

**ACUTE GASTROENTERITIS IN RWANDAN  
CHILDREN UNDER FIVE YEARS OF AGE  
INVESTIGATED BY REAL-TIME PCR**

JEAN-CLAUDE KABAYIZA

Department of Infectious Diseases  
Institute of Biomedicine  
Sahlgrenska Academy at University of Gothenburg



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Jean-Claude Kabayiza 2014  
jckaba@yahoo.fr

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*To my family*



# ACUTE GASTROENTERITIS IN RWANDAN CHILDREN UNDER FIVE YEARS OF AGE INVESTIGATED BY REAL-TIME PCR

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## ABSTRACT

Acute gastroenteritis is a major cause of illness and death among children in developing countries. Knowledge about the aetiology is important to make the right priorities regarding preventive measures, and for the recommendation to use or not use antibiotics. The objective of this thesis was to investigate causes of acute diarrhea in children in Rwanda by real-time PCR targeting a wide range of infectious agents.

By analysing 326 paired faecal samples we found that rectal swabs provided equal rates of PCR detection of 10 different pathogens as usual stool samples, and correlating Ct values indicated that rectal swabs also may be used for quantitative measurements.

PCR findings in 544 children with acute diarrhea and 162 controls showed a higher prevalences in children with than without diarrhea only for rotavirus and the enterotoxigenic *E. coli* (ETEC-estA) (42% vs. 2%, and 21% vs. 9%). Other agents were detected at similar rates in sick and healthy children (adenovirus, 39% vs. 36%; ETEC-eltB, 29% vs. 30%, *Campylobacter*, 14% vs. 17%, *Shigella*, 13% vs. 10%). Lower Ct values for ETEC-estA, *Shigella* and norovirus GII indicate that measuring pathogen concentration in faeces may help to identify clinically relevant infections.

At least one pathogen was detected in 92% of 880 children with diarrhea. Rotavirus and ETEC-estA were associated with more severe dehydration, *Shigella* with bloody diarrhoea and higher CRP, and concentrations in faeces of rotavirus, ETEC-estA and *Shigella* were associated with more severe symptoms. Rotavirus and ETEC-estA were more common in younger, *Shigella* more a common in older children. Antibiotics were given to 42% of children, mainly those with fever and more severe dehydration, and without any logical connection with the causative organism.

The conclusions of this thesis are (i) that rectal swabs are as good as conventional stool samples for pathogen detection by PCR, (ii) that rotavirus, ETEC-estA and *Shigella* were the major causes of gastroenteritis, (iii) that higher concentrations of rotavirus, ETEC-estA, *Shigella* and norovirus GII were associated with symptoms, and that Ct value cut-offs for these agents improved identification of them as causes of disease, (iv) that antibiotics were used extensively and in a seemingly irrational manner, and (v) highly sensitive multiple real-time PCR was efficient and informative and that its use in future studies may provide valuable new information about the clinical significance and epidemiology of these infections.

This thesis is based on the following papers:

I. Jean-Claude Kabayiza, Maria E Andersson, Christina Welinder-Olsson, Tomas Bergström, Gregoire Muhirwa and Magnus Lindh. Comparison of rectal swabs and faeces for real-time PCR detection of enteric agents in Rwandan children with gastroenteritis. *BMC Infectious Diseases* 2013, 13:447.

II. Jean-Claude Kabayiza, Maria E Andersson, Staffan Nilsson, Cyprien Baribwira, Tomas Bergström, Gregoire Muhirwa, Magnus Lindh. Real-time PCR identification of agents causing diarrhoea in Rwandan children under five years of age. Submitted manuscript.

III. Jean-Claude Kabayiza, Maria E Andersson, Staffan Nilsson, Cyprien Baribwira, Gregoire Muhirwa, Tomas Bergström, Magnus Lindh. Clinical and epidemiological characteristics of microbes causing more severe infectious diarrhoea identified by real-time PCR in children under five years of age in Rwanda. Manuscript.

**AKUT GASTROENTERIT HOS RWANDISKA BARN UNDER FEM ÅRS  
ÅLDER UNDERSÖKT MED REALTIDS-PCR**  
JEAN-CLAUDE KABAYIZA

Akut gastroenterit är en viktig orsak till sjukdom och död bland barn i utvecklingsländer, och kan orsakas av ett stort antal olika smittämnen. Ökad kunskap om etiologin är viktig, bland annat för att göra rätt prioritering avseende preventiva insatser, såsom införande av vaccin mot rotavirus, förbättrad vattenförsörjning, avloppssystem och hygien, och för rekommendation om användning av antibiotika. Ett stort antal studier har tidigare genomförts, men ofta har antalet undersökta mikrober varit begränsat, eller har metoder med begränsad känslighet använts. På senare år har utvecklingen av så kallade molekylära tekniker förbättrats så att ett stort antal olika smittämnen kan undersökas samtidigt med hög träffsäkerhet och hög analytisk känslighet, men denna teknik har ännu inte använts i studier av diarré hos barn i utvecklingsländer.

Det övergripande målet med denna avhandling var att undersöka orsaker till akut diarré hos barn i Rwanda. De specifika syftena i avhandlingens tre delarbeten var att (i) genom jämförelse av 326 parade prov undersöka om ett enkelt pinnprov från ändtarmen (rektalsvabb) kan användas som alternativ till sedvanligt avföringsprov, (ii) undersöka trolig orsak till diarré genom att jämföra resultat av nukleinsyraanalys avseende ett stort antal smittämnen i avföring från 544 barn med akut diarré och 162 barn som inte haft diarré under de senaste 14 dagarna före provtagning, och (iii) undersöka epidemiologiska faktorer, klinisk bild och antibiotika-användning i relation till mikrobiella fynd hos 880 barn med akut diarré.

I det första delarbetet fann vi att rektalsvabb var likvärdig med vanligt avföringsprov vad avser påvisande av 10 olika smittämnen (rotavirus, norovirus GII, adenovirus, *Cryptosporidium*, *Shigella*, *Campylobacter*, och fyra typer av *Escherichia coli*). Studien visade även relativt god överensstämmelse mellan provtyperna avseende så kallade Ct-värden, vilket ger stöd för att rektalsvabb även kan användas för kvantitativa mätningar.

I det andra delarbetet fann vi högre förekomst av rotavirus och enterotoxigena *E. coli* (ETEC-estA) hos barn med jämfört med utan diarré (42% vs. 2%, respektive 21% vs. 9%). Andra agens påvisades lika ofta hos sjuka som hos friska (adenovirus, 39% vs. 36%; ETEC-eltB, 29% vs. 30%; *Campylobacter*, 14% vs. 17%; *Shigella*, 13% vs. 10%), men med hjälp av Ct-värdenas kvantitativa information kunde, förutom rotavirus, även ETEC-estA, *Shigella* och norovirus GII identifieras som de viktigaste orsakerna till gastroenterit. Resultaten tyder dessutom på att brytpunkter för Ct-värdet kan användas för att identifiera kliniskt relevanta infektioner.

I det tredje delarbetet påvisades minst en patogen hos 92% av 880 barn med diarré, varav 37% hade rotavirusinfektion. Rotavirus och ETEC-estA var associerade med signifikant högre andel barn med kräkning och uttalad dehydrering, medan *Shigella* var associerad med blodig diarré och högre CRP. För rotavirus, ETEC-estA och *Shigella* sågs signifikant samband mellan högre koncentration i faeces (lägre Ct-värde) och mer uttalad dehydrering. Rotavirus och ETEC-estA var vanligare hos yngre barn, *Shigella* vanligare hos äldre barn. Antibiotika gavs till 42% av barnen, främst till barn med feber och mer uttalad dehydrering och utan logiskt samband med orsakande organism.

Avhandlingens slutsatser är (i) att rektalpinnprov är lika bra som vanligt avföringsprov för smittämnesanalys med PCR-teknik, (ii) att rotavirus, ETEC-estA och *Shigella* och i mindre utsträckning norovirus GII var de viktigaste orsakerna till gastroenterit, (iii) att det fanns samband mellan förekomst av, eller svårighetsgrad av, symtom och koncentrationen av rotavirus, ETEC-estA, *Shigella* eller norovirus GII, (iv) att brytpunkter för dessa agens (utom rotavirus) påtagligt förbättrar specificiteten och identifieringen av dem som trolig sjukdomsorsak, (v) att antibiotika används i hög utsträckning och på ett till synes irrationellt vis, samt (vi) att metoden med samtidig analys av ett stort antal agens med högkänslig realtids-PCR var effektiv och informativ och att dess användning i framtida studier kan komma att bidra med värdefull ny information om dessa infektioners kliniska betydelse och epidemiologi.



## **LIST OF ABBREVIATIONS.**

PCR: Polymerase chain reaction  
ETEC: Enterotoxigenic Escherichia coli  
CRP: C-reactive protein  
AGE: Acute gastroenteritis  
RNA: Ribonucleic acid  
NSP: Non-structural protein  
VP: Viral protein  
HBGA: Human Blood Group Antigen  
FUT2: Fucosyl transferase 2  
EIA: Enzyme immunoassay  
LT: Heat labile toxin (of ETEC)  
ST: Heat stable toxin (of ETEC)  
CF: Colonizing factor  
GEMS: Global enteric disease multicentre study  
EPEC: Enteropathogenic Escherichia coli  
DNA: Deoxyribonucleic acid  
MGB: Minor groove binding  
ROC: Receiver operating characteristics  
IQR: Interquartile range  
ORS: Oral rehydration solution  
OR: Odds ratio

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# INTRODUCTION

## BACKGROUND

Acute gastroenteritis (AGE) means inflammation of the intestinal mucosa. It is characterized by the onset of diarrhoea with or without vomiting, fever or abdominal pain. The aetiology of acute gastroenteritis is variable with two main entities: infectious and non-infectious, with the former being the most common. In children acute diarrhoea may have a non-enteric origin, including a range of other infections such as urinary tract infection, pneumonia, otitis media and bacterial sepsis. Non-infectious diarrhoea can result from the intake of toxic food, chemicals, lactose or gluten intolerance, or malignancy.

