discipline
are the way to life.

Proverbs 6: 20-23

Virulence factors and clonal relatedness of enterotoxigenic *Escherichia coli* (ETEC) isolated from children with diarrhoea in Bolivia

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Enterotoxigenic *Escherichia coli* (ETEC) is one of the major causes of diarrhoea in children under five years of age in developing countries and travellers to these settings. To cause disease, ETEC must be able to colonise the small intestine and produce heat-stable enterotoxin (ST) or heat-labile toxin (LT) or both toxins. The attachment of ETEC is mediated by different fimbrial antigens, known as colonisation factors (CFs); at least 22 CFs have been identified to date. Besides determination of the toxins and CFs, serotyping has been used to identify and characterize ETEC. However, there is a lack of rapid and simple standardized methods to detect ETEC in many laboratories. In recent years, PCR-based assays have been increasingly common and due to their simplicity and commercially available reagents for diagnostic purposes with the ability to obtain good specificity and sensitivity.

In order to speed up the detection of CFs by PCR, we have established a multiplex PCR assay for detection of ETEC CFs, using previously established CF primers. The published CF primers were assembled into four panels designed to amplify 19 CFs in four PCR reactions. This test was used to amplify on two ETEC strain collections from Bolivia and Bangladesh isolates from children with diarrhoea.

We have also determined the relation between enterotoxins, CFs and serotypes as well as the antimicrobial resistance patterns in 43 ETEC strains isolated from hospitalized children with acute diarrhoea in Bolivia during the period 2002 to 2006. Among the ETEC isolates tested, 30 were positive for LT, 3 for STh and 10 for LT/STh. Sixty-five percent of the strains expressed one or more of the CFs; the most common ones were CS17 (n=8) and CFA/I (n=8). The most common serotypes were O8:H9 LT/CS17 (n=6) and O78:HNM LT/ST CFA/I (n= 4); 67% of the strains were resistant to one or several of the antimicrobial agents tested for.

Phylogenetic analyses are used to localize outbreaks of ETEC disease and to trace disease over time in different geographic regions. Multilocus sequencing Typing (MLST) method has recently been used to determine the genetic variations and clonal lineages of this pathogen. We have investigated 24 ETEC strains isolated from US adult travellers and infected resident children in Mexico and Guatemala that were infected with ETEC strains. These strains expressed ST/CS6 and 7 MLST sequence types (ST), being the most common: ST-398 (n=10), ST-182 (n=6) and ST-278 (n=4) expressing STp and carrying genetically identical CS6 sequences the cause of disease.

We also determined the genetic relatedness of ETEC strains obtained from a cohort of children with diarrhoea in Bolivia. We found 2 different LT/CS17 clones in ETEC strains; one of them had a specific molecular signature and persisted in La Paz from 2002 to 2005. By using the MLST method and sequencing the CS17 operon, we compared the Bolivian LT/CS17 isolates to Bangladeshi LT/CS17 ETEC strains isolated from children with diarrhoea. A common clone was identified which was identical to LT/CS17 strains isolated in subsequent studies in 2007 and 2009 in Bolivia indicating that this is a persistent clone that circulated in Bolivia for at least eight years.

Finally, we analysed toxin production in 54 ETEC strains collected during 2 summer periods in Bolivia and compared these results to Bangladeshi, Egyptian and Guatemalan ETEC strains. Findings showed a high production of toxins in Bangladeshi and Egyptian ETEC strains, followed by the Guatemalan and a low production in Bolivian strains. No association between severity of disease and toxin production was found among Bolivian ETEC strains.

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ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by the Roman numerals (I – V):

I. **Rodas C**., Iniguez V., Qadri F., Wiklund G., Svennerholm A-M. and Sjöling Å. Development of Multiplex PCR assays for detection of enterotoxigenic *Escherichia coli* (ETEC) colonisation factors and toxins.
J Clin Microbiol 2009; 47(4):1218-1220

II **Rodas C.,** Mamani R., Blanco J., Blanco J.E., Wiklund G., Svennerholm A-M., Sjöling Å., and Iniguez V. Enterotoxins, colonisation factors, serotypes and antimicrobial resistance of enterotoxigenic *Escherichia coli* (ETEC) strains isolated from hospitalized children with diarrhoea in Bolivia.
Accepted for publication in Brazilian Journal of Infectious Diseases

III Nicklasson M., Klena J., **Rodas C**., Torres O., Bourgeouis AL., Svennerholm A-M. and Sjöling Å. Enterotoxigenic *Escherichia coli* multilocus sequence types in Guatemala and Mexico.
Emerging Infectious Diseases 2010; 16(1):143-6.

IV **Rodas C.**, Klena J., Nicklasson M., Iniguez V. and Sjöling Å. Clonal relatedness of enterotoxigenic *Escherichia coli* (ETEC) strains expressing LT and CS17 isolated from children with diarrhoea in La Paz, Bolivia.
Submitted

V **Rodas C.**, Iniguez V., Svennerholm A-M. and Sjöling Å. 2010. Clinical isolates of enterotoxigenic *Escherichia coli* (ETEC) from children in Bolivia cause severe diarrhoea but produce comparatively low levels of the heat labile (LT) and heat stable (ST) enterotoxins.
Submitted

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ABBREVIATIONS.

ARFs	ADP-ribosylating factors
A/E	attaching and effacing
ADD	acute diarrhoeal diseases
ADP	adenosis diphospate
adk	adenylate kinase
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
CF	colonisation factor
CFA	colonisation factor antigen
CFs	colonisation factors
CS	coli surface
DNA	deoxyribonucleic acid
DAEC	diffusely adherent *Escherichia coli*
E. coli	*Escherichia coli*
ELISA	enzyme-linked immunosorbent assay
ETEC	enterotoxigenic *Escherichia coli*
EAEC	enteroaggregative *Escherichia coli*
EPEC	enteropatogenic *Escherichia coli*
EIEC	enteroinvasive *Escherichia coli*
EHEC	enterohemorragic *Escherichia coli*
EAEC	enteroadherenth *Escherichia coli*
fumC	fumarate hydratase
GC	guanylate cyclase
GM1	monosialotetrahexosylganglioside
gyrB	DNA gyrase
H-NS	histone-like nucleoid structuring protein
HUS	Hemolityc Uremic Syndrome
IBMB	Instituto de Biologia Molecular y Biotecnologia
icd	isocitrate/isopropylmalate dehydrogenase
LB	Luria Bertani culture medium
LT	heat-labile toxin
LPS	lipopolysacaride
MAb	monoclonal antibodies
mdh	malate dehydrogenase
MLEE	multilocus enzyme electrophoresis
MLST	multilocus sequencing typing
OPD	orthophenylenediamine
ORT	oral rehydration therapy
PFGE	pulsed-field gel electrophoresis
purA	adenylossuccinate dehydrogenase
ST	heat-stable toxin
ST	sequence type
STh	human heat-stable toxin
STp	porcine heat-stable toxin
PCR	polymerase chain reaction
RAPD	random amplification of polymorphic DNA
recA	ATP/GTP binding motif
RT-PCR	real time polymerase chain reaction
RFLP	restriction fragment length polymorphism

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1. INTRODUCTION.

1.1. Diarrhoeal diseases are a major health problem.

Acute diarrhoeal diseases are still recognized as one of the major causes of child morbidity and mortality worldwide after pre and postnatal deaths and acute respiratory diseases (Black *et al.,* 2010). Diarrhoeal infections are estimated to contribute to the death of 1.3 to 6 million children per year mainly in developing countries in Asia, Africa and Latin America (Torres *et al.,* 2001; Shah *et al.,* 2009; Weil *et al.,* 2009; Cheun *et al.,* 2010; Nweze, 2010; Black *et al.*, 2010).

In developing countries children might suffer from 2 to 12 episodes of diarrhoea per year, usually with the highest frequency during the first 2 years of life (Qadri *et al.,* 2000a). Repeated severe or persistent diarrhoea can damage the epithelium and reduce nutrient uptake which can cause malnutrition (Guerrant *et al.,* 2008). Micronutrient deficiencies, for example vitamin A and zinc, which are important for the development of the immune system (Wieringa *et al.,* 2004), are very common in developing countries and deficiency in vitamin A and zinc generally increases the morbidity of diarrhoea (Rahman *et al.,* 2001). This is possibly due to a higher bacterial load that can attach on the surface of the intestinal mucosa in immunocompromised malnourished children (Qadri *et al.,* 2005).

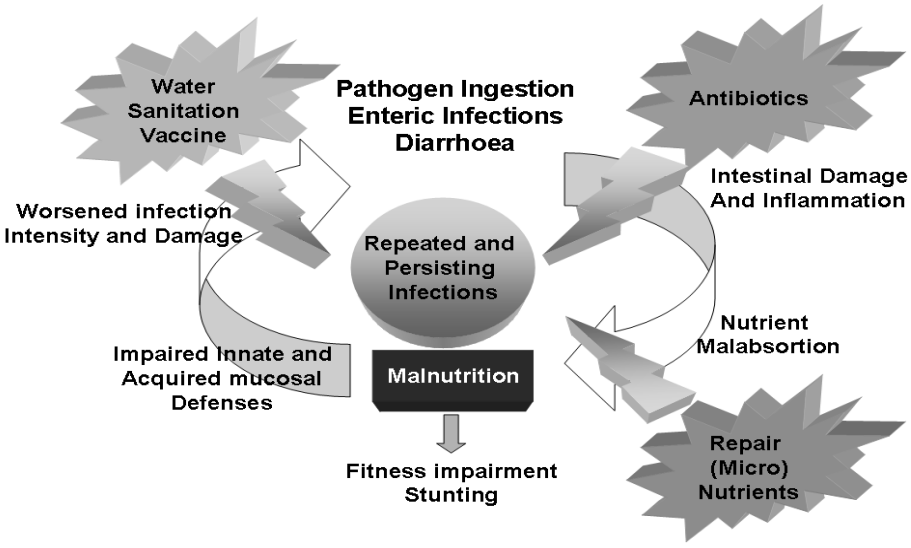

Figure 1. The vicious cycle between malnutrition and diarrhoea and intervention strategies to break it.

These cycles of increased susceptibility and repeated infections can cause malnutrition and in the worst cases stunting on the child (Checkley *et al.,* 2008) (Figure 1). Older children and adults that suffer from diarrhoea are often unable to attend school or go to work and hence diarrhoeal diseases may have an important socioeconomic impact on entire populations in endemic areas.

1.2. Severe diarrhoea is food and water-borne and mainly cased by virus and bacteria.

Diarrhoea is usually caused by pathogenic bacteria, virus or by protozoa and is believed to be transmitted mainly by contaminated food and water. The most prevalent pathogens associated to severe diarrhoea are: Rotavirus, diarrhoeagenic *Escherichia coli, Vibrio cholerae, Salmonella spp*, *Shigella spp* and *Campylobacter jejuni* (Birmingham *et al.,* 1997; Thielman *et al.,* 2004). Rotavirus is the main cause of diarrhoeal cases worldwide and every year cause an estimated number of 600.000 deaths mainly in children under 5 years old (Parashar *et al.,* 2003; Widdowson, 2009).

Among the bacterial pathogens, diarrhoegenic *Escherichia coli* strains are one of the important causes of childhood diarrhoea around the world, especially in developing countries (Clarke, 2001). Outbreaks of cholera (caused by *V. choleare*), shigellosis (*Shigella*) and typhoid fever (*Salmonella*) most often occur in resource poor populations such as refugee camps and groups living in shanty towns with insufficient access to clean drinking water adding to the burden of disease among the most vulnerable individuals in poor countries.

Diarrhoea can be classified into three major types: acute watery diarrhoea, dysentery (bloody diarrhoea) and persistent diarrhoea. Acute watery diarrhoea can cause severe dehydration that could lead to hospitalization and death. Enterotoxigenic *Escherichia coli* (ETEC) and rotavirus are the most common agents associated with this type of diarrhoea in children, affecting mostly infants and children less than 2 years. *Vibrio cholerae* can cause outbreaks of dehydrating diarrhoea in all ages and fatal cases in the absence of immediate rehydration. Dysentery is more associated with *Shigella spp*, which causes death through bacteremia or hypoglycemia. Persistent diarrhoea, which by definition lasts longer than 14 days, can severely affect the nutritional status and hence mortality, more so than acute watery diarrhoea. Enteroaggregative *Escherichia coli* (EAEC) and the protozoa *Cryptosporidium spp.* have mainly been associated with persistent diarrhoea (Kosek *et al.,* 2003).