Acute infectious gastroenteritis affects mainly children under five years of age, and especially those below 2 years of age because of the immaturity of intestinal immunity. Worldwide 3-5 billion cases of diarrhoea occur per year. The annual mortality in children less than five years old in developing countries has decreased during the last 20 years, due mainly to the introduction of oral rehydration therapy (ORT), from 4.5 million deaths to 1.8 million deaths, but the morbidity remains high (Liu *et al.*, 2012). Two thirds of the mortality still occurs in developing countries, including Rwanda (Walker *et al.*, 2013),

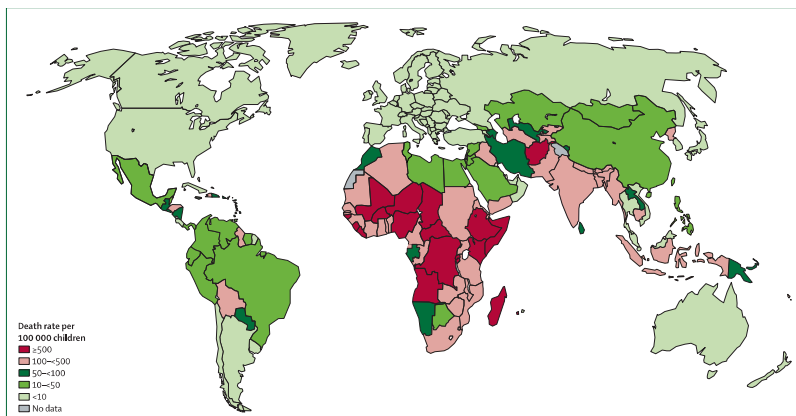


Figure 1. Annual deaths per 100,000 children due to diarrhoea.

In Rwanda, diarrhoea was the third cause of mortality in 2005, with 21% of deaths per year in children under five years of age (Anonymous, 2008). The microbial agents causing these deaths are not known, because the aetiologies of acute gastroenteritis in Rwanda have not been much studied. Although rotavirus vaccines might be less effective in sub-Saharan Africa (Armah *et al.*, 2012; Walker *et al.*, 2013) the introduction of rotavirus vaccination in Rwanda in June 2012 will probably reduce morbidity and mortality. It is important to investigate the role of rotavirus among other causes of acute gastroenteritis in Rwandan children before and after the introduction of the vaccine, in order to evaluate the effect of vaccination, as well as for planning preventive health interventions in general.

In developing countries, it is challenging to define the microbiological aetiology of diarrhoea due to the large number of potential causes, and the high rate of multiple and asymptomatic infections. The development of PCR based methods allows study of a wide range of enteropathogens with a high sensitivity and specificity (Amar *et al.*, 2007; Platts-Mills *et al.*, 2012). Molecular methods have previously been used mainly for detection of viruses (Wolffs *et al.*, 2011), but increasingly also for bacterial causes of diarrhoea (Buchan *et al.*, 2013; Iijima, 2004; McAuliffe *et al.*, 2013; Nadkarni, 2002; Ojha *et al.*, 2013; Vu *et al.*, 2004). The introduction of molecular methods targeting a wide range of causative agents may improve our knowledge about causes of diarrhoea in developing countries, because previous studies have often focused on a few pathogens or used conventional methods. Molecular methods that allow simultaneous analysis of a large number agents may provide more accurate information about causes of infectious diarrhoea (Liu *et al.*, 2013), but the high sensitivity may also lead to results that are difficult to interpret (Robins-Browne & Levine, 2012). Recently, some studies have shown that pathogen load by real-time PCR could help to determine the clinical relevance of detected pathogens. (Barletta *et al.*, 2011; Dung *et al.*, 2013; Lindsay *et al.*, 2013; Phillips *et al.*, 2009)

Thus, updated information on enteric infections in developing countries, including co-infections and relation to clinical symptoms and epidemiological factors, is needed for paediatricians and policy makers. In this project we investigated aetiologies of diarrhoea in Rwandan children by real-time PCR methods in conjunction with epidemiological and clinical context. The first part of the thesis evaluates methods for sampling stool for real-time PCR. Stool samples may be difficult to obtain, especially in children under five years of age. Rectal swabs are more practical for stool sampling, and have been widely used for culture, but studies on the utility of rectal swabs for molecular methods are lacking. In the second part of the thesis, we developed and applied a multiple real-time PCR to identify the aetiologies of acute infectious diarrhoea in Rwandan children. We used this assay to compare detection rates and pathogen loads in patients and healthy controls, and then to investigate the association between the disease markers and the pathogen load as well as the management of the patients.

## CLINICAL FEATURES OF ACUTE GASTROENTERITIS

The main symptom of acute gastroenteritis is diarrhoea. The word diarrhoea is derived from Greek meaning flow through, and refers to increase in water, volume, frequency and decrease in consistency of the stool due to imbalance of secretion and absorption of water and salts in the intestine. The World Health Organisation (WHO) defines diarrhoea as the passage of three or more loose or watery stools per day, but in breastfeeding infants, diarrhoea is considered when they have more than 6 to 8 stools per day. Nevertheless, normal stool can be difficult to define, and one may consider diarrhoea as having more than normal stools for that person. The presence of blood or mucus in the stool as well as signs of dehydration are important for the definition of diarrhoea regardless of the frequency, volume and consistency of the stool, and diarrhoea is also categorized according to its duration and cause. Acute diarrhoea lasts for less than 2 weeks (14 days), persistent diarrhoea more than 2 weeks, and some refer to chronic diarrhoea when duration is more than 30 days. Both infectious and non-infectious diarrhoea may be further classified according to its pathophysiological mechanism (Field,

2003). *Osmotic diarrhoea* may occur when bile salt or disaccharides are not properly resorbed in the small intestine, for example as a result of lactase deficiency. *Secretory diarrhoea* is due to overstimulation of the intestinal secretion and is characterized by large volumes of watery diarrhoea. *Exudative diarrhoea* results from a mucosal damage by inflammation, and may contain blood, pus and proteins, and also accumulation of water and electrolytes in the lumen secondary to the hydrostatic pressure in blood and lymph vessels. Invasive enteric infections like Shigella infection can cause this type of diarrhoea. *Motility disturbance diarrhoea* is usually caused by increased intestinal motility, but decreased intestine motility can also lead to diarrhoea secondary to bacterial overgrowth.

Infectious diarrhoea often involves more than one of these pathogenic mechanisms (Field, 2003) and may result in loss of excessive fluids, leading to electrolytic imbalance and collapse of the circulatory system, which can be life threatening in absence of intervention. Dehydration can also be due to vomiting, which along with fever or abdominal cramps, often accompanies infectious diarrhoea. Vomiting may be considered as a protective mechanism that serve to remove harmful substance from gastrointestinal tract, and may be induced by toxins produced by enteropathogens stimulating the enteric or central nervous system (Andrews & Sanger, 2013).

## MODES OF TRANSMISSION

The main route of transmission of enteropathogens is faecal-oral through the ingestion of contaminated food or fluids or by direct person-to-person contact. The factors that increase the transmission of enteropathogens in developing countries include contaminated water and food, poor sanitation and hygiene, lack of breastfeeding, malnutrition, deficiencies in micronutrients like zinc or vitamin A, crowded environment, and living close to domestic animals. The key reservoirs of human enteropathogens are food, water and humans, but some of these infections (e.g. Salmonella, Campylobacter and Yersinia) are zoonoses that are transmitted from live animals, or by unsafe preparation of food.



Children less than 2 years of age are particularly exposed to enteric pathogens, because of poor hygiene of hands and feet, and their explorative behaviour. When exposed, they often develop gastroenteritis due the lack of immunity induced by previous infections. The transition from breastfeeding, which provides some protection against enteropathogens (Golding *et al.*, 1997), to formula or cow milk also increases the risk of gastroenteritis.

## CAUSATIVE AGENTS

There are a wide range of infections that can cause acute gastroenteritis, including viruses (rotavirus, norovirus, astrovirus, sapovirus, adenovirus), bacteria (Shigella, Escherichia coli, Campylobacter, Salmonella, Vibrio cholerae, Yersinia enterocolitica, Aeromonas), and protozoa (Cryptosporidium, Entamoeba histolytica, Giardia intestinalis). In addition, Clostridium difficile may induce diarrhoea when the antibiotic treatment alters the intestinal microbial balance, and bacterial toxins may cause gastroenteritis without enteric infection (e.g. Staphylococcus aureus). In developed countries, viruses are the major cause of acute infectious diseases, whereas bacteria, in particular E. coli and Shigella, are common in developing countries. Table 1 describes the proportions of detected agents identified in some of the clinical studies, of which most have focused on only some of the agents that may cause disease, and only some include a control group.

Table 1. Detection rates in clinical studies of children with diarrhoea, with or without control group

	N (patients/ controls)	Adenovirus %	Astrovirus %	Norovirus %	Rotavirus %	Cryptosporidium %	Campylobacter %	EPEC %	ETEC %	Salmonella %	Shigella %	
Multicountry	3640/3279	4/2			16/2	11/7	11/7	9/6	16/5	3/2	11/1	(Huilan <i>et al.</i> , 1991)
Hanoi	111/111				17/4.5	1.8/1.8	1.8/1.8		3.6/4	3.6/2.7	5.4/0	(Hien <i>et al.</i> , 2007)
Thailand	236/236	16/2	3/1		18/3	2/5	22/25	15/14	10/6	6/9	9/0.4	(Bodhidatta <i>et al.</i> , 2010)
Bangladesh	814/814				20/1.5	1.4/0.4	17/13	12/5.4	17/8.8		9.2/3.0	(Albert <i>et al.</i> , 1999)
Cambodia	600/578	4.4/0.5	0.3/0	6.7/3.2	26/0.5	2.6/1.6	6.2/8.6	11/6.8	12/4.3	13/16	5.2/1.7	(Meng <i>et al.</i> , 2011)
Burkina Faso	283/60	5/0			30/2		2/0	8/0	4/0	9/2	6/0	(Bonkougou <i>et al.</i> , 2013)
Kenya	432/432				10/3	1/0.5	1/0.5		1.5/1.0		11/1	(Swierczewski <i>et al.</i> , 2013)
Vietnam	291/291	4/1	12/1		31/3	4/0	4/0	9/4	3/0	7/1	9/1	(Bodhidatta <i>et al.</i> , 2007)
Vietnam	1419/609			18.2.7	47/							(My <i>et al.</i> , 2013)
Ghana	243/124	28/31	4.5/1.6	9.5/8.9	55/12	0.4/0.8	0.8/0.8			2.4/0	1.6/0.8	(Reither <i>et al.</i> , 2007)
Tanzania	451				24 dry/ 4 rainy				7 dry/16 rainy		24 dry/13 rainy	(Vargas <i>et al.</i> , 2004)
Egypt	356				17	11	5				2	(El-Mohamady <i>et al.</i> , 2006)
China	811	2.7	1.2	18	26							(Chen <i>et al.</i> , 2013)
Chile	1913			18	26							(O'Ryan <i>et al.</i> , 2010)
Multicountry	485			9.7	29							(Rackoff <i>et al.</i> , 2013)
Libya	780			18	32							(Abugalia <i>et al.</i> , 2011)
Burkina Faso	309				32			9.7		3.2	5.8	(Nitiema <i>et al.</i> , 2011)

## ROTAVIRUS

Rotavirus is the major cause of acute gastroenteritis in children below five years of age in both developed and developing countries, and was first identified in humans in 1973 (Bishop *et al.*, 1973). The outcome and consequences of rotavirus disease are more severe in low-income countries (Temu *et al.*, 2012). Rotavirus is transmitted by faecal-oral route, and is common also in countries with good sanitation and access to clean water. Before the introduction of rotavirus vaccination, rotavirus was associated with more than 400,000 deaths per year among children under 5 years of age, mainly in developing countries, in particular in Africa (O’Ryan *et al.*, 2005; Parashar *et al.*, 2009). The fraction of gastroenteritis that is due to rotavirus varies between studies and seasons. In a compilation of African studies between 1974 and 1992, the proportions with rotavirus ranged between 14% and 55%. This agrees well with more recent African studies, which have reported rates from around 20% (Abebe *et al.*, 2014; El-Mohamady *et al.*, 2006; Khagayi *et al.*, 2014; Kwambana *et al.*, 2014; Temu *et al.*, 2012) to more than 30% (Abugalia *et al.*, 2011; Bonkougou *et al.*, 2010; Nitiema *et al.*, 2011; Odiit *et al.*, 2014), or even above 50% (Binka *et al.*, 2011; Enweronu-Laryea *et al.*, 2014; Reither *et al.*, 2007), depending on geographic area, age distribution and season. Similar rates have been reported from Asian countries (Bodhidatta *et al.*, 2010; Chen *et al.*, 2013; Huilan *et al.*, 1991; Meng *et al.*, 2011; My *et al.*, 2013; Vu Nguyen *et al.*, 2006).

Rotavirus is a member of Reoviridae family. It is a non-enveloped virus with a double-stranded RNA genome divided in 11 segments with  $\approx 18,500$  basepairs in total. The name derives from Latin word “rota” meaning wheel, because the virus has a wheel shaped appearance on electron microscopy.

Rotaviruses are classified according to their antigenic specificities into serogroups and serotypes (Santos & Hoshino, 2004). There are seven serogroups of Rotavirus, referred to as A through G. Humans are infected by serogroups A, B and C, with serogroup A causing more than 90% of infections. Each genomic segment encodes a structural protein (VP1,

VP2, VP3, VP4, (VP5+VP8), VP6 and VP7) or a non-structural protein (NSP1, NSP2, NSP3, NSP4, and NSP5). VP4 and VP7 are important because their sequence variability defines the serotypes of rotavirus A. The glycoprotein VP7 defines G-types, and the VP4 protein defines P-types. There are 14 known G-types, of which nine infect humans. P types can be classified serologically into P serotypes, or genetically into P genotypes. So far, 14 P serotypes have been described, of which nine have been found from humans (P1, P2A, P3, P4, P5A, P7, P8, P11 and P12), and 23 P genotypes (written with brackets) have been described, of which ten have been found in humans (P[3]–P[6], P[8]–P[11], P[14] and P[19]). The genes coding for G and P are present on different segments they may reassort when two rotavirus strains infect the same cell, creating strains with new P-G combinations. Thus, rotaviruses with a range of P-G combinations have developed and been identified, of which four (P[8]G1, P[4]G2, P[8]G3 and P[8]G4 are considered to cause >80% of rotavirus diarrhoea among children worldwide (Santos & Hoshino, 2004). In Africa however, only 50% of the rotavirus infections are caused by these types, and otherwise uncommon types, some of which might be acquired from animals, are found in significant numbers (Seheri *et al.*, 2014).

Rotavirus infects mature enterocytes cells of the mid and upper part of the villi in the small intestine, and cause damage to these cells, which leads to villous epithelium atrophy. Because these enterocytes normally secrete lactase into the small intestine, this leads to milk intolerance due to lactase deficiency, which has been considered characteristic for rotavirus infection. However, increasing evidence point at the importance also of secretory diarrhoea caused by the enterotoxin effect of NSP4 rather than the malabsorption diarrhoea described above. The NSP4 seems to stimulate the enteric nervous system, leading to the induction of intestinal water and electrolyte secretion (Hagbom *et al.*, 2011; Lorrot & Vasseur, 2007).

The immunologic mechanisms responsible for protection against infection by rotavirus are not well known. The first infection with

rotavirus produces a homotypic neutralizing antibody response, with heterotypic responses in subsequent infections, but there are also cellular immunity-mediated responses for example against NSP4 (Johansen *et al.*, 1999). Thus, after the first, usually symptomatic rotavirus infection, immunity develops and becomes reinforced by repeated exposures (Thapar & Sanderson, 2004), often leading to abortive or asymptomatic infections. Consequently, symptomatic infection rates are highest in children under two years of age and decrease progressively with age. Infections in newborns, although common, are often associated with mild or asymptomatic disease, and the most severe symptoms occur in children six months to two years of age and those with compromised or absent immune system functions (Parashar *et al.*, 2009).

Immunity can also be induced by vaccination. Two vaccines have been available for several years, Rotarix by GlaxoSmithKline and RotaTeq by Merck, and recently a third vaccine, Rotavac, produced in India, was introduced. Rotarix is a live attenuated vaccine based on a G1P[8] rotavirus strain. RotaTeq is also a live vaccine taken orally, but it contains five rotaviruses produced by reassortment. Four of these express different VP7 (serotypes G1, G2, G3, or G4) from a human rotavirus strain and the attachment protein VP4 of type P7 from bovine rotavirus. The fifth virus expresses VP7 (serotype G6) from a bovine rotavirus and VP4 of type P1A from the human rotavirus. Rotavirus vaccination has had a dramatic impact on rotavirus infections in Central America, and is recommended by WHO since 2009 to be part of the general childhood vaccination. These vaccines also seem to be effective in African populations, but there is some concern that the protection may prove less effective in African children, partly because of mismatch between the subtype represented by the vaccine and the subtypes of rotaviruses circulating in this region (Bhutta *et al.*, 2013; Lopman *et al.*, 2012; Seheri *et al.*, 2014).

## **NOROVIRUS**

Norovirus is an important cause of viral gastroenteritis in adults as well as children. Its impact appears to be greater in industrialized countries, but several recent reports indicate that it is a common cause of gastroenteritis in children under five years of age in developing countries (Bodhidatta *et al.*, 2007; Dey *et al.*, 2007; Meng *et al.*, 2011; Swierczewski *et al.*, 2013). On the other hand, norovirus infections in children in developing countries are frequently asymptomatic (Ayukekbong *et al.*, 2013).

Noroviruses are highly contagious and they affect individuals of all age groups. They are transmitted by faecally contaminated food and water, by person-to-person contact, or via aerosols (Moreno-Espinosa *et al.*, 2004).