1.3. Pathogenic *Escherichia coli.*

Escherichia coli (*E. coli*), a member of the *Enterobacteriacea* family, is a Gram negative facultative anaerobic bacilli which normally colonises the gastrointestinal tract of human infants within a few hours after birth as part of the human normal flora and establishes a relation between host and bacteria for a common benefit. Since *E. coli* is a part of the normal intestinal flora it is considered as an indication of faecal contamination when it is present in water and food (Humbert *et al.,* 2000).

Although *E. coli* can cause disease in immunocompromised individuals or when entering the blood (Glauser *et al.,* 1984), it is regarded to be a commensal bacterium. However, there are many pathogenic *E. coli* strains that can cause different diseases in animals and humans (Nataro & Karper, 1998; Donnenberg *et al.,* 2001). The pathogenic *E. coli* usually harbours pathogenic plasmids or pathogenic islands within the genome that carry the virulence properties. Most of the pathogenic *E. coli* are transmitted via faecal-oral routes from person to person through water or food and pathogenic *E. coli* can produce different clinical diagnosis, such as diarrhoea, urinary tract infections and kidney infections (Humbert *et al.,* 2000).

The *E. coli* strains that cause diarrhoea can be classified into six categories based on their mechanism of pathogenesis and clinical diagnostics: Enterotoxigenic *E coli* (ETEC) which is commonly known as traveller's diarrhoea and causes acute watery diarrhoea, Enteropathogenic *E coli* (EPEC) which causes attaching and effacing (A/E) lesions resulting in osmotic diarrhoea, Enteroinvasive *E coli* (EIEC) which causes a *Shigella*-like dysentery, Enterohemorrhagic *E coli* (EHEC) which causes hemorrhagic colitis or hemolytic-uremic syndrome (HUS), Enteroaggregative *E coli* (EAEC) is primarily associated with persistent diarrhoea in children in developing countries, and diffusely adherent *E coli* (DAEC) which may induce inflammatory bowel diseases. ETEC, EPEC, EAEC, and DAEC colonise the small intestine while EIEC and EHEC preferentially colonise the large bowel prior to causing diarrhoea (Rodriguez, 2002) (Figure 2).

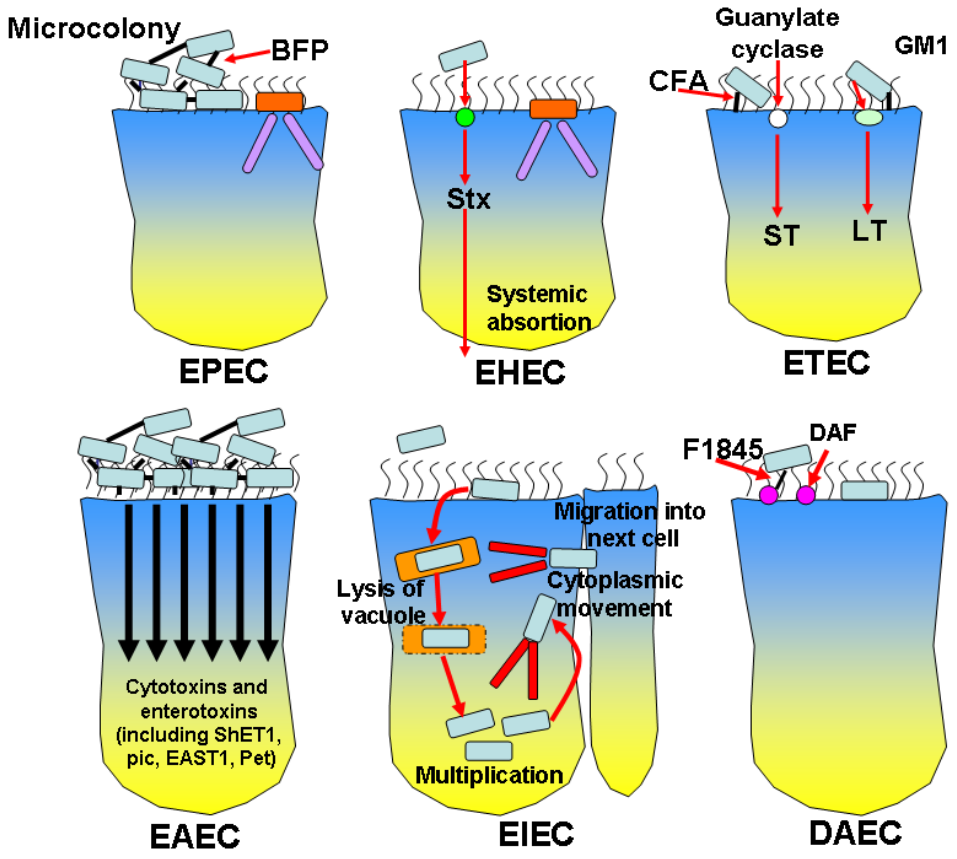

Figure 2. Pathogenic schemes of diarrhoeagenic *E. coli*. The six recognized categories of diarrhoeagenic *E. coli* each have unique features in their interaction with eukaryotic cells (modified from Nataro and Kaper, 1998).

1.4. Enterotoxigenic *Escherichia coli* (ETEC).

The focus of this thesis is on ETEC which is responsible for the majority of pathogenic *E. coli*-mediated cases of human diarrhoea worldwide. ETEC causes watery diarrhoea, which can range from mild, self limiting disease to severe purging disease. ETEC is an important cause of childhood diarrhoea in the developing world where sanitation and clean supplies of drinking water are inadequate and it is the main cause of diarrhoea in travellers to developing countries (Nataro *et al.,* 1998; Crossman *et al.,* 2010). It is estimated that there are around 200 million incidences of ETEC infection every year with an estimated number of 380.000 deaths in children under five years of age (Wennerås and Erling, 2004; Qadri *et at.*, 2005).

1.5. Virulence factors of ETEC.

ETEC is mainly characterized by two types of virulence factors: the enterotoxins [Heat stable toxin (ST) and Heat labile toxin (LT)] and the colonisation factors (CFs) which mediate adherence to the enterocytes of the intestine (Figure 3).

ETEC attaches to specific receptors on the surface of enterocytes of the intestinal lumen by virtue of their CFs which are commonly hair-like fimbriae. More than 22 types of fimbrial antigens, called coli surface (CS) antigens or colonisation factor antigens (CFAs) have been described (Gaastra and Svennerholm, 1996). When ETEC colonises the surface of the small bowel mucosa by virtue of the CFs, it elaborates enterotoxins, which give rise to intestinal secretion (Turner *et al.,* 2006; Nataro and Kaper, 1998).

ETEC produces two toxins: a heat-stable toxin (ST) and a heat-labile toxin (LT). Although different strains of ETEC can secrete either one or both of these toxins, the illness caused by each toxin is similar (Blackburn *et al.,* 2009). Without the colonisation factor adhesins, ETEC would probably be eliminated by the peristaltic movement of the small intestine resulting in less diarrhoea even if the enterotoxins are produced (Gaastra and Svennerholm, 1996; Kaper & Nataro, 1998; Qadri *et al.,* 2005; Turner *et al.,* 2006).

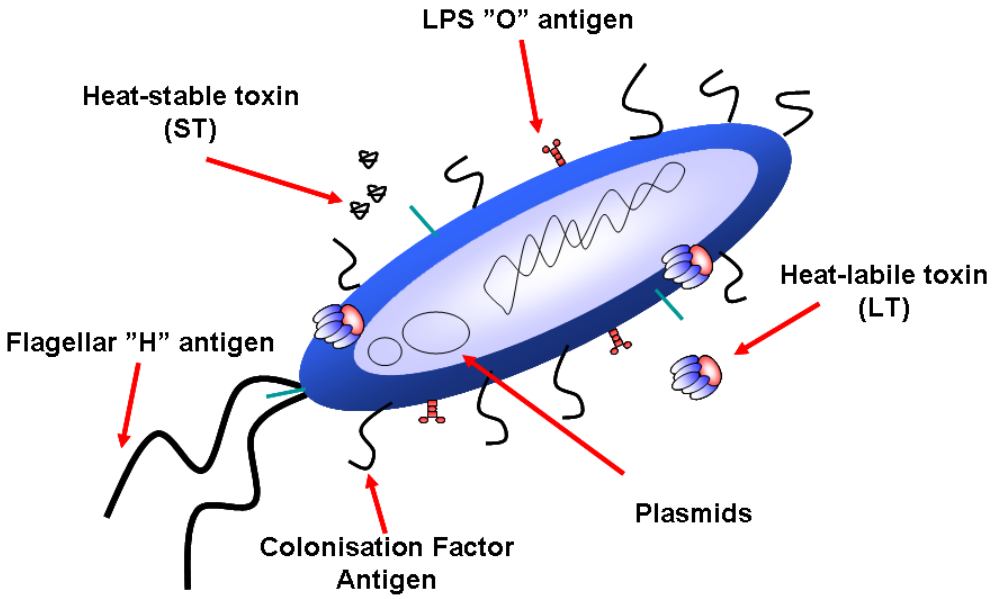

Figure 3. Scheme of the components of enterotoxigenic *Escherichia coli* (ETEC)

1.5.1. Toxins.

1.5.1.1. Host activity and bacterial regulation of the LT toxin.

The LT enterotoxin is a 86 kDa AB_5 toxin with homologous activity, immunogenicity and features as the cholera toxin (the proteins share 82% amino acid homology). LT is composed of five B subunits that bind to the enteric GM1 ganglioside receptors in the intestinal epithelium, and a single enzymatically active A subunit whose ADP-ribosylating activity leads to activation of cellular adenylcyclase and an increase in cAMP, efflux of chloride ions and water and subsequent watery diarrhoea (Freytag and Clements, 1999) (Figure 5).

LT is encoded by the *eltAB* operon which is regulated by the global bacterial regulators CRP and histone-like nucleoid structuring protein (H-NS) (Trachman and Maas, 1998; Robins-Browne and Harltland, 2002; Bodero and Munson, 2009). The translated peptides pass through the inner membrane by the Sec dependent pathway (Mudrak and Kuehn, 2010). Once in the periplasm, the subunits are rapidly assembled to the mature form of the AB_5 holotoxin in a process that is DsbA dependent protein (Tauschek *et al.,* 2002). The type II secretion system in the outer membrane of the gram negative ETEC is essential for LT secretion through the outer membrane (Figure 4A).

The mechanism by which LT is secreted by ETEC into the extracellular space is still controversial. Early studies showed that LT remains associated to the membrane and that only a minor proportion of the produced LT is secreted into the extracellular medium (Hirst *et al.,* 1984; Sanchez and Holmgren, 2005). Recent studies have proposed that after secretion through the outer membrane, the toxin binds to lipopolysaccharide (LPS) on the extracellular surface of the bacteria through the B subunits (Horstman and Kuehn, 2002; Horstman *et al.,* 2004) and that the main delivery of the toxin is through the release of outer membrane vesicles loaded with LT on their surface and periplasmic interior (Kuehn and Kesty, 2005). However, this has been debated by others (Sanchez and Holmgren 2005; Jansson *et al.,* 2009). Still, we and others have found that LT is indeed secreted into the exterior in some strains (Lasaro *et al.,* 2008).

1.5.1.2. Host activity and bacterial regulation of the ST toxin.

The ST is an 18-amino acid (STh initially isolated from humans) or 19-amino acid (STp initially isolated from pigs but causing disease in humans) highly folded peptide which also causes disruption of chloride channels in the cell leading to secretory diarrhoea (Figure 5).

ST is expressed in about 66% of ETEC strains, either alone or in combination with LT, and thus is significantly responsible for the worldwide disease burden of ETEC (Qadri *et al.,* 2000a). The ST toxin is encoded by different genes for STh and STp and at least STh has been shown to be under catabolite repression through the regulation of CRP (Bodero and Munson, 2009). The ST genes are transcribed into "preproprecursors" (Rasheed *et al.,* 2006) but the short mature ST is secreted through the TolC channel and folded into its mature form by 4 cystein bridges (Figure 4B).