Norovirus induced diarrhoea is typically limited in time, with symptoms lasting a few days in most cases. However, severe forms have been reported in individuals at high risk: young children, the elderly, malnourished and immunocompromised persons (Bok & Green, 2012). It is not well known whether human norovirus infections induce any lasting protective immunity (Simmons *et al.*, 2013), or to what extent immunity protects against exposure to different strains. This is important because noroviruses are highly genetically and antigenetically diverse, a complexity that is a big challenge for the development of an efficient norovirus vaccine (Rackoff *et al.*, 2013).

Noroviruses are non-enveloped, single-stranded RNA viruses belonging to Calciviridae family. They are classified in five genogroups (GI –GV), three of which (GI, GII, and GIV) infect humans, with genogroup II being most strongly associated with diarrhoea illness in all age groups (Moreno-Espinosa *et al.*, 2004). The genogroups are further subdivided into genotypes: 8 in GI, 17 in G2, 2 in G3, and 1 each in G4 and G5 (Zheng *et al.*, 2006).

The development of norovirus infection depends on the presence of histo-blood group antigen (HBGA). These antigens are present on the surface of red blood cells, as well as intestinal epithelium, in a manner dependent of the expression of genes coding for enzymes involved in glycosylation. Persons with certain mutations in the gene coding for one of these enzymes, fucosyl transferase 2 (FUT2), so called non-secretors, have a natural resistance to infections with norovirus II.4, which is the most common norovirus type in humans. Because synthesis of AB0 blood group antigens involves fucosyl transferases, susceptibility of norovirus infections can be predicted from the blood group, with blood group 0 individuals (who are “secretors”) being susceptible, whereas blood group B are less likely to be infected (Rydell *et al.*, 2011).

### **SAPOVIRUS**

Sapovirus, like noroviruses, belongs to the Caliciviridae family and may infect humans and swine (Hansman *et al.*, 2007a). The prototype virus was identified at an outbreak of diarrhoea in a kindergarten in Sapporo in 1977. There are five genotypes, GI-GV, of which all but GIII infect humans. Sapovirus can infect both children and adults, and may cause outbreaks of diarrhoea, in particular in small children (Hansman *et al.*, 2007b) . It is not known if sapovirus is important as cause of diarrhoea among African children.

### **ADENOVIRUS**

Adenovirus infections are very common in children, but their importance as cause of gastroenteritis is insufficiently known. Adenoviruses are divided in 6 genogroups (A-F), further classified in more than 50 subtypes or serotypes. Of these, subtypes 40-41 belonging to genogroup F have been associated to diarrhoea. Thus, in most previous studies that have included adenovirus, the detection has been limited to types 40/41 by the use of assays (usually EIA) that are specific for these types. The detection rates have been 5-10% in children with diarrhoea (Aminu *et al.*, 2007; Bodhidatta *et al.*, 2007; Vu *et al.*, 2004), including a study applying PCR (Verma *et al.*, 2009), and lower in controls without

diarrhoea . It is not well known if other types may cause diarrhoea, as suggested by a small study (Faden *et al.*, 2011).

### **ASTROVIRUS**

Astrovirus is a non-enveloped RNA virus that was identified in 1975 by electron microscopy in children with diarrhoea. The virus is non-enveloped with a positive sense, single-stranded RNA, and belongs to the Astroviridae family. Several studies have shown that astrovirus is a relatively common cause of gastroenteritis in children, with most cases presenting in children below 4 years of age. Its role as cause of diarrhoea in African countries is still insufficiently studied, but one study detected astrovirus in 4.5% of children with diarrhoea, as compared with 1.6% of controls (Reither *et al.*, 2007), indicating that this virus might be an important aetiology.

### **ENTEROTOXIGENIC E. COLI (ETEC)**

The importance of toxin producing *E. coli* as cause of diarrhoea was discovered in India more than 60 years ago. There are two types of ETEC. One produces heat labile toxin (LT), an 84 kDa protein with several subunits and similarities to the cholera toxin. The gene coding for LT is called *eltB*, and as this gene was detected by PCR in our studies, the agent was called ETEC-*eltB*. The other type produces heat stable toxin (ST), which is a peptide, only 19 amino acids in size. The gene coding for ST, called *estA*, was targeted by PCR in our studies, and this agent was called ETEC-*estA*.

Numerous studies of ETEC have been performed, usually with methods based on bacterial culture and identification of the toxin by serology or other assays, and the detection rate of ETEC in children with diarrhoea typically ranges between 5% and 15%, whereas rates in children without diarrhoea usually are lower, typically around 5-10%. Differences between studies may be due to differences in age or season, as well as differences between presence of so called colonising factors (CF), which seem to influence virulence by promoting binding of *E. coli* to the mucosa. ETEC infections are frequently acquired early in life, both ETEC-LT and -ST, the former being most common (Rao *et al.*, 2001;



Steinsland *et al.*, 2002). The majority of studies indicate that ETEC producing ST are more strongly associated with diarrhoea than ETEC producing LT (Qadri *et al.*, 2005). This was also the case in a recent global multicentre study (GEMS) in which ETEC-ST was one of the main aetiologies (causing 5-10% of cases), whereas ETEC-LT was detected at similar rates in patients and controls (Kotloff *et al.*, 2013).

### **ENTEROPATHOGENIC E. COLI (EPEC)**

*E. coli* that do not produce toxins or have invasive properties may still cause diarrhoea by other mechanisms. Such enteropathogenic *E. coli* (EPEC) were previously defined on the basis of pattern of adherence to cells in tissue culture, with attaching and effacing (A/E) lesions being characteristic (Ochoa & Contreras, 2011). They are now usually classified by molecular techniques that include identification of genes coding for bundle forming pilus (*bfpA* gene) and intimin (*eae* gene). Typical EPEC carry both the *bfpA* and *eae* genes, whereas atypical EPEC code only for *eae*.

EPEC have been investigated in numerous studies, and have usually been found in 5-10% of children with diarrhoea. However, EPEC has also frequently been found in children without diarrhoea, so its importance as cause of diarrhoea is controversial. One recent study indicate that quantification by real-time PCR targeting the *eae* gene may serve to distinguish EPEC infections that cause diarrhoea (Barletta *et al.*, 2011).

### **SHIGELLA**

*Shigella* is one of the most important causes of diarrhoeal disease among children in developing countries. Previous studies have shown that 5-10% of these cases are caused by *Shigella*, with a higher proportion among children older than 2 years and lower rates in the youngest age group (Kotloff *et al.*, 1999), and similar rates have been observed during the last decade (Bonkougou *et al.*, 2013; Hien *et al.*, 2008; Mandomando *et al.*, 2007; Seidlein *et al.*, 2006), including a recent multicentre study (Kotloff *et al.*, 2012).

*Shigella* is genetically very similar to *E. coli*, and may be considered as *E. coli* with certain phenotypic characteristics (Peng *et al.*, 2009). Despite

this, it is still classified as a separate genus that is separated into four species, *S. dysenteriae*, *S. flexneri*, *S. sonnei* and *S. boydi*. Genomic analysis of prototype strains has revealed considerable differences between the four *Shigella* species as well as between *Shigella* and *E. coli* in both the chromosomal DNA and plasmid DNA, including a number of recombinations (Yang, 2005).

In Africa, *S. dysenteriae* and *S. flexneri* are most important, because they are more frequent and have great clinical impact by causing invasive infection of the colon, a type of infection that may induce bloody diarrhoea, i.e. dysentery. *S. dysenteriae* may also produce Shigatoxin, which may cause additional complications, including renal damage (Lee *et al.*, 2010). It has been estimated that *Shigella* causes more than 150 million diarrhoea episodes and more than one million deaths annually (Kotloff *et al.*, 1999). In most clinical studies, *Shigella* has been identified by means of bacterial culture and additional phenotyping analyses. During recent years, molecular methods have been introduced as an alternative to culture in epidemiological studies (Ojha *et al.*, 2013; Vu *et al.*, 2004). Most of these assays use the multicopy invasion plasmid gene as target for a general *Shigella* PCR (Lindsay *et al.*, 2013; Sethabutr *et al.*, 2000; Vu *et al.*, 2004). The greater sensitivity of PCR should be an advantage, but may also result in higher detection rates in healthy individuals or those with mild disease (Gatei *et al.*, 2006; Wang *et al.*, 2010). Recently, it was reported that quantification by real-time PCR may improve specificity and thus identification of clinically relevant *Shigella* infections (Lindsay *et al.*, 2013).

A limitation of PCR based on *ipaH* is that *Shigella* cannot be separated from enteroinvasive *E. coli* that also may carry this gene, but considering the close relatedness between *Shigella* and this type of *E. coli* this might be of taxonomic rather than clinical importance. Another limitation is that *ipaH* PCR does not provide *Shigella* species. The published genomes of the different *Shigella* species provide information that could be used to design methods for identifying *Shigella* species by PCR. Some such methods have been presented, but their accuracy has not yet been established (Ojha *et al.*, 2013).

## **SALMONELLA**

Salmonella is an important cause of diarrhoea in developed countries where it often occurs as a zoonosis and frequently originates from infected poultry (Coburn *et al.*, 2009). Its epidemiology among children in developing countries is less well studied, but it appears to be an significant cause of gastroenteritis, detected in 5-10% of children and less often in healthy controls (Bonkougou *et al.*, 2013; Hien *et al.*, 2007; Zaidi *et al.*, 2013).

Salmonella are Gram negative bacteria classified in two species, *S. enterica* and *S. bongori*. The former is divided into six subspecies of which *S. enterica enterica* causes essentially all human disease, and is further separated into  $\approx 50$  serogroups on the basis of somatic (O) antigen, and  $\approx 2,300$  serovars (serological variants) on the basis of flagellar (H) antigen. Virulence factors and the likelihood of different serovars to induce “typhoid” disease, i.e. invasive infection, “non-typhoid disease” (mainly gastroenteritis) or asymptomatic infection remain to be clarified.

During recent years several PCR based methods for detection of Salmonella in faeces or food have been published (Alvarez *et al.*, 2004; Buchan *et al.*, 2013; Malorny *et al.*, 2007). Such methods may target genes common for all serogroups, or be specific for certain serogroups or serovars. However, the most suitable genes for identification of Salmonella causing gastroenteritis or other clinically relevant infections in humans are not yet established, but will hopefully be revealed by analysis of the increasing number of published genomes (Gordienko *et al.*, 2013). This is important, because currently available methods for serological or molecular classifications are too complicated for clinical diagnostic use (Shi *et al.*, 2013).

## **CAMPYLOBACTER**

Campylobacter is a major cause of diarrhoeal illness in industrialized countries, where it often is foodborne and usually affects adults.

Campylobacter infections are however much more common in developing countries, where they mainly affect children (Blaser, 1997). In these societies, the source of infection is not well known, but it is

likely that keeping animals near or inside living areas and poor hygiene at preparation and cooking of meat (in particular poultry) contribute to the high infection rate. Several studies have reported similar detection rates in children with and without diarrhoea, suggesting that *Campylobacter* is not pathogenic in children. Careful studies, including also children below 6 months of age, however indicate that the initial infection usually is symptomatic, whereas subsequent infections are not, probably as a result of acquired immunity (Rao *et al.*, 2001). The higher rates and levels of antibodies in children from Bangladesh compared with those in United States also support early exposure and acquisition of immunity in developing countries (Blaser *et al.*, 1985). *Campylobacter* are Gram-negative bacteria belonging to the *Campylobacteriaceae* family. They require special media and conditions for culture, and PCR has proven a useful alternative for detection (Konkel *et al.*, 1999). There are several species infecting humans, but only two are important as causes of gastroenteritis, with over 80-85% of cases being caused by *C. jejuni* and 10-15% by *C. coli*. (Blaser, 1997) The infection resides in ileum, jejunum, as well as in colon, and seems to induce non-characteristic diarrhoea in children in developing countries, whereas adults in industrialised countries often present with abdominal pain, fever and bloody diarrhoea reflecting invasive colitis that may last for 7 days or more.

## **YERSINIA**

*Yersinia enterocolitica* is a well-known cause of gastroenteritis in industrialized countries, considered as a zoonosis acquired from preparation or consumption of food, in particular pork. It may cause diarrhoea in children (Qouqa *et al.*, 2011), but it has not been reported as an important cause for childhood gastroenteritis. To some extent this might reflect that it has not been investigated or analysed by insufficiently sensitive assays. Possibly, the application of molecular methods which are superior to bacterial culture (Zheng *et al.*, 2007), might reveal *Yersinia* infection in a greater proportion of children with diarrhoea.

## **VIBRIO CHOLERAE**

Infections caused by *Vibrio cholerae* are important because they can be lethal by rapidly inducing severe watery diarrhoea and loss of fluids. Severe infections are caused by certain serotypes and appear in epidemics, in particular in dense populations in tropical regions. Diarrhoea is caused by production of cholera toxin, which impairs the enterocytes ability to resorb salt and water, leading to pronounced losses of water. Severe epidemics have become rather rare, but are still a significant risk when large number of people gather, for example in refugee camps (Swerdlow *et al.*, 1997). After the earthquake in Haiti 2010, a severe outbreak of cholera occurred, which originated from peacekeeping troops from Nepal, and spread to infect more than 500,000 and kill more than 7,000 people (Frerichs *et al.*, 2012). This illustrates the threat from this infection, but fortunately severe outbreaks are uncommon, and in most studies of childhood diarrhoea *Vibrio cholerae* has been a rare finding. The infection can easily be detected by real-time PCR, for example targeting the cholera toxin gene (Blackstone *et al.*, 2007; Shirai *et al.*, 1991).

## **CRYPTOSPORIDIUM**

*Cryptosporidium* is a protozoa that forms oocysts, which after ingestion release sporozoites that infect enterocytes. There are several species that infect different hosts. *C. hominis* infects only humans, whereas *C. parvum* infects humans as well as animals, including cattle. In developed countries, the importance of foodborne and waterborne infections has become increasingly recognised (Putignani & Menichella, 2010). In developing countries, *Cryptosporidium* infections in children is a well-known and important health problem, both as a frequent cause of acute gastroenteritis and because protracted infections are common (Tumwine *et al.*, 2003), may impair growth (Mølbak *et al.*, 1997), and be life threatening in malnourished or HIV infected children. Among immunocompromised children infection with other species, such as *C. meleagridis* and *C. felis*, also occur. Recent studies, including a global multicentre study, indicate that *Cryptosporidium* is one of the most important causes of acute diarrhoea among children, detected in  $\approx 10\%$

of cases (Agnew *et al.*, 1998; Khan *et al.*, 2004; Kotloff *et al.*, 2013; Tumwine *et al.*, 2003). In African studies *C. hominis* has constituted  $\approx$  80% of cases and *C. parvum* 15-20% (Gatei *et al.*, 2006; Tumwine *et al.*, 2003). Cryptosporidium infection may cause diarrhoea that is accompanied by fever and vomiting, and may lead to dehydration that requires hospitalisation. Diagnostics has previously been performed by microscopy, but detection by PCR is probably more reproducible and sensitive (Chalmers & Katzer, 2013; Haque *et al.*, 2007).

### **ENTAMOEBIA HISTOLYTICA AND GIARDIA INTESTINALIS**

These protozoa are well-known causes of diarrhoea in developing countries. *Giardia* typically causes mild and protracted diarrhoea (Muhsen & Levine, 2012), whereas *Entamoeba histolytica* may cause both acute and prolonged diarrhoea, sometimes with blood (dysentery) due to invasive colitis (Haque *et al.*, 2003). By tradition these infections are diagnosed by microscopy, but PCR methods have been introduced and appear to be accurate and improve distinction between different species of *Entamoeba* (Fotedar *et al.*, 2007; Guy *et al.*, 2003; Haque *et al.*, 2007; Roy *et al.*, 2005).

### **DIAGNOSTIC METHODS IN MICROBIOLOGY**

*Bacterial* culture has been in use for a long time and is still the main diagnostic method for many diarrhoeagenic agents. It has the advantage that it can be performed without advanced equipment and may provide information about antibiotic resistance. However, for some agents special conditions are required, the sensitivity may be limited, and the time to result is relatively long. *Virus* culture is also in general too slow or too insensitive for diagnostic use. For rotavirus there are rapid antigen detection assays with acceptable sensitivity, but for adenoviruses and caliciviruses sensitivity of such assays is insufficient. *Protozoa* have by tradition been detected by direct microscopy, which can be performed locally without advanced equipment. The accuracy of this method is however uncertain and depend on the skill and experience of the technician.

Molecular based diagnostic assays provide rapid and reliable identification of pathogens, and have improved the diagnostics of all types of microbial agents. Polymerase chain reaction (PCR) is the main molecular method used in microbiology (Yang & Rothman, 2004). By this method a short sequence of DNA of the target pathogen is amplified in vitro. This is achieved by adding nucleotides, primers designed specifically for the target DNA, and a thermostable TaqDNA polymerase to nucleic acids extracted from the clinical sample, and then heat and cool the sample for 30-45 cycles to allow repeated copying of the targeted segment. If the target is RNA, a reverse transcription step is included prior to the cyclic amplification.

In traditional PCR, the test result, i.e. presence of target DNA, is identified by gel electrophoresis. In real-time PCR the amplicon is instead detected by a camera that registers fluorescence once per cycle. This light is emitted either by fluorescent molecules that bind unspecifically to DNA (usually SYBR green), or by so-called probes that emit light of a certain wavelength when hybridised to the target. The advantage with the latter is that it greatly increases the specificity of the assay. Other important advantages with real-time PCR are:

- reduced hands-on time
- no risk of contamination from amplified products
- high sensitivity without need of so-called nesting
- possibility to quantify by means of the so-called Ct value

The latter option is the reason that this technique also is called qPCR (quantitative PCR). Quantification is based of the fact that the logarithm of the pathogen load is inversely proportional to amplification cycle when the fluorescent signal can first be identified. This variable is an integer that is called threshold cycle, Ct, and its mere value gives an indication of the concentration of the target gene in the sample. Thus, because 40-42 cycles usually are required to amplify 1 target gene copy to a fluorescence level that can be detected, a Ct value of 40 reflects presence of 1-3 copies in the reaction volume if amplification is effective, and this typically corresponds to a target concentration  $\approx$  100-300 copies/mL of original specimen (depending of dilution procedures during sample preparation). Accordingly, a Ct value of 30 corresponds to

a target concentration that is  $\approx 1,000$  times higher (because  $2^{10} = 1,024$ ), i.e.  $\approx 300,000$  copies/mL. To obtain an accurate quantification, the Ct value has to be related either to a reference gene or to a “standard curve”. The latter is actually a line based on the Ct values obtained for known amounts of the target gene, and this technique is used when absolute quantification (copies per mL) is wanted. In other cases it is sufficient or more relevant to relate the Ct for the target gene to a reference gene present in the sample. This could be an RNA molecule that is expressed in a stable manner or a DNA sequence that is present in a useful way, usually as two copies per cell, thus allowing an estimate of the number of target genes per human cells in the sample.