The main receptor for the secreted ST toxin is a transmembrane enzyme, guanylate cyclase (GC) located in the apical membrane of the intestinal cells. When ST binds to GC it promotes an increase in intracellular levels of cyclic guanosine monophosphate (cGMP). The increase in cGMP allows activation of CFTR through phosphorylation-dependent cGMP protein kinase II generating an increase in salt and water secretion and inhibition of sodium absorption via the apical Na/H channel (Nair and Takeda, 1998; Vaandrager, 2002) (Figure 5).

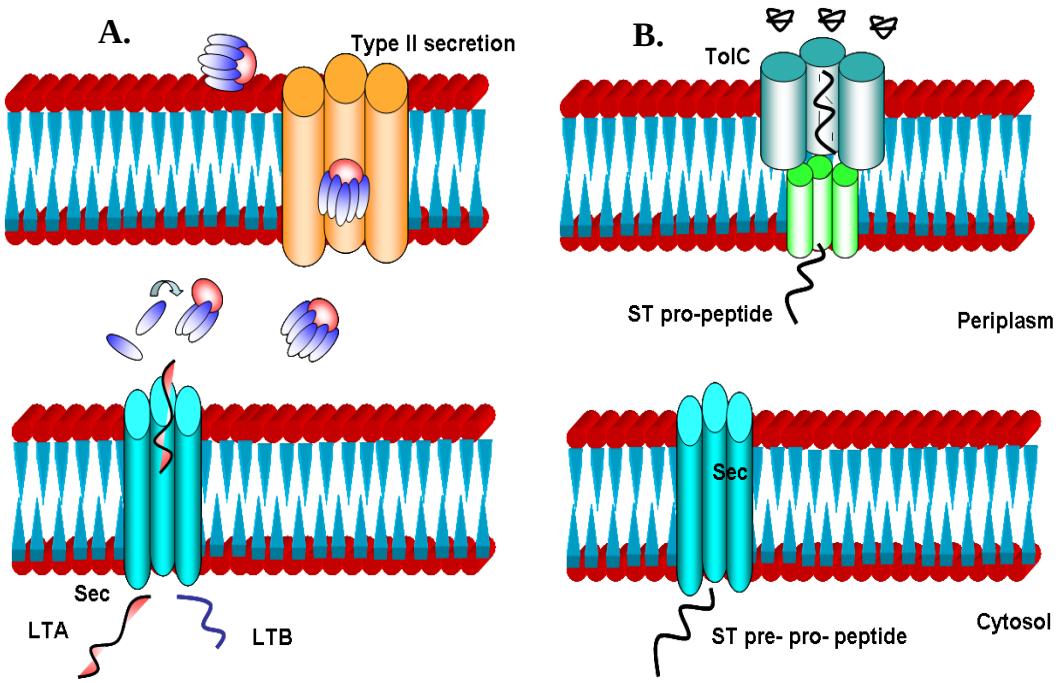

Figure 4. Mechanisms of assembly and secretion of A. LT and B. ST enterotoxins through the inner and outer membranes of the gram-negative ETEC

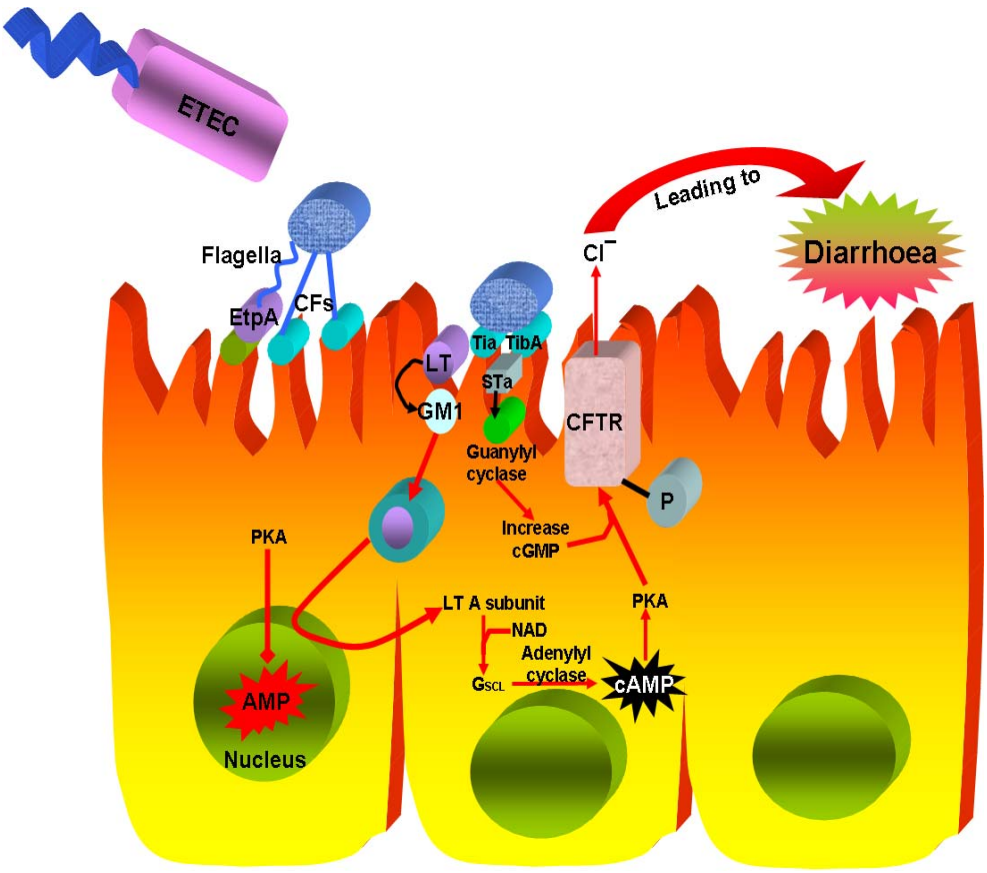

Figure 5. Mechanism of virulence of enterotoxigenic *Escherichia coli* (ETEC)

1.5.2. Colonisation Factors.

More than 22 CFs have been recognized in human ETEC (Table 1); however, around 30% of the ETEC strains isolated worldwide still lack detectable CFs (Paper I, Steinsland *et al.*, 2003). The CFs are mainly fimbrial proteins organized in polymeric structures composed by subunits called pilins (Gaastra and Svennerholm 1996; Qadri *et al.*, 2005). Within the wide range of CFs, the most common ones are: CFA/I, CS1 to CS6, CS7, CS14, CS17, and CS21 (Qadri *et al.*, 2005).

Table 1.Characteristics of the CFs in human ETEC strains

CFs	Morphology (nm)	Size of the subunit (nm)	Toxins
CFA/I	F (7)	15.0	LT/ST, ST
CS1	F (7)	16.8	LT/ST
CS2	F (7)	15.3	LT/ST
CS3	F (2, 3)	15.1	LT/ST, ST
CS4	F (6)	17.0	LT/ST, ST
CS5	H (5)	21.0	LT/ST, ST
CS6	nF	14.5	LT/ST, LT, ST
CS7	H (3-6)	21.5	LT/ST, LT, ST
CS8	F (7)	18.0	LT
CS12	F (7)	19.0	LT/ST
CS13	F	27.0	LT
CS14	F (7)	15.5	LT/ST, ST
CS15	nf	16.3	ST
CS17	F (7)	15.5	LT
CS18	F (7)	25.0	LT/ST
CS19	F (7)	16.0	LT/ST
CS20	F (7)	20.8	LT/ST
CS21	F (7)	22.0	LT/ST, LT, ST
CS22	F (ND)	32.5	LT

CS= Coli surface antigens; F= Fimbrial; nF = non-fimbrial; ND = not-determined; H=helicoidal; f=fibrilar
(Modified from Gaastra and Svennerholm, 1996).

The interaction between ETEC-CFs and their receptors appear to be host-specific-oligosaccharide dependent expressed on the surface of mammal cells (Jansson *et al.,* 2009; Tobias *et al.,* 2010). Therefore, CFs expressed by human ETEC strains are different from the ETEC strains that infect animals *i.e* cattle and pigs (Torres *et al.,* 2005).

The genes that code for most CFs are found on plasmids that also encode the enterotoxins, although in some cases they might be found on separate plasmids. The expression of the determinant gene is regulated by environmental factors, *i.e.,* the degree of growth of the bacteria, temperature, (since it has been observed that CFs are expressed only at temperatures above 25º C, and that the presence of particular substances such as bile salts, which increase the expression of specific CFs). The production of most of the CFs depends on the presence of a transcriptional activator protein, such as Rns or CfaR which belong to the AraC family of transcriptional activators (Gaastra and Svennerholm, 1996).

Most of the CFs are encoded by plasmid operons that have a similar structure and organization. Usually four or more genes are required for the expression and assembly, *i.e.,* the major subunit and the minor subunit which build up the CF, the periplasmic chaperone which protects the assembly of the major and minor subunits in the periplasm and the usher which assembles and exports the CF through the outer membrane. Some CF operons also encode regulators that directly activate transcription of additional chaperones (Sanchez and Holmgren, 2005; Anantha *et al.,* 2004).

1.6. Isolation and identification of ETEC.

The screening or surveillance of ETEC is performed by culturing of stool samples. *E. coli* can be recovered easily from clinical specimens on non-selective or selective media at 37°C under aerobic conditions. In stool samples selective growth on agar plates which favours members of the *Enterobacteriaceae* family and permits differentiation of *E. coli* on the basis of morphology is commonly used (Balow *et al.,* 1991). Selective media includes MacConkey agar or EMB agar.

The detection and characterization of clinical ETEC isolates are usually performed through phenotypic and genotypic methods. The phenotypic tests used for detection of toxins and CFs are based on recognition of monoclonal antibodies (MAbs) and the genotypic assays are usually based on Polymerase Chain Reaction (PCR) and Real time PCR (RT-PCR) (Sjöling *et al.,* 2007; Lothigius *et al.,* 2008; Paper I).

Although the detection of the toxins is sufficient to identify ETEC strains, toxin positive strains should be tested for the presence of CFs, particularly in epidemiological studies by dot blot tests using specific MAbs to discriminate between different types of CFs or PCR (Steinsland *et al.,* 2006). ETEC strains to be tested by dot blot usually are grown on CFA agar containing bile salts, because the CFs CS5, CS7, CS14, CS8 and CS17-CS19 require the presence of bile for their phenotypic expression. On the other hand, CS21 must be detected on blood agar and tested separately (Gutierrez-Cázares *et al.,* 2000; Qadri *et al.,* 2000; Sjöling *et al.,* 2007). The traditional phenotypic dot blot test is performed only for 12 CFs (CFA/I, CS1 to CS6, CS7, CS12, CS14, CS17 and CS21) (Sjöling *et al.,* 2007).

1.7. Serotyping of ETEC.

The O antigen, which is the variable polysaccharide part of the lipopolysaccharide (LPS) present in the outer membrane of gram negative bacteria, can be determined and used for characterization of all gram negative bacteria (Figure 6). Studies in different countries have shown a large number and variation of serotypes in ETEC isolates both geographically and over time. At the present, there are over 170 recognized *E. coli* 'O' antigens around the world and many strains that can not be categorized for having unknown serotypes; the way to characterize them is using a standard rabbit antisera, which is easily applicable to a human seroepidemiological survey for the presence of antibodies to a range of *E. coli* 'O' antigens (Tabaqchali *et al.*, 1978).

However, in ETEC the O6 group is the most common, being present in approximately 16% of all isolates and in general, O6, O78, O8, O128 and O153 are present in half of the ETEC isolates (Wolf, 1997). The O serogroup hence can provide additional epidemiological information about the variety of *E. coli* strains distributed in different geographical areas (Wolf, 1997). The H serogroup defines the characteristics of the flagellar antigens. A total of 34 H serogroups have been associated with ETEC including H12, H16, H21, H45 and H9 which are commonly found in more than half of the ETEC isolates worldwide (Wolf, 1997).