### **FALSE POSITIVITY**

False positive results may be the result of contamination from other samples, and is mainly a risk if samples with very high pathogen load are analysed in parallel with samples with low target load. The risk of this type of contamination can be reduced by very careful sample preparation and strict laboratory procedures. False positive reactions may also be caused by inaccurate assay design, that is, if primers or probe are not sufficiently specific for the target. The risk of this false reaction is mainly a problem for targets for which there is not enough information in GenBank or other databases. From a clinical point of view positive reactions in samples from healthy persons are sometimes classified as “false positive”, even if the test has identified the target of interest.

### **FALSE NEGATIVITY**

False negative reactions may be obtained if the pathogen is present in lower concentration than detection limit of the assay. The risk of this is much lower for PCR than for assays with poorer sensitivity. False negative results can still occur if substances in the sample inhibit the amplification that is the basis of the high sensitivity of PCR. False negative reactions can also be obtained if target genes are lost during storing or preparation of the sample, or if the reagents used for PCR are not present in optimal concentrations. A number of procedures can be applied to avoid false negative results. A positive control representing



target genes of each PCR should be included to identify false negatives due to problem related to PCR reagents (master mix). The impact of inhibitors can be reduced by purifying the nucleic acids using efficient extraction protocols. In modern diagnostics this is achieved by use of robots that purify nucleic acids by means of silica bound to magnetic particles or membranes. The potential impact of inhibitors can also be avoided by diluting the sample, because even moderate dilution markedly reduces the effect of inhibitors. Often it is enough to dilute 1:4 to prevent inhibition. We diluted the samples 1:10 prior to PCR, and observed that this effectively prevented inhibition.

An additional way to avoid false negative results is to add an internal control to the sample prior to extraction and amplification, and then analyse this target gene by real-time PCR run in parallel with the other reactions. A disadvantage with this approach is that if the internal control reduces the number of target genes that can be analysed.

### **CLINICAL SIGNIFICANCE OF PCR RESULTS**

The high sensitivity of PCR is in general an advantage, but detection of pathogens present at low concentration may be difficult to interpret. This is relevant if the pathogen of interest is present in low concentration in healthy persons and at high concentration in sick persons. If this is the case, then a method with lower sensitivity might be more accurate. It is however unlikely that clinically relevant concentrations of all pathogens would match the diagnostic performance of a certain assay. Instead, the diagnostic performance could be optimised by using quantitative assays that may be applied with a cut-off that distinguishes infections causing disease from those that may be present also in healthy persons. The latter strategy is in focus in this thesis.

### **THE COST OF MOLECULAR BASED METHODS**

The cost of molecular diagnostics is main challenge in countries with limited resources, where the prevalence of diarrhoea is high. The development of molecular methods that are affordable and easy to use also for personnel without long training (preferably with integrated nucleic acid extraction and real-time PCR) is of high priority.

## **AIMS**

The overall goal of this work was to investigate causes of acute gastroenteritis in Rwandan children under 5 years of age.

The specific aims were:

- To compare rectal swabs and conventional faeces samples as specimen for real-time PCR of a range of enteric agents
- To analyse by means of real-time PCR diarrhoeagenic agents in faecal specimens from children with or without diarrhoea in order to identify the probable causes of acute gastroenteritis
- To analyse associations between real-time PCR findings and clinical and epidemiological data in children with acute gastroenteritis

## METHODS

### PATIENTS AND CONTROLS

In two field studies we included children seeking care for acute diarrhoeal disease at 5 health centres, 3 district hospitals and 2 university teaching hospitals in Rwanda (representing all levels of Rwandan health care system) during repeated study periods from November 2009 to June 2012 as to cover both rainy and dry seasons. Rwanda is a small densely populated (420/km<sup>2</sup>) country in the Great Lakes region of Central East Africa, and has a tropical climate with two dry seasons and two rainy seasons. Temperature varies between 19 to 27 °C over the year. The proportion of households with access to improved sanitation has increased from 59% to 75% over the past five years. The gross domestic product per capita is 730 USD and public health care is supported by a community based medical insurance system since 1999.

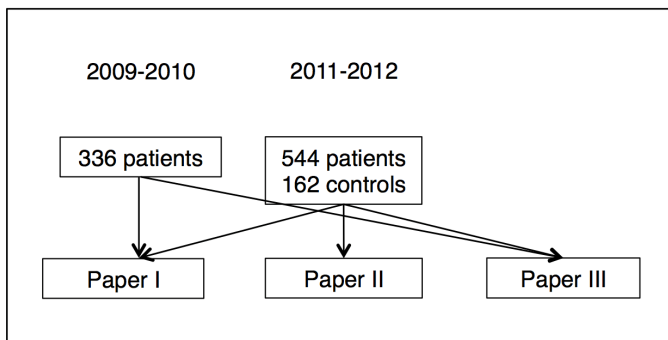


Figure 2. Overview of the inclusion of 1042 study subjects.

The inclusion criteria of the studies were age  $\leq 5.0$  years and diarrhoea with duration of less than 96 hours (with or without vomiting or fever), and exclusion criteria were non-enteric acute infections, severe malnutrition and AIDS. In the second field study, children without diarrhoea were also included as a control group (which was the basis for the analyses in Paper II). The controls were included from the same geographic area as patients by nurses at health centres and health workers

in the community with the criteria that they would be in the same age range as patients, and would not have had any diarrhoea or fever during the last 14 days. The number of study subjects enrolled in the field studies, and their inclusion in the three studies is described in Figure 2. The age distribution among patients and controls is shown in Figure 3.

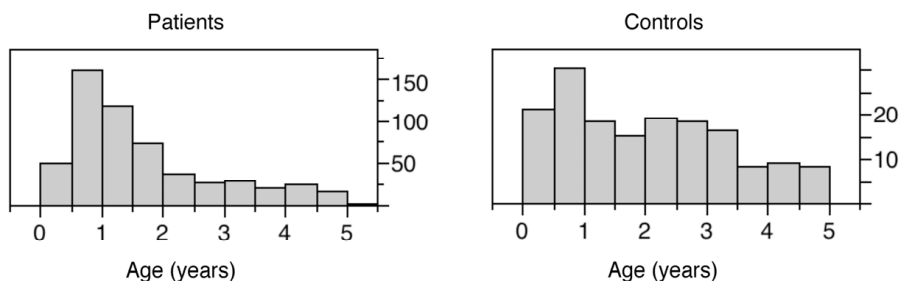


Figure 3. Age distribution of 544 patients and 162 controls (Paper II).

## SOCIOECONOMIC AND CLINICAL CHARACTERISTICS

The following factors of potential importance for diarrhoeal disease were registered: Area of living, type of water supply, type of toilet, body temperature, CRP, vomiting, stool frequency, degree of dehydration, type of rehydration therapy, and zinc and antibiotic treatment.

## STOOLS SAMPLE COLLECTION

Stool was collected as a rectal swab (Copan Regular Flocked Swab 502CS01, Copan Italia Spa, Brescia, Italy) in a tube with 1 mL of sterile saline, or as 2 mL of faeces. The samples were sent to a local laboratory for storage at  $-80^{\circ}\text{C}$  until transport to the Department of Infectious Diseases at University of Gothenburg, Sweden, where molecular testing was performed.

## MICROBIAL AGENTS AND TARGET SEQUENCES

The targets for real-time PCR are described in Table 2. Amplified regions of viruses were located to conserved genomic regions, using established primers and probes. Samples reactive for adenovirus were run by an additional PCR targeting only types 40/41. The non-viral targets were selected for the purpose of this study and included most of the

Table 2. Primers and probes targeting RNA or DNA

	Mix	Probe type	Target (gene/region)	Reference
Norovirus GII	1	Conventional	pol-capsid junction	(Nenonen <i>et al.</i> , 2009)
Rotavirus	1	MGB	non-structural protein 3	(Pang <i>et al.</i> , 2004)
Astrovirus	2	Conventional	pol-capsid junction	(Gustavsson <i>et al.</i> , 2011)
Sapovirus	2	MGB	pol-capsid junction	(Oka <i>et al.</i> , 2006)
Norovirus GI	3	MGB	pol-capsid junction	(Nenonen <i>et al.</i> , 2009)
Campylobacter jejuni	4	MGB	fibronectin-binding protein	(Konkel <i>et al.</i> , 1999) <sup>b</sup>
Yersinia enterocolitica	5	MGB	enterotoxin Yst precursor	(Ibrahim <i>et al.</i> , 1997) <sup>b</sup>
Vibrio cholerae	6	MGB	cholera toxin A subunit	(Shirai <i>et al.</i> , 1991) <sup>b</sup>
Salmonella spp	7	MGB	outer membrane protein C	(Alvarez <i>et al.</i> , 2004) <sup>b</sup>
ETEC-estA	7	MGB	heat-stable enterotoxin	(Stacy-Phipps <i>et al.</i> , 1995) <sup>b</sup>
ETEC-eltB	8	Conventional	heat-labile enterotoxin	(Victor <i>et al.</i> , 1991) <sup>b</sup>
Shigella spp	8	Conventional	invasion plasmid antigen H	(Sethabutr <i>et al.</i> , 2000) <sup>b</sup>
Cryptosporidium parvum /hominis	9	MGB	oocyst wall protein	(Haque <i>et al.</i> , 2007) <sup>a</sup>
EPEC eae	10	Conventional	intimin	(Beaudry <i>et al.</i> , 1996) <sup>b</sup>
EPEC bfpA	10	Conventional	bundle-forming pilus	(Gunzburg <i>et al.</i> , 1995) <sup>b</sup>
Adenovirus	11	Conventional	hexon	(Heim <i>et al.</i> , 2003)
Adenovirus 40/41 <sup>c</sup>	12	Conventional	hexon	

MGB, minor groove binding. <sup>a</sup> Modification. <sup>b</sup> Adaptation to realtime PCR. <sup>c</sup> Run as complementary analysis.

bacteria associated with diarrhoea, as well as *Cryptosporidium*. *Aeromonas* was not included, but it does not seem to be an important aetiology in African countries (Kotloff *et al.*, 2013). The performance of each of the new PCRs was evaluated by analysing serial dilutions of

plasmids containing synthetic target genes. Using this strategy it was confirmed that duplex or triplex analysis did not significantly compromise the amplification efficiency.