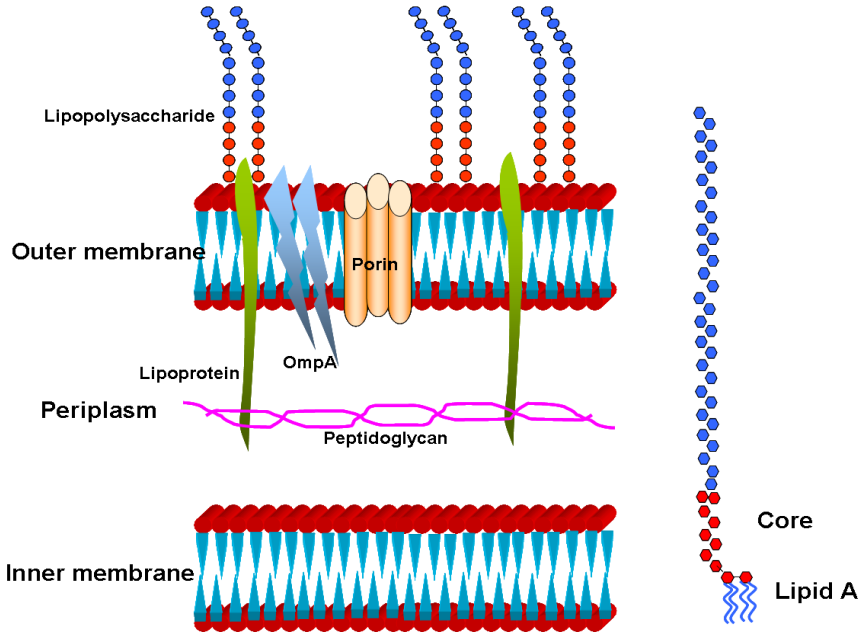

Figure 6. Membrane of *E. coli* strains showing LPS (lipopolysaccaride) with variable O antigens

1.8. Phylogeny of ETEC.

E. coli strains are generally found in all mammalian hosts. These versatile organisms were originally separated into five groups: A, B1, B2, D and E (Turner *et al.,* 2006b; Perez *et al.,* 2010). Only 39.2% of the genes in the pangenome of *E. coli* are present in all strains showing the high degree of genomic diversity within these species. The discrimination between isolates of bacterial species (molecular typing) is central to many aspects of clinical microbiology. Many epidemiological studies are concerned with the relationships between isolates that are recovered within a short period of time, from an individual, a hospital or a community (Tenover *et al.,* 1997).

There are various ways to determine whether bacterial strains are genetically related. Most methods use sequence variations in chromosomal genes to determine if strains share a common ancestor. Species are usually determined by sequencing of the variable regions of the16S rRNA encoding genes but intraspecies variation requires analysis of several genes or polymorphic DNA variations. Common methods include: Randomly Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphisms (RFLP), Repetitive Sequence-Pairbased PCR (REP-PCR), Pulsed Field Gel Electrophoresis (PFGE) (Romling *et al.,* 1992), Enterobacterial Repetitive Intergenic Consensus Sequence-Based PCR (ERIC-PCR), Multilocus Enzyme Electrophoresis (MLEE) (Selander *et al.,* 1986), analysis by either Southern blot or polymerase chain reaction (PCR) (Persing *et al.,* 1993), Multilocus Variation Analyses (MLVA) or Multilocus Sequence Typing (MLST) (Akopyanz *et al.,* 1992a; 1992b; Kawamata *et al.,* 1996; Osorio *et al.,* 2000; Thoreson *et al.,* 2000).

MLST is primarily a method for the identification of clusters of isolates with identical or highly related genotypes (clones or clonal complexes). It is a nucleotide sequence-based approach for characterization of strains of bacterial species, or other microbial species. MLST is based on the analysis of the sequences of internal fragments of a certain number of chromosomal house-keeping genes for each strain of a particular specie (Spratt, 1999; Wirth *et al.,* 2006). The sequences of each fragment are compared with all the previously identified sequences (alleles) in the locus and subsequently are assigned allele numbers. The combination of the seven allele numbers defines the allelic profile of the strain (the strain specific genotype) and each different allelic profile is assigned as a sequence type (ST), which is used to describe the strain (Maiden *et al.,* 1998).

For *E. coli* there are two major different MLST schemes available on public databases: the shigatox and the Achman scheme which uses the 7 house-keeping genes: *adk* (adenylate kinase), *fumC* (fumarate hydratase), *gyrB* (DNA gyrase), *icd* (isocitrate/isopropylmalate dehydrogenase), *mdh* (malate dehydrogenase), *purA* (adenylosuccinate dehydrogenase) and *recA* (ATP/GTP binding motif) (Figure 7).

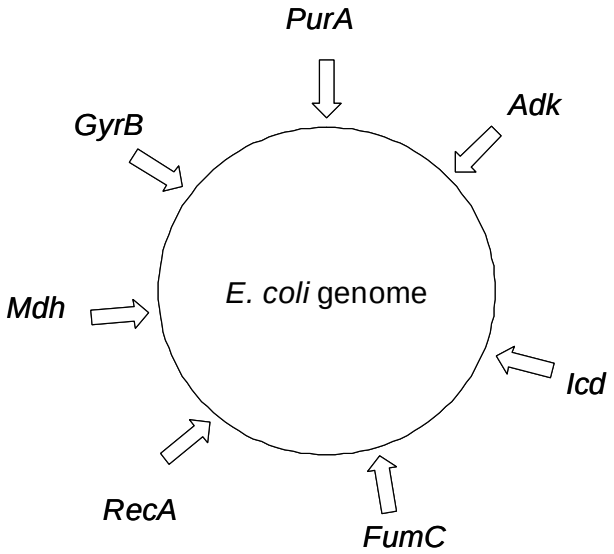

Figure 7. The seven house-keeping genes for *E. coli* strains (*adk, icd, fumC, recA, mdh, gyrB* and *purA*)

Recent studies of the phylogeny of ETEC show that ETEC strains are not restricted to a particular phylogenetic group (Turner *et al.,* 2006a, 2006b). There is no apparent association between types of toxin with a particular phylogenetic group. Many ETEC strains have the same toxin and CFs genotypes clustered in groups, that are not necessarily related phylogenetically which shows that there might be multiple and independent acquisition of virulence genes during evolution. Most ETEC strains are thus distributed within all groups of the *E. coli* lineage.

1.9. Use of antibiotics and antimicrobial resistance in ETEC.

In recent years, an increasing use of antimicrobials in treatment of pathogens associated with diarrhoea has been noticed (Sack *et al.,* 1997; Tjaniadi *et al.,* 2003). This is most probably due to increased self-medication prior to seeking medical care in countries where antibiotic drugs

are freely available in local shops (Putman *et al.,* 2000). In addition, strains from young children were found to be more resistant than strains isolated from older children, which may indicate that young children are more often treated with antibiotics because they tend to show a higher number of infections than older children (Putman *et al.,* 2000).

Studies in different geographical regions show an increased antimicrobial resistance pattern to commonly used antibiotics such as Tetracycline, Streptomycin, Amoxicillin, Ampicillin, Gentamicin, Trimethoprim-Sulfamethoxazole and Sulphonamide in *E. coli* strains associated with diarrhoea (Shaheen *et al.,* 2004, Vicente *et al.,* 2005; Al-Gallas *et al.,* 2007; Mandomando *et al.,* 2007). Resistance to Ampicillin and Gentamicin is probably attributed to that both antimicrobials are being used to treat pneumonia in children under 2 years (Mandomando *et al.,* 2007). It has also been observed that Chloramphenicol, fluoroquinolones and third generation cephalosporins are still effective for most pathogens. However, the use of Chloramphenicol and fluoroquinolones are not recommended in young children (Mandomando *et al.,* 2007).

Although antimicrobials are still frequently used to treat acute diarrhoeal diseases (ADD), the treatment should be focus on preventing or curing dehydration and prevent malnutrition. Oral rehydration therapy (ORT) is the method of choice to replace loss of fluids and electrolytes in children with acute diarrhoea in most cases. Not least since the antimicrobial resistance pattern is an emerging problem worldwide. However, there are situations where the use of antimicrobial substances may be necessary, for instance, children with malnutrition, immunodeficiency or serious illness, young infants, suspected sepsis and patients with prolonged bacterial diarrhoea. In some cases the use of antidiarrhoeal drugs can help to reduce the effect of the infection. The most populars are opiate derivatives that exert their action by reducing motility and slowing intestinal transit. Also, the use of bismuth subsalicylate has a direct antibacterial effect, and racecadotril which is an inhibitor of enkephalinase that acts through a decrease in the intestinal secretion (Jiménez *et al.,* 1998).

1.10. Diarrhoeal diseases and ETEC in Bolivia.

The focus of this thesis was to determine the impact of ETEC in Bolivia and also to investigate the toxin and CF profiles in relation to serotype and genetic background as determined by MLST and sequencing of virulence factors.

In Bolivia, acute diarrhoea is one of the main causes of mortality and morbidity in children. Data from 2002 shows that 29% of children under 5 years of age suffered from acute diarrhoea at least once every year and 46% needed medical help (Instituto Nacional de Estadística, 2002). In Bolivia, ADD are present in around 30% of the total child population under 5 years of age, producing more than 12 000 deaths every year (Romero *et al.,* 2007). ADD are the second leading cause of child mortality. This situation is exacerbated by the impact of chronic malnutrition, which affects 28% of children under three years (Gutierrez *el al.,* 2004).

Lately, Bolivia has improved in health care services to children mainly because of external help from other countries and help from the government. However, child mortality is still very high and the socioeconomic levels in different parts of the country as well as geographic and cultural boundaries are still the main obstacles to reduce child mortality. The most prevalent pathogens in Bolivian children associated with diarrhoea are rotavirus, *Shigella spp*, *Salmonella spp*, pathogenic *E. coli*, and *Campylobacter spp* (Instituto Nacional de Estadística, 2004). The main risk factor that contributes to diarrhoeal diseases is living conditions in poor areas where the level of hygiene is low and there is reduced access to clean water (UNICEF, 2008). In La Paz city, the majority of the population lives in slum areas where the sanitary infrastructure is deficient which contributes to a large spread of pathogenic microorganisms.

In Bolivia, there is still a lack of information about aetiology and prevalence of diarrhoea. Other studies made in La Paz and Sucre in children under 5 years of age with diarrhoea showed that EPEC was the most frequent microorganism isolated (14%), followed by ETEC (3%) and EIEC (2%) (Utsunomiya *et al.,* 1995). Another early study in La Paz in children under 3 years of age with diarrhoea reported EPEC with a prevalence of 10.9%, followed by ETEC (3.1%) and EIEC (3.1%) (Akira *et al.,* 1997). In a more recent study made in La Paz in children with diarrhoea under 5 years of age, EPEC and EHEC had a prevalence of 6.3% and 0.4%, respectively (Sanchez, 2002).

In this study, we performed an initial survey on the present prevalence of ETEC in Bolivia and the methodology for detection and characterisation of ETEC was improved by novel methods. Finally, an additional in-depth analysis of strains that circulated in Bolivia in recent years was performed.

2. AIMS OF THE STUDY.

The overall aim of this study was to determine if there was any association between virulence factors of enterotoxigenic *Escherichia coli* (ETEC) and severity of disease induced by this organism in hospitalized children with diarrhoea in Bolivia. The specific aims were:

- To develop improved PCR methods for detection of the ETEC enterotoxins and colonisation factors (CFs) and transfer such methods for detection of ETEC to Bolivia.

- To characterize ETEC strains from hospitalized children under 5 years of age with acute diarrhoea in Bolivia, with regard to enterotoxins, CF profiles, serogroups and resistance to antimicrobial agents.

- To determine the genetic relatedness of ETEC strains using Multi Locus Sequence Typing (MLST) as well as the DNA sequence of colonisation factors in order to follow the persistence and dissemination of genetically related ETEC strains in Latin America.

- To evaluate a possible association between different virulence properties of clinical ETEC isolates and severity of disease in Bolivia and compare production of ETEC enterotoxins in strains isolated from Bolivia and other highly endemic ETEC countries.

3. MATERIAL AND METHODS.

3.1. Collection and analysis of clinical samples for presence of ETEC.

Children under 5 years of age seeking care for diarrhoeal disease at three different hospitals in La Paz, Bolivia were selected for the study. The children samples were collected between 2002 to 2009. Ethical permission was obtained from the Bolivian Ethical Committee in La Paz. Parents seeking care at the hospitals were asked to participate in the study upon arrival to the hospitals and gave oral consent for analysis of stool samples from their children and for using anonymous clinical information. Ethical clearance has also been obtained by The Regional Ethical Board of Gothenburg, Sweden (Ethics Committee Reference No: 088-10).

Clinical data were obtained using a standardized pro-forma from the Health Ministry in Bolivia and included age, gender, date of onset of illness, symptoms and clinical signs, *i.e.,* characteristics of stools, abdominal pain, vomiting (number per day and duration), fever and extent of dehydration, if the patient received any antimicrobials before and during hospitalization, type of treatment for dehydration (Plan A, B or C) and if they had another disease such as acute respiratory infections.

Five to ten grams (or millilitres) of faeces were collected in plastic containers. At the same time, a cotton swab was rolled and moistened in the fresh sample and included in a tube with Carry-Blair transport medium. Both samples were submitted to the laboratory in boxes with ice packs for further analysis. The samples were examined in less than 4 hours after their collection. Once the stools samples have reached the laboratory, they were grown in MacConkey agar plates to test for growth of *E. coli* at 37°C overnight. Rotavirus (Romero *et al.,* 2007) and presence of other pathogenic *E. coli* categories (EAEC, EPEC) were also tested.

3.1.1. Detection of ETEC enterotoxins by GM1 ELISA.

Freshly collected stool samples obtained from hospitalized children in La Paz, Bolivia were planted onto MacConkey agar and the plates were incubated at 37°C overnight. Five lactose-fermenting colonies from stool samples culturally resembling to *E. coli* were tested for presence of ETEC toxins and CFs. The detection of LT and ST was carried out by ganglioside GM1 enzyme-linked immunoabsorbent assay (ELISA) (Svennerholm and Wiklund, 1983; Svennerholm *et al.,* 1986).

3.1.2 Detection of CFs by dot blot.

The remaining portion of each colony on MacConkey agar plate that tested positive for the toxin(s) was plated on colonisation factor antigen agar (CFA agar) with and without bile salts (McConnel *et al.,* 1989; Binsztein *et al.,* 1991) and plates were incubated at 37°C overnight. For each sample, enterotoxin positive *E. coli* colonies from CFA and CFA plus bile were tested for the expression of CFA/I, CS1, CS2, CS3, CS4, CS5, CS6, CS7, CS8, CS12, CS14, CS17 and CS21 by a dot blot assay using specific monoclonal antibodies (MAbs) for the different CFs (Lopez-Vidal and Svennerholm, 1990; Qadri *et al.,* 2000; Sjöling *et al.,* 2007).

3.1.3 Determination of LT and ST toxins and CFs by Multiplex PCR.

E. coli colonies from the CFA plates were also boiled for 5 minutes in double distilled H_2O and diluted to a concentration of 10-100 ng/µl. Single and multiplex PCR reactions for detection of toxins and colonisation factors were initially performed as described previously (Sjöling *et al.,* 2007) and subsequently as described in paper I using the improved multiplex assays.

3.2 Culture conditions and phenotypic analyses of ETEC.

The ETEC strains were cultured in Luria Bertani (LB) Broth or CFA media in the absence or presence of 1% glucose at 150 rpm at 37ºC. Samples for quantification of toxin production as determined by ELISA (Paper V), antibiotic resistance testing (Papers II and IV) and motility tests (Paper V) were taken after overnight growth.

3.2.1 Quantitative ELISA.

For the analysis of toxin production and secretion in the strains we used a quantitative GM1 ELISA. Since the cell surface lipid ganglioside GM1 is the main receptor for LT, the binding of LT to immobilized GM1 on microtiter plates (Nunc, Roskilde, Denmark) is widely used in assays for LT quantitation, *i.e.* GM1-ELISA while an inhibition GM1-ELISA is used for quantitation of ST toxin. The LT and ST levels were estimated in culture supernatants and in sonicated pellets of ETEC grown in LB at 37˚C using rCTB (0.3 µg/ml) (SBL) as reference for LT and ST-ref 881108 (0.3 nmol/ml) for ST toxin. For LT, we used the anti-CTB/LTB MAb LT 39:13:1 in a dilution 1/100 in 0.1% BSA-PBS-Tween; for the ST toxin, it was used the ST 1:3 960424 MAb diluted 1/600 in 0.1% BSA-PBS. After a 3-fold dilution was made, for both toxins, an addition of anti-mouse Ig-HRP conjugate diluted in 0.1% BSA-PBS-Tween was performed. The plates were developed with Orthophenylenediamine (OPD)

dissolved in sodium citrate with H_2O_2 added. The amount of LT secreted was determined by multiplying the titer of each sample with the sensitivity. The sensitivity was obtained by dividing the concentration of the reference with the titer of the reference. The inhibition ELISA for detection of ST in based on a comparison between the dilution of the sample and the dilution of the ST reference with a 50% inhibition.

3.2.2 Antibiotic resistance, serotyping and motility tests.

The antibiotic disc test was used to determine antibiotic resistance in a subset of the strains in Gothenburg while the majority of the strains were tested in Bolivia as described in Papers II and IV. The serotyping was performed in Spain in collaboration with Drs Blanco and Blanco; the procedures are described in Paper II. The motility test was performed on semisolid LB agar plates (0.3%). A drop of bacteria was placed in the centre of each agar plate and the dissemination of bacteria was measured after 3 hours and after 24 hours. Results in Paper V are based on the motility after 3 hours.

3.3 PCR analyses.

PCR applications have been a major part of the methodologies used in this thesis. PCR is based on the denaturation of the two complementary DNA strands at 94-96°C followed by annealing of specific primers at 50-57°C and subsequent activation of the DNA polymerase and elongation of the PCR products at 72°C which is optimal for most commercially available polymerases. For each of the PCR reactions used in the studies (Papers I, II, III, IV and V) we performed optimisation of the PCR conditions to obtain the best results. Especially for the multiplex PCR assays, determination of optimal conditions is important and amplification at high altitudes, for example in The Instituto de Biología Molecular y Biotecnología (IBMB) in La Paz required additional optimisation and different annealing temperatures than amplification at the sea level. Primers for PCR were designed by available programs on the net (*e.g* Primer3; frodo.wi.mit.edu/) and ordered from Eurofins MWG (Ebersberg, Germany).

3.3.1 Sequencing analysis.

Briefly, MLST is a nucleotide sequence based technique that determines or characterizes strains of different bacterial species using the sequences of internal fragments of seven house-keeping genes. Specific PCR products were amplified and purified using the PCR purification kit (Qiagen, Hilden Germany). Three main public databases for MLST analysis of the *E. coli* genome are available: www.shigatox.net, http://mlst.ucc.ie/mlst/dbs/Ecoli and The Pasteur Institute http://www.pasteur.fr/recherche/genopole/PF8/mlst/. Analysis of the seven *E. coli* genes used for MLST and sequence analysis of the genes encoding colonisation factors CS6 and CS17 were carried out on PCR products. The concentrations of the purified fragments were measured on a Nanodrop and sent for sequencing at Eurofins MWG (Ebersberg, Germany).

The obtained sequences using both the forward and reverse primers were analysed by BLAST (www.ncbi.nlm.nih.gov/BLAST) and assembled and compared using Bioedit and/or ClustalW. The electropherograms of the nucleotide peaks were visually inspected and edited if necessary. The MLST analysis was performed using the Mega4 software (Tamura *et al.*, 2007). Phylogenetic trees were created using the neighbour-joining method with the Kimura 2-parameter substitution model and branches were evaluated using the bootstrapping method with 1000 replications. Branch values below 70% were viewed as non-significant.

4. RESULTS AND COMMENTS.

4.1 Determination of ETEC toxin and colonisation factor profiles in children with diarrhoea in La Paz (Papers I, II, IV and V).

4.1.1. Collection of stool samples.

The main objective with this thesis was to determine the prevalence of ETEC in infants and toddlers with diarrhoea in La Paz, Bolivia and to determine virulence characteristics of the isolated ETEC strains. The studies were based on analyses of stool samples isolated from totally 853 children with diarrhoea from 2002 to early 2006 (Papers I, II and IV) and 496 samples collected during 2 years (2007-2008 and 2008-2009) in the summer period in Bolivia (December to March) (Papers IV and V). Totally 47 ETEC strains isolated from 2002-2006 and 48 strains from 2007- 2009 were analysed in Papers I, II, IV and V.

All strains came from children under 5 years of age with diarrhoea: 15 were outpatients and 80 were hospitalized children. Samples were collected in 3 different hospitals in La Paz: Boliviano Holandés Hospital, Materno Infantil Hospital and Del Niño Hospital.

4.1.2. Development of new methods for detection and characterisation of ETEC.

ETEC is commonly detected by selective growth on MacConkey agar followed by analysis of *E. coli*-like colonies by GM1-ELISA for detection of the LT and ST toxins and dot blot for detection of CFs (Svennerholm *et al.,* 1983; Svennerholm and Wiklund, 1986; Sjöling *et al.,* 2007). Recently PCR methods for detection and quantification of the ETEC toxins have been developed in our laboratory (Bölin *et al.,* 2006; Sjöling *et al.,* 2007; Lothigius *et al.,* 2008). One of our initial aims was to further improve the available PCR methods for fast and reliable detection of both the toxins and CFs by molecular methods.

A multiplex PCR assay for detection of the LT, STh and STp toxins has been described previously in our laboratory and this method was implemented in collaborating labs in developing countries such as Bangladesh and Bolivia. However, the detection of STh was not optimal in this assay. Therefore, we first developed an improved multiplex PCR for detection of the ETEC toxins by a change of the primers that amplified the STh toxin (Paper I).

We also developed multiplex assays for the amplification of 19 confirmed ETEC CFs in four PCR reactions (Paper I). The previously published primers for CFA/1, CS1, CS3, CS4, CS5, CS6, CS7, CS8, CS12, CS13, CS14, CS15, CS17, CS17/19, CS20 and CS22 (Sjöling *et al.*, 2007) and new primers for CS2, CS18 and CS21 were first tested for their specific amplification of a set of reference strains. Each specific CF primer only gave one PCR product of the correct length for the respective reference strain. Therefore, the 19 primer pairs were assembled into four panels (Figure 7) and tested in multiplex PCR against the reference strain collection. The methods were verified on a set of Bolivian ETEC strains obtained from 2002-2008 and Bangladeshi ETEC strains isolated from children in 2002-2003.

The assays were also transferred to IBMB in La Paz where it was optimised for PCR amplification at high altitudes and established as a routine method for rapid detection of ETEC toxins and CFs in the surveillance program of this pathogen. We also used these multiplex PCR assays in subsequent analyses of Bolivian ETEC strains isolated from 2007 to 2010 (Papers II, IV, V and ongoing studies) as well as for analyses of ETEC strains from other countries.