The reproducibility of the assay was evaluated by re-analysing (both extraction and PCR) triplicates of samples in which multiple (4-6) pathogens had been detected. This showed that all targets were detected in all triplicate reactions, with the exception of a few that had Ct values above 38 in the original PCR. At re-testing (after freeze-thawing), the Ct values were a mean of 0.55 cycles higher (range  $-0.29 - 1.71$ ) than at the original PCR, and the standard deviation between the triplicates was  $0.35 - 1.36$  (mean 0.56). The potential impact of inhibition was studied by spiking samples with a known amount of seal herpes virus (PhHV1) prior to extraction and comparing Ct values. When 24 faeces and 24 rectal swabs were analysed after such spiking and extraction, significant inhibition was not detected in any case.

The content of human DNA in faeces and rectal swabs was studied by analysing a human gene, betaglobin. Such analysis of 24 faeces samples and 23 rectal swabs showed that human DNA was detectable in all rectal swabs (median Ct=27.8, range 24.1-37.1), but in only 12 out of 24 faeces samples and at lower concentration (median Ct= 36.8). This difference might be relevant for detection of pathogens that may be present in or adhere to mucosal cells in rectum.

## SAMPLE PREPARATION AND NUCLEIC ACID EXTRACTION

Approximately 250  $\mu\text{L}$  of faeces were dissolved in 4.5 mL of saline and centrifuged 5 min at 750 x g. Then, 250  $\mu\text{L}$  of dissolved faeces or 250  $\mu\text{L}$  of rectal swab fluid were mixed with 2 mL of lysis buffer, and this volume was used for extraction of total nucleic acid in an EasyMag instrument (BioMerieux, Marcy l'Étoile, France). The nucleic acids were eluted in 110  $\mu\text{L}$ , and 5  $\mu\text{L}$  of this were used for real-time PCR. These procedures correspond to an approximate dilution of faeces to 1:10 prior to PCR. The dilution of rectal swab samples depends on the specimen volume contained in the swab, but typically was 1:10 to 1:100. These dilutions effectively prevent the potential impact of factors that might inhibit amplification.

## REAL-TIME PCR

Amplification was performed in an ABI7900 instrument (Applied Biosystems, Foster City, CA) in 11 parallel 20  $\mu$ L-reactions containing oligonucleotides (Table 1) and Taqman Fast Virus 1-step Mastermix (ABI, for RNA targets) or Universal Mastermix (ABI, for DNA targets). A two-step amplification (15 s 95°C, 60 s 56°C) was run for 45 cycles after an initial 10 min denaturation 95°C and 30 min reverse transcription at 46°C. Plasmids containing the target regions for all agents were amplified in parallel with patient specimens to verify the performance of each target PCR (mastermix control).

## C-REACTIVE PROTEIN

C-reactive protein (CRP) is a marker for the early inflammatory response, and has been used for decades to detect severe infections and distinguish bacterial and viral infection. It has not been much used for diagnostics in patients with gastroenteritis, but there is data suggesting that CRP could be useful for identifying patients with invasive, bacterial infections that might require antibiotic treatment (Cadwgan *et al.*, 2000). CRP levels were measured at a local laboratory in Rwanda by the NycoCard assay (Medinor, Lidingö, Sweden) according to the manufacturer's instruction. Briefly, 5  $\mu$ L of capillary blood were diluted and 50  $\mu$ L of diluted samples were added to a reaction device, followed by the addition of one drop of conjugate and after 30 s one drop of washing solution, and measurement in NycoCard Reader II.

## ETHICAL COMMITTEE APPROVAL

The study was approved by the regional ethical review board in Gothenburg and by the ethical committee at National University of Rwanda. An informed consent was obtained from carers of each child included in the study.

# RESULTS AND DISCUSSION

## PAPER I

Detection rates by PCR were similar in 326 paired rectal swabs and faeces samples from children with or without diarrhoea as shown in Figure 4. If detection in either faeces or rectal swab was considered as true ("gold standard"), the sensitivity ranged between 73% and 92%, with no significant difference between sample types (Figure 4 and 5). Ct values in faeces and rectal swabs correlated significantly ( $P < 0.01$  for all agents,  $R^2$  ranging between 0.31 and 0.85). For most agents the Ct values were 1-2 cycles lower in faeces, indicating that the amount of specimen in general was higher in faeces (Figure 6). An explanation to this might be that much of the specimen on rectal swabs is lost during retraction from rectum. For adenovirus and *Campylobacter*, Ct values were however lower (i.e. microbial content higher) in rectal swabs. This might reflect that these agents are present not only in faeces but also, and in high amount, in or adhered to the rectal epithelium. This possibility was supported by our results of real-time PCR targeting betaglobin (a marker for human cell content), which showed higher detection rates and lower Ct values for betaglobin DNA in rectal swabs than faeces, suggesting that epithelial cells are present at higher numbers in rectal swab samples as compared with faeces.

Rectal swabs have been used for decades as an alternative to faeces for culture of bacteria (McFarland *et al.*, 1987), and their utility for molecular analyses has been supported by recent reports. Two small studies indicated that rectal swabs were adequate for detection of *Clostridium difficile* (Kundrapu *et al.*, 2012; Shakir *et al.*, 2012). Another relatively small study showed that detection of viruses by PCR was equal in rectal swabs and faeces (Gustavsson *et al.*, 2011), and a recent study described that rectal swabs could also be used for quantification of bacterial genes (Lerner *et al.*, 2013). Our data extend these findings because both sick and healthy children were included, a greater number of paired samples were compared, and ten different pathogens were analysed. The very similar detection rates in rectal swabs



and faeces confirm that the rectal swab is a reliable and useful way of collecting stool specimen for PCR detection. The relatively good correlation between Ct values obtained in rectal swabs and faeces **suggests** that rectal swabs can be used also for quantitative estimates of enteric microbes.

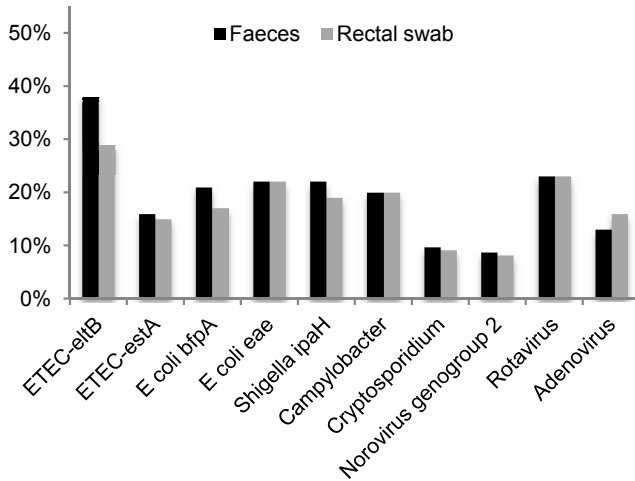


Figure 4. Detection rates in faeces and rectal swabs from patients.

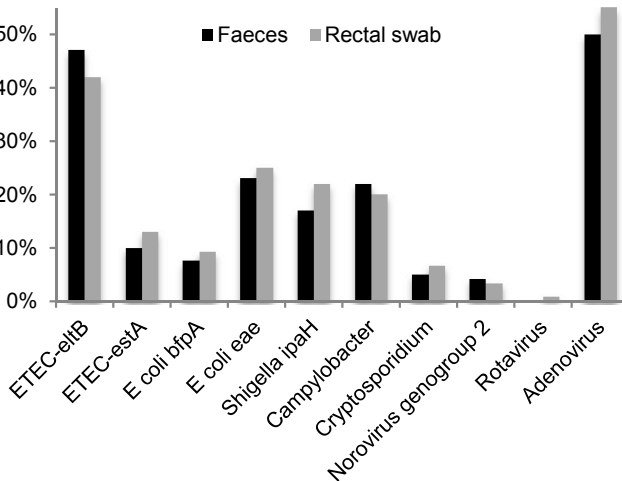


Figure 5. Detection rates in faeces and rectal swabs from controls.

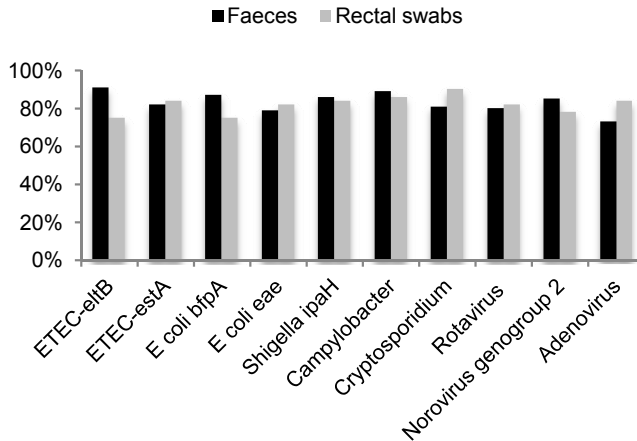


Figure 6. Sensitivity for PCR detection in faeces or rectal swabs (if sensitivity in either is considered true positive).