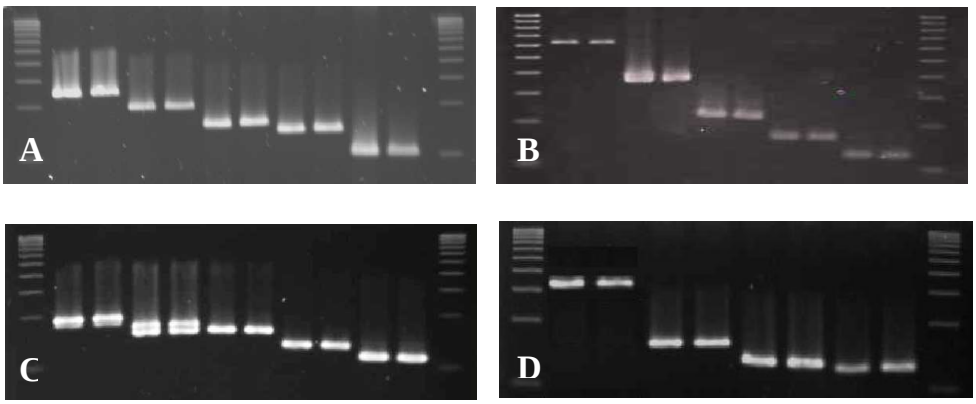

Figure 7. ETEC CF reference strains amplified in duplicate with the indicated multiplex PCR panel (panels I to IV). (A) Panel I. Lanes: 1 and 2, CS1 (243 bp); 3 and 4, CS4 (198 bp); 5 and 6, CS7 (154 bp); 7 and 8, CS12 (137 bp); 9 and 10, CS3 (100 bp). (B) Panel II. Lanes: 1 and 2, CS21 (630 bp); 3 and 4, CS2 (368 bp); 5 and 6, CS5 (226 bp); 7 and 8, CFA/I (170 bp); 9 and 10, CS17 (130 bp). (C) Panel III. Lanes: 1 and 2, CS19 (195 bp); 3 and 4, CS8 (166 bp); 5 and 6, CS6 (152 bp); 7 and 8, CS15 (130 bp); 9 and 10, CS20 (114 bp). (D) Panel IV. Lanes: 1 and 2, CS18 (362 bp); 3 and 4, CS13 (178 bp); 5 and 6, CS14 (162 bp); 7 and 8, CS22 (127 bp). Molecular size ladders are on each side of each panel

4.1.3. Identification of toxins.

When using the new multiplex PCR assays on Bolivian strains (Paper I) the LT-only toxin gene was found in 38/65 strains (58.5 %), combinations of LT and STh and LT and STp were found in 13 strains (15.5 %) and 4 strains (6%), respectively and STh-only in 13 (20 %) of the ETEC strains. Similar results were obtained using the old and the new versions of the ETEC toxin multiplex PCR, although the new assay was considerably easier to interpret (Paper I). In our next study on ETEC strains isolated from 2002-2006 (Paper II), LT-only was found in 70% of the strains (n=30), followed by LT/STh in 23% (n=10) and STh-only in 3 strains (7%). However, during the following 3 years (2007-2009), LT/ST strains were more common (40.5%), followed by LT-only (33.3%) and finally ST-only in 26% of the 42 analyzed strains (Paper V).

In general, the three toxin profiles LT/ST, ST-only and LT-only are found at equal frequencies worldwide although fluctuations do occur, for instance ST-only strains were reported to be more prevalent in Bangladesh (Qadri *et al*., 2000a; Qadri *et al*., 2007), Indonesia (Oyofo *et al.,* 2002), and Egypt (Weirzba *et al.,* 2006), while studies performed in Argentina (Viboud *et al.,* 1999), Brazil (Bueris *et al.,* 2007), Guinea-Bissau (Steinsland *et al.,* 2002) and Perú (Nirdnoy *et al.,* 1997) showed that LT-only was more prevalent. Finally in studies in Mexico (Cravioto *et al*., 1990) and Uruguay (Torrez *et al*., 2001), a combination of LT and ST was reported as the most prevalent toxin profile in these regions. These variations show the difference in geographical regions of distribution of toxins in ETEC but also the differences in the scheme and design of the studies which reflect the necessity to do coordinated studies and use similar detection methods to have a better understanding of the most prevalent toxins.

The most prevalent toxin profile, in general, in Bolivia was LT-only strains. However, several studies have demonstrated that LT only-producing ETEC strains are less important as pathogens and are not associated with severe diarrhoea (McConnell *et al.,* 1986; Gaastra and Svennerholm, 1996; Qadri *et al.,* 2007). However, in Bolivia as well as in several other Latin American countries, LT-only ETEC strains are frequent and seemingly cause severe disease (Viboud *et al.,* 1999; Bueris *et al.,* 2007; Nirdnoy *et al.,* 1997; Paper II, Rivera *et al.,* 2010).

4.1.4. Identification of CFs.

There is a wide variation in the reported prevalence of ETEC isolates that express CFs (which varies from 33% to 69%), as described in reports from different countries (Wolf, 1997; Qadri

et al., 2000). It is, however, difficult to compare the prevalence since different procedures have been used. In our studies using highly specific and sensitive methods we found that 35% of the Bolivian strains were CF negative and this is a figure that is comparable to most other studies showing the CF prevalence (Girón *et al.,* 1995; Sommerfelt *et al.,* 1996; Qadri *et al.,* 2007).

In the Bolivian ETEC strains, the most common CF combinations detected with the multiplex PCR panels were: CFA/I, CS21, CS14, CS17, CS1+CS3, CS2+CS3, CS7, CS12, and CS6 (Papers II and V). All these CFs were also mostly detected by dot blot indicating that the genes were transcribed and translated into their fibrillar or protein structures. The only exception was CS21. The CS21 pilus is encoded by the *lngA* operon and is also known with the name Longus (Girón *et al.,* 1996; Gaastra and Svennerholm, 1996; Gutierrez-Cazarez *et al.,* 2000).

When setting up the PCR assays, we tested several CS21 primers including the previously published CS21 primers (Sjöling *et al.,* 2007) but we got inconclusive results that probably were due to sequence variation in this CF (Steinsland *et al.,* 2003; Gomez-Duarte *et al.,* 2007). Because of this variability of the CS21 operon, we used the primers described by Pichel and collaborators (Pichel *et al.,* 2002) that anneal to conserved regions of the operon and amplify a 617 bp long product. When using this primer pair, we found that 34% of the strains were positive by the multiplex PCR but only half of them were positive by dot blot (Paper I). These results may be due to the observed variability in the coding region for CS21 and may lead to an underestimation of CS21 when using phenotypic methods.

The high prevalence of PCR-positive CS21 strains among the Bolivian and Bangladeshi strains (Papers I, II and V) showed an agreement with results in other regions, including Mexico, Brazil, Bangladesh and Argentina which reported frequencies of CS21 in 36.5% (Nishimura *et al.,* 2002), 33% (Pichel *et al.,* 2002), 29% (Qadri *et al.,* 2000b) and 20.7% (Gutierrez-Cazares *et al.,* 2000), respectively.

Interestingly in the initial studies we also found an unexpected high prevalence of LT/CS17 strains in Bolivia and this prompted us to further investigate whether certain LT/CS17 clones circulated in Bolivia.

4.2. Analysis of the genetic background by MLST, serotyping and antimicrobial resistance patterns (Papers II, III and IV).

4.2.1 Multilocus Sequence typing (MLST).

We used to use Multilocus Sequence Typing, MLST to determine if the Bolivian LT/CS17 strains were clonal descendent (Paper IV) but to set up the method we first analysed a set of ST/CS6 strains isolated from Mexico and Guatemala (Paper III).

We used the Achtman scheme available at http://mlst.ucc.ie/mlst/dbs/Ecoli. Using this scheme, we amplified the 7 house-keeping genes: *adk*, *fumC, gyrB, icd, mdh*, *purA* and *recA*. Around 450 to 600 bp internal fragments of each gene were analysed and sequenced from both strands using a DNA sequencer. The forward and reverse sequences were the aligned and compared, and a defined part of the coding sequence for each gene was used. For each house-keeping gene, an allele number was assigned to each polymorphic gene sequence. Strains were then characterized by their allelic profiles (or genotype) for the 7 genes and were referred to as an unique sequence type (ST) and given a ST- number.

4.2.2 MLST and CF sequencing in ST/CS6 Guatemalan and Mexican ETEC strains.

We set up the MLST and used it to analyze 24 ETEC strains that expressed ST/CS6 that were isolated from children and travellers to Mexico and Guatemala (Paper III). Seven MLST sequence types were observed in the 24 ST/CS6 ETEC strains from Guatemala (n=20) and Mexico (n=4).

Twenty strains belonged to three major MLST sequence types: ST-398, ST-182 and ST-278. The last four strains belonged to ST-443 (n=1) and three novel MLST sequence types which were submitted to the MLST database and designed as new genotypes with the designations ST-712, ST-726 and ST-727. All ETEC strains from children clustered into MLST sequence type ST-398 except the strain E874, which was ST-443. The ETEC strains from adult travellers clustered into three major genotypes ST-398, ST-182 and ST-278 (Paper III; Figure 8).

To further verify that the strains found within the different sequence types were identical, we decided to analyse the sequence of the colonisation factor CS6. Sequencing of a 540bp long internal region of the CS6 (*cssABCD*) operon which has previously been shown to be very polymorphic in LT-only strains (Nicklasson *et al.*, 2008) was performed. We found that all STp strains had identical CS6 sequences while the only STh strain in the collection had a different sequence (Paper III).

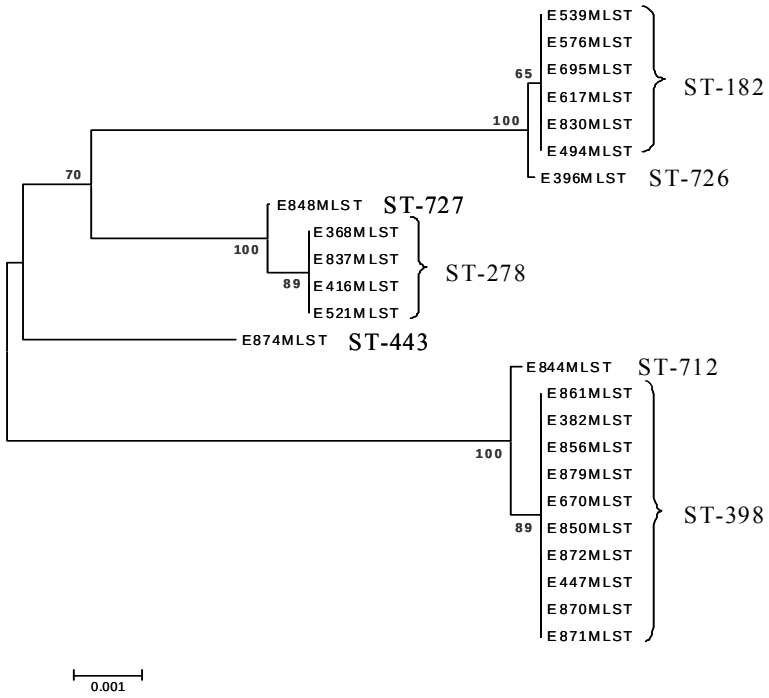

Figure 8. Dendrogram of the 24 ETEC strains from Mexico and Guatemala sequences with their respective MLST sequence types.

4.2.3 MLST in Bolivian ETEC strains that expressed LT/CS17.

Next, we wanted to analyze the genetic relatedness of ETEC strains isolated from children with diarrhoea in Bolivia. We found a high frequency of LT/CS17 Bolivian ETEC strains isolated between 2002 to 2006 (Paper I and Paper II) and for most of these strains we also had the serotype including the O sugar antigens from the LPS (O) and the flagellar antigens (H) as well as the antimicrobial resistance patterns for 10 antibiotics (Paper II).

Therefore, we wanted to see if the increase frequency of LT/CS17 strains in Bolivia was due to the emergence of one virulent clone that persisted in La Paz. The LT/CS17 strains from 2002-2006 as well as four additional LT/CS17 strains isolated in subsequent studies (Paper V) were analysed by MLST and compared to other Bolivian ETEC strains with other toxin/CF profiles (Figure 9).

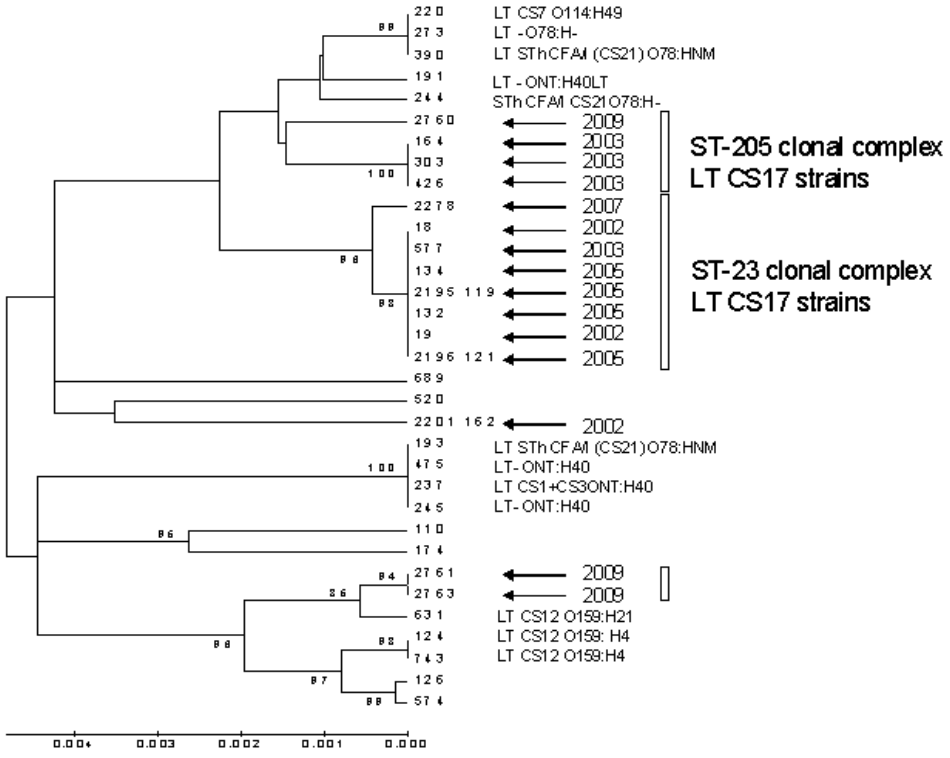

Figure 9. Dendogram of Bolivian strains: LT CS17 strains (indicated by arrows and year of isolation) and their respective ST types and clonal complexes in relation to other ETEC strains isolated in Bolivia.

Eighteen MLST sequence types were found to be present in the analyzed strains: 9 of them were already known STs and the other 9 were not described previously and were submitted to the MLST database and given a new ST number. Two major genetically related sequence type groups containing LT/CS17 clustered together: ST-423 (n=7) and ST-443 (n=3). The biggest group, ST-423, was mainly O8:H9 and resistant to several antibiotics including Ampicillin, Chloramphenicol, Ampicillin Sulbactam and Trimethoprim Sulfamethoxazole. These strains were isolated from early 2002 to late 2005 and hence persisted for at least four years in La Paz.

The closest related strain to this group was the 2278 ETEC strain which was isolated in 2007 and belonged to the new MLST sequence type 1990. The ST-1990 only differs from ST-423 by three nucleotide changes in the *gyrB* gene and since the definition of a clonal complex is that 6 out of 7 gene alleles are identical between strains this strain also belongs to the same clonal complex as ST-423. We hence believed that this strain was related and probably derived from the original ST-423 strains (Figure 9). The same fenomena was found in the ST-443 strains where an additional strain isolated in 2009 belonged to the same clonal complex and hence probably was closely related to these strains. Unfortunately we did not have the serogroup for either the 2278 strain not the ST 205 clonal complex to further verify this relationship.

Interestingly we found that the LT/CS17 strains in Bolivia belonged to several unrelated MLST sequence types and hence the increased frequency of LT/CS17 strains in Bolivia was not due to a single emerging clone but to several strains/clones with the same virulence profiles that circulate independently of each other in Bolivia. This finding is in contrast to other studies which have indicated that ETEC strains with identical virulence profiles are very closely related genetically (Steinsland *et al.,* 2003) but confirmed studies suggest that ETEC virulence has been acquired by *E. coli* with different genetic background at several occasions during evolution (Turner *et al.,* 2006b).

4.2.4. Serotyping of the Bolivian ETEC isolates.

Observations of the association of some serotypes with disease initially suggested that bacterial populations are clonal (Orskov & Orskov, 1983). In the study that was conducted in Guatemala and Mexico, we only analyzed the MLST sequence types and the operon that encodes CS6 which gave us an overview of how genetically related the ST/CS6 strains (among children and travellers) were (Paper III). Therefore, in addition to determine clonality and to add a further dimension when attempting to describe the genetic relationship among ETEC strains that were collected in Bolivia, we improved our analysis by adding data of serotypes and antimicrobial resistance that could lead us to discriminate clones among ETEC strains (Paper IV). The predominant serogroups of the Bolivian ETEC strains were: O6, O8, O78, O114, O120, O148, O153 and O159 and H9, H16, H40 and H45 (Paper II). The O serogroup has previously been used to differentiate between pathogenic and commensal *E. coli* (Wolf, 1997). However, in recent years, classification of pathogenic *E. coli* has involved enterotoxins and CF expression more than the O serotype. However, O serogroup provides

worthwhile epidemiological information about the variety of *E. coli* strains in different geographic regions (Wolf, 1997; Pacheco *et al.,* 1998). The predominant LT/CS17 ST-423 clone was mainly O8:H9 (n = 6 out of 7) and this serotype is common in ETEC isolates and usually found in strains that express CFA/I or CS17 (Wolf, 1997).

4.2.5 Sequencing of the CS17 genotype.

Although the LT/CS17 strains in Bolivia were genetically different, the main clonal complex consisting of ST-423 and ST-1990 that persisted in La Paz for 4 to 6 years was interesting. To further evaluate if ST-1990 was a descendant of the ST-423 strains we next sent out to sequence parts of the CS17 operon to determine if ST-423 and the 2278 ETEC strain carried the same virulence plasmid. We used the coding gene sequence of *csbABCD,* the operon that encodes CS17 and its assembly proteins, and amplified parts of the usher, the minor, the major and the chaperone units using primers described in Paper IV. We found that the sequence was quite conserved in the operon but was able to identify one polymorphic nucleotide in the usher sequence. This A to G polymorphism did not change the amino acid sequence and was found exclusively in the ST-423 strains, the 2278 strain and in one additional LT/CS17 MLST sequence type (ST-1991) isolated in 2009.

Since the virulence properties of ETEC are mainly plasmid-encoded, we hence hypothesised that the ST-23 clonal complex persisted for at least 6 years in La Paz and kept the same virulence plasmid but the strain 2278 evolved into a different MLST type by three nucleotide changes in the *gyrB* gene some time between late 2005 and 2007. We also believe that the two ST-1991 strains isolated in 2009 have acquired the virulence plasmid from ST-423 and then emerged as new LT/CS17 ETEC strains. Our data are still inconclusive but opens up for interesting studies on the evolution and transmission of pathogenic clones.

4.2.6. Antimicrobial resistance patterns in ETEC isolated from Bolivia.

Results for the antimicrobial susceptibility testing of the Bolivian ETEC strains are shown in Papers II and IV. Ampicillin resistance was found in 53%, followed by Ampicillin-Sulbactam with 47%, Trimetoprim-Sulfametoxazole in 32.5% and Tetracycline with 27%. Chloramphenicol showed moderate resistance of 21%. Hence the ETEC strains isolated in Bolivia presented multidrug resistance but Nalidixic acid, Ciprofloxacin, Gentamicine and Cefoxitine still had very good activity against this microorganism.

It was reasonable to predict that this multiresistance pattern in ETEC strains might emerge in developing countries where these classical antibiotics (Ampicillin, Tetracycline, and Trimethoprim-Sulfamethoxazole) have been widely used. It has been shown that the treatment for ETEC with antibiotics, specifically Trimetoprim Sulfametoxazole, decreases the duration and intensity of disease (Black *et al.,* 1982). However, in our study, ETEC presented high levels of resistance to this antimicrobial agent (Figure 10). Based on different interviews to parents of children who were hospitalized, revealed that patients with ETEC diarrhoea are often given antibiotics together with rehydration therapy. This is done to reduce the duration of hospital stay as well as to decrease of transmission to the environment. However this might be a problem since ETEC seems to have gained increased resistance to common antibiotics used for treatment of diarrhoea.

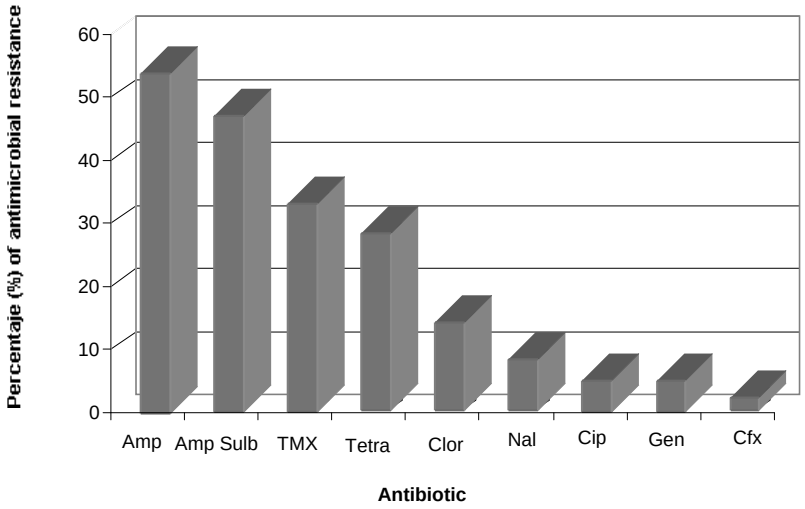

Figure 10. Percentage of antimicrobial drug resistance in the ETEC strains isolated from Bolivia. Amp = Ampicilline, Amp-Sulb= Ampicilline – Sulbactam, TMX = Trimetoprim Sulfametoxasole, Tetra = Tetracycline, Clor = Chloranphenicol, Nal = Nalidixic acid, Cip = Ciprofloxacine, Gen = Gentamicine, Cfx = Cefoxitine

In the past 20 years, a lot of information in relation to the mechanism and dissemination of antimicrobial resistance has been published. The investigations of the evolution of antimicrobial resistance have focused on the clonal transmission of pre-existing mutant genes. Later on, the impact of genetic determinants transmitted horizontally and the role of recombination of resistance genes have been studied (de la Cruz and Davies, 2000).

Resistance genes can also be exchanged between different bacterial species (Martinez and Baquero, 2002). Many antimicrobial resistant genes are localized in plasmids and transposons that allow transfer between different bacterial species. Therefore, the resistance genes can be spread among bacterial populations infecting both animals and humans (Davies, 1994).

In the past few years, a third mechanism for dissemination of antimicrobial resistance genes has been found. This mechanism involves a DNA element that links the integration of resistance genes through a recombinant mechanism in a specific site. This DNA element, called integron, is found as part of the transposons of the *Tn21* and *Tn7* family or independent in many plasmid groups (Levesque *et al.*, 1995). Although in this study we did not analyse the mechanisms of antimicrobial resistance gene transmission, we looked at the resistance pattern in the ST-23 clonal complex. We found that the virulence properties, which are also plasmid encoded seemingly might be more stable than antibiotics resistance genes within a clone since resistant phenotypes, appeared and disappeared within the same clonal complex isolated in different years (Paper IV) (Figure 11).

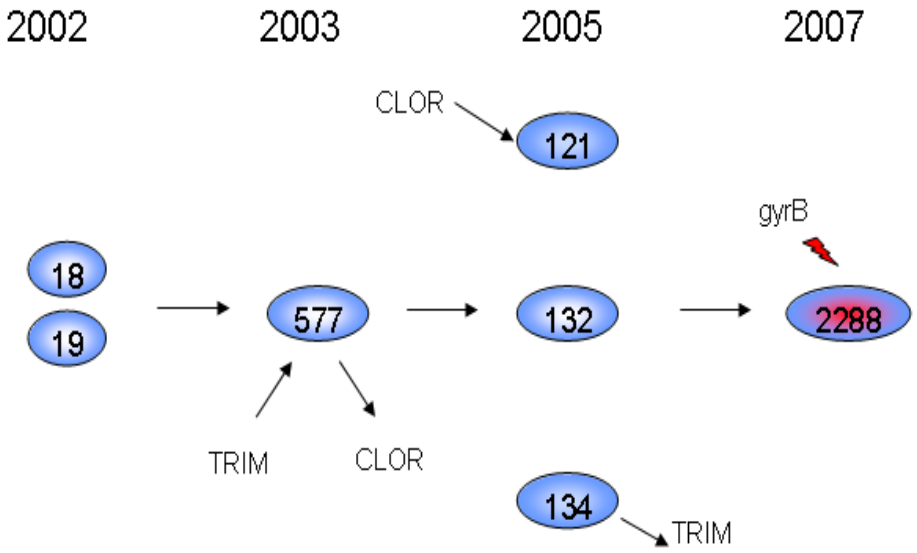

Figure 11. Proposed model of clonal evolution and loss or gain of Choramphenicol and Trimethoprim resistance of the ST-423 LT/CS17 O8:H9 strains within the ST-23 clonal complex.

4.3 Correlation of virulence properties and severity of diarrhoea produced by ETEC (Paper V).

ETEC diarrhoea might range from mild to severe watery diarrhoea which can be fatal. The relation between virulence factors and severity of disease in ETEC is still not fully evaluated. Although LT-only strains have been proposed to be associated with mild diarrhoea (Qadri *et al.,* 2007), LT strains in Latin America and other places have also been isolated from severely ill patients. Hence we wanted to determine the virulence properties of Bolivian ETEC strains in order to analyse if there was any association to severity of disease.

ETEC strains isolated from children with diarrhoea during the summer peaks (2007-2008 and 2008-2009) were isolated and characterized in terms of the toxin type and CFs expressed as well as toxin production and motility. Clinical data was gathered and analysed. Totally 42 ETEC strains from children with ETEC only were isolated and analysed for virulence properties in relation to clinical data (Paper V). The duration of diarrhoea among the patients was usually between 7 to 12 days. The majority of the patients suffered a febrile stage (n=27), and consistence of diarrhoea was recorded where the majority of the patients presented liquid stool samples (89 %). There were no patients that presented bloody diarrhoea.

4.3.1. Production and secretion of toxins in Bolivia.

The production of LT and ST toxin in both the pellet and the supernatant of each ETEC strain was analysed by ELISA and compared with the severity of diarrhoea in order to see if there was any correlation among these two groups. Bolivian ETEC strains were not high producers of LT toxin since the average production did not exceed 100 ng/ml (Figure 12A). No significant association was found when comparing mild and moderate diarrhoea with severe infection. These results were analyzed based on the levels of LT in the bacterial pellet fraction since LT toxin is usually associated with the periplasmic or extracellular surface of the bacteria although some traces can be also found in the supernatant (Hirst *et al.,* 1984; Sanchez and Holmgren, 2005).

When analysing the ST production in the Bolivian ETEC strains no statistically significant differences could be demonstrated between the values of produced ST by ETEC strains and the severity of diarrhoea in the infected individuals. Again, we found that Bolivian strains were not very high ST toxin producers based on the results obtained in the supernatant of each strain (Figure 12B). Finally we analysed the strains for an association between presence of

toxin, presence or absence of CFs and motility with clinical data but we could not find any association with severity of disease.

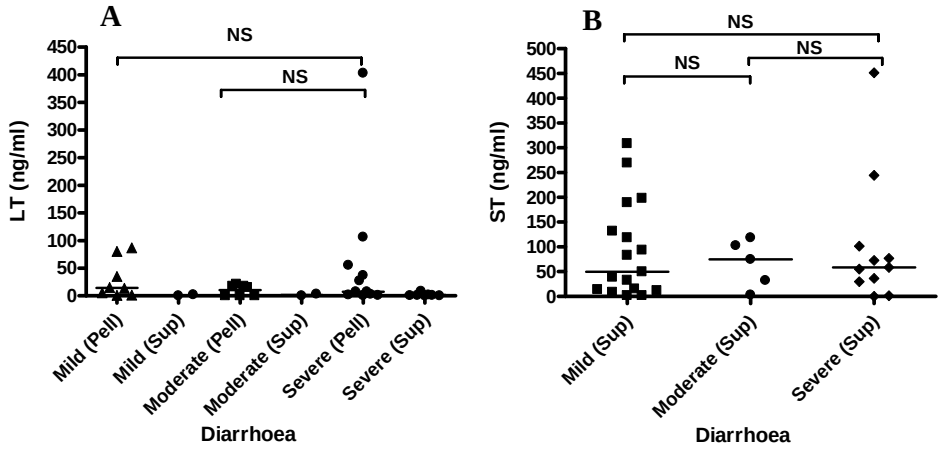

Fig 12. Production of toxins by Bolivian ETEC strains in sonicated bacteria (Pell) and supernatant (Sup) in relation to severity of disease A. LT toxin **B.** ST toxin

4.3.2. Production and secretion of toxins in other countries (Bangladesh, Egypt and Guatemala in relation to Bolivia).

Since we found that the toxin production in Bolivian ETEC strains seemed to be low compared to what we usually see in clinical ETEC isolates, we decided to analyse the toxin production on ETEC strains from children with diarrhoea in other countries where ETEC is highly endemic. In Bangladesh, the prevalence of ETEC has been reported to be 31% (Black *et al.,* 1981; Faruque *et al.,* 1998), in Egypt between 27% to 52% (in the suburbs and in the north-east of the country) (Oyofo *et al,* 1995; Zaqui *et al.,* 1986) and in Guatemala around 21% (in a village) to 27% (hospitalized cases) (Torres *et al.,* unpublished), In Bolivia the prevalence of ETEC was found to be lower and around 7 to 9.5% (Paper II and ongoing studies).

When comparing the LT production by the Bangladeshi and Egyptian strains using the Mann-Whitney test (non-paired parameters), no significant difference was found. However, there was highly significant differences between the LT production by the Guatemalan and the Bolivian strains (p=0.0012). Moreover, a more significant value was found when comparing Bangladeshi (p=0.0002) and Egyptian (p=0.0005) strains in relation to the Bolivian ETEC strains. In all the strains from all the countries, LT production into the supernatants was low,

not exceeding 30ng/ml for any of the strains tested. A significant difference was also found when comparing the production of ST by the Bolivian strains with the Bangladeshi, Egyptian and Guatemalan strains ($p < 0.0001$) (Paper V).

We found that ETEC strains from Bolivia produced significantly lower levels of LT and ST toxin in comparison with other countries but still, the children were generally severely ill. These results could perhaps be due to different bindings of the MAbs in the GM1 ELISA test to different epitopes and that could be a reason of sequencing the toxins and see if there is a difference in the interaction toxin-receptor-production of toxin.

However, another explanation is that since we could not establish any association with bacterial factors and severity of disease, the difference may be in the host. In fact, one tempting reason is that the blood group antigens in native Indian children that were included in the study play an important role. Native Indians in Bolivia and Peru are almost universally blood group O and in cholera this group has been linked to less incidence of diarrhoea (as we found for ETEC in Bolivia and also in Peru) but more severe symptoms once an infection is established (Harris, 2005). Since the ETEC strains in Bolivia produced significantly less toxins but still caused severe disease one might speculate that the native population is more susceptible to ETEC (and cholera) diarrhoea than individuals from other continents. However this needs to be proven in subsequent studies.

5. GENERAL CONCLUSIONS.

This is the first comprehensive study of ETEC infections in children from La Paz, Bolivia with characterization of toxin and CF profiles as well as other virulence characteristics. Our results were based on ETEC strains isolated from children under 5 years with diarrhoea seeking care at 3 different hospitals which provide a wide insight into the prevalence of ETEC in young population in this area. The children were mostly less than 2 years of age and from low socioeconomic living conditions suggesting that these children are the more susceptible to diarrhoeal diseases.

ETEC is considered one of the main bacterial agents causing acute diarrhoeal disease in children under 2 years of age in developing countries and in travellers from developed countries to endemic areas. In this study, we analyzed more than 1000 children with diarrhoea and found that the prevalence of ETEC in Bolivia was only 7-9% (Paper II and ongoing studies). This figure differs from other developing countries like Bangladesh that report 14-16% (Qadri *et al.,* 2000, Qadri *et al.,* 2007), Argentina 18.3% (Viboud *et al.,* 1999) and Nicaragua 38% (Paniagua *et al.,* 1997; Reyes-Navarrete, 2010). However, our studies include mainly young children with severe disease who were hospitalized for diarrhoea and therefore do not reflect the prevalence of ETEC in the population as a whole. Still we can conclude that ETEC cause severe disease in Bolivia.

To be able to study ETEC strains more efficiently, we developed methods for fast screening of *E. coli* colonies by PCR and multiplex PCR (Paper I). The methods were designed to be easily adapted to laboratory conditions in developing countries and specifically adapted to be used at high altitudes. The multiplex PCR methods developed for both the toxins and CFs are very sensitive and specific assays for detection and identification of ETEC in *E. coli* strains and easy to use for rapid surveillance of ETEC. The methods are currently used in our laboratory and have been transferred to different institutions in Bolivia, Bangladesh and soon also to China. We used the methods to determine the toxin and CF profiles of ETEC in Bolivia and found that although the general toxin/CF profiles were similar to other ETEC strains found all over the world, there was a higher proportion of LT-only strains expressing the colonisation factor CS17 in Bolivia than in other locations.

We analysed the genetic profile on a subset of Bolivian LT/CS17 strains using MLST and found that several of them circulated in La Paz during 2002-2009 demonstrating that genetically unrelated strains can carry the same virulence profile (Paper IV). We could also show that strains with the same MLST profile can have different virulence properties. This suggests that ETEC virulence plasmids do not require a specific genetic *E. coli* background and, therefore that independent horizontal transfer events may result in the generation of a greater diversity of isolates (Hien *et al.,* 2008). Hence our data support multiple and independent acquisition of virulence genes that has been suggested (Turner *et al.,* 2006a, and b) but also that clonal expansion might occur that create persistent genetically related strains that circulate in a specific area over years (Papers III and IV). If such clones also circulate at different continents remains to be investigated.

We also found that clonally related strains with similar virulence profiles (toxin/CFs) can have different antibiotic resistance patterns (Paper IV). This finding might suggest that persistent 'clones' can pick up and loose mobile elements carrying antibiotic resistance genes over time. In general the Bolivian ETEC strains showed an alarmingly great diversity of multidrug resistance that probably is due to the frequent use of antibiotics commonly bought among the population such as Ampicillin, Tetracycline and Trimetoprim-Sulfamethoxasole. These multiresistant patters can be explained by the presence of several resistance genes that are transferred between ETEC strains and/or from other categories of pathogenic diarrhoeagenic *E. coli.* However, an important aspect to consider is that cases of acute diarrhoea usually do not require antibiotic treatment unless the child has malnutrition, immunodeficiency, suspected sepsis or prolonged diarrhoea. In Bolivia, on the other hand, these criteria are not taken into account and improper use of antibiotics probably increases the resistance levels in both pathogenic and commensal strains which may cause a high frequency of treatment failures when antibiotic treatment is necessary. In this sense, the high resistance to sulfametoprim, the antibiotic most commonly used for treatment of gastroenteritis in Bolivia, requires a review for its use and should only be administered under strict control on the basis of information of sensitivity pattern of the circulating strains to prevent further spread of resistant bacteria.

Finally, we sought to determine if we could correlate virulence properties of the Bolivian ETEC strains to severity of disease. Although we analysed several properties such as production and secretion of the enterotoxins, presence of colonisation factors and motility, we did not find any association with severity. In another study, LT-toxin production and its secretion were intended to be linked with severity of disease but they could not establish a clear association (Lasaro *et al.,* 2006). These results might suggest that host genetic factors determine the outcome of disease and although we speculate that blood group factors might play a role in the Bolivian native children who are mostly blood group O, this hypothesis needs further investigation. We could however determine that, for some reason, the Bolivian strains are significantly less able to express and produce the LT and ST enterotoxins than comparable ETEC strains isolated from other endemic countries but still the Bolivian strains produce severe disease in children.

In conclusion, the studies presented in this thesis have provided a better view on the prevalence, virulence properties and dissemination of ETEC strains in Bolivia and has also highlighted the important emerging threat of multiresistant pathogenic bacteria that increases worldwide. This project might be helpful for planning of further studies performed in Latin America and may also affect treatment approaches and vaccine development strategies.

6. ACKNOWLEDGMENTS.

From experience I can tell you that these last pages of a PhD thesis are the most widely read pages of the entire publication. It is here where you think that you will find out whether you have meant something in the life of the PhD student. While this may be true to some level, you have to weigh my verdict with the disturbingly low level of sanity left in this PhD student after several years of studying a bacteria that causes diarrhoea in children due to the production of toxins (summary of the entire thesis…if you read it!). But, I would like to take this opportunity to thank a lot of people who supported me during my PhD studies and made my life in Gothenburg an unforgettable time. First of all I would like to acknowledge my supervisors who helped me during these years:

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