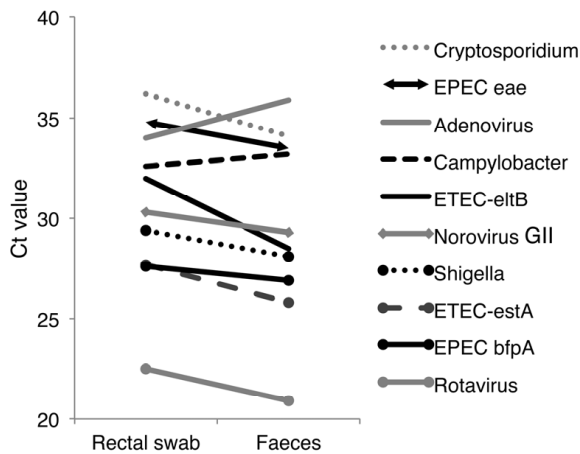


Figure 7. Mean Ct values in samples positive by PCR in faeces and rectal swab.

## PAPER II AND PAPER III

### General

Papers II and III describe the frequency of a wide range of diarrhoeagenic agents and analyse their pathogenic importance by applying a broad real-time PCR assay on faecal samples from children with or without acute diarrhoea.

Paper II compares detection rates and pathogen load estimates (Ct values) among 706 children, of whom the 544 with diarrhoea more often were boys (61.5% vs. 47.5% and younger (15 vs. 23 months) than the 162 healthy controls. At least one pathogen was detected in 94% of children with diarrhoea and 79% of healthy controls. The high detection rate among healthy children can be explained by both the high sensitivity of PCR and the large number of pathogens that our assay targets, and probably reflects the great exposure to enteric pathogens that children in developing countries encounter. The lack of symptoms in these cases may have different explanations (Levine & Robins-Browne, 2012), but probably acquired immunity is an important factor.

Table 3. Detection rates by real-time PCR

	Patients	Controls	OR	P value
Rotavirus	43%	3.1%	23.5	<0.0001
ETEC-estA	21%	10%	2.42	0.001
Norovirus GII	8.1%	4.3%	1.95	0.12
Shigella	13%	11%	1.22	0.59
EPEC bfpA	10%	8.0%	1.24	0.64
Astrovirus	4.8%	3.1%	1.58	0.51
Adenovirus	40%	42%	0.91	0.64
Cryptosporidium	3.1%	3.7%	0.84	0.80
ETEC-eltB	29%	33%	0.83	0.33
Campylobacter	15%	19%	0.77	0.27
EPEC eae	22%	29%	0.69	0.07
Salmonella	5.3%	10%	0.48	0.03
Norovirus GI	2.8%	7.4%	0.35	0.02
Sapovirus	3.7%	11%	0.31	0.0006

OR, odds ratio. <sup>a</sup> Fisher's exact test.

As shown in Table 3, only rotavirus and ETEC-estA were significantly more common in patients than in controls. In multiple regression analysis (Table 4) including detection rates for several agents as well as age and

gender, also norovirus GII and Shigella were associated with diarrhoea, whereas sapovirus and norovirus GI were significantly more rare in children with diarrhoea.

**Table 4. Multiple regression analysis of detection rates**

	OR	P value
Rotavirus	23.4	<0.0001
ETEC-estA	2.74	0.0014
Norovirus GII	2.79	0.0094
Shigella	1.79	0.0042
Sapovirus	0.26	0.0008
Norovirus GI	0.26	0.0065

OR = odds ratio. <sup>a</sup> Patient/control was dependent variable, PCR detection (yes/no), gender and age (continuous) were independent variables. Only agents with P values < 0.05 were included in the final analysis that is shown here.

**Table 5. Detection rates in cases that were PCR negative for rotavirus**

	Patients n=311	Controls n=157	OR	P value
Shigella	20%	11%	2.02	0.026
ETEC-estA	16%	10%	2.65	0.007
EPEC bfpA	14%	8%	2.40	0.024
Norovirus GII	12%	4%	2.79	0.020
Astrovirus	5%	1%	7.29	0.020
Cryptosporidium	5%	3%	1.42	0.530
Adenovirus	42%	42%	1.19	0.428
ETEC-eltB	32%	34%	0.95	0.814
EPEC eae	28%	30%	0.70	0.140
Campylobacter	13%	19%	0.64	0.136
Salmonella	6%	11%	0.45	0.047
Sapovirus	5%	11%	0.24	0.001
Norovirus GI	2%	8%	0.20	0.004

OR, odds ratios, and P values were calculated by multiple logistic regression analysis that included all agents (positive vs. negative) as well as age and gender.

Because rotavirus was predominant we performed separate analyses of rotavirus negative cases. This comparison is interesting also because it might predict the spectrum after introduction of rotavirus vaccination. As shown in Table 5, this comparison showed that in addition to Shigella, ETEC-estA and norovirus GII, also EPEC bfpA and astrovirus were associated with diarrhoea.

The pathogen loads were significantly higher (i.e. Ct values were lower) for Campylobacter, norovirus GII and ETEC-estA, tended to be higher for, Cryptosporidium, rotavirus and Shigella, as shown in Table 6 and Figure 8.

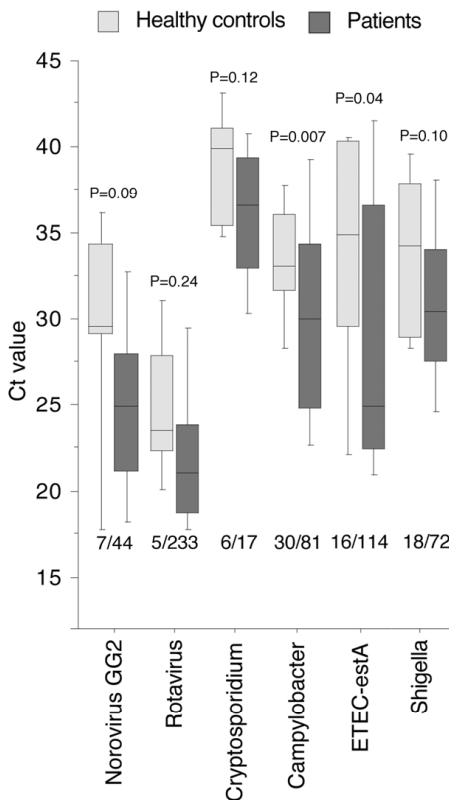


Figure 8. Box plot showing Ct values for the pathogens with differences between patients and controls. The box shows median, 25th and 75 percentile, bars indicate 10th and 90th percentile. P values are shown above, number of patients below the boxes.

Table 6. Median Ct values by real-time PCR

	Patients	Controls	P value <sup>a</sup>
Norovirus GII	24.9	29.5	0.03
Rotavirus	21.1	23.5	0.09
Campylobacter	30.0	33.0	0.01
ETEC-estA	24.9	34.9	0.04
Shigella	30.4	34.0	0.06

<sup>a</sup> Comparison of Ct values by Mann-Whitney U test.

Analyses of Ct values (including ROC analyses) allowed us to identify Ct value cut-offs. Application of these cut-offs resulted in an improved identification of causative agents in terms of greater odds ratios and lower P values (Tables 7 and 8). Interestingly, the cut-off for norovirus (Ct 29) was almost identical to the cut-off presented in a previous report (Phillips *et al.*, 2009). The cut-off for Shigella (Ct 28) is difficult to compare to that suggested in a previous report (Lindsay *et al.*, 2013), because the cut-off in that study was defined as a level of Shigella DNA copies per total faecal DNA (by spectrophotometry).

Table 7. Ct value cut-offs

	Cut-off	Proportion identified by cut-off	OR	P value <sup>a</sup>
Rotavirus	45	43%	23.5	<0.0001
ETEC-estA	28	13%	8.0	<0.0001
Norovirus GII	29	6.1%	10.4	0.003
Shigella	28	3.9%	6.5	0.038
Campylobacter	26	4.0%	6.8	0.040

OR, odds ratio. <sup>a</sup> Fisher's exact test.

Table 8. Multiple regression analysis based on Ct below cut-off

	OR	P value
Rotavirus	26.6	<0.0001
ETEC-estA	7.8	<0.0001
Norovirus GII	18.3	<0.0001
Shigella	12.0	0.0005
Campylobacter	5.5	0.056

OR = odds ratio. <sup>a</sup>Patient/control was dependent variable, Ct value below/above cut-off, gender and age (continuous) were independent variables.

Some kind of standardisation will be needed in order to obtain quantifications that can be compared between laboratories, but measuring total DNA by spectrophotometry does not seem practical. Another group suggested an alternative approach, by relating target DNA to total 16S coding DNA (Lambertz *et al.*, 2008). This type of standardisation should be feasible, and would probably be more accurate than only using the Ct value, because the Ct value alone is influenced by sample dilution, extraction efficiency, PCR efficiency, and how the amplification plot is interpreted. Despite this, Ct values seem to be quite useful even if not normalised, probably because the impact of the mentioned factors is moderate (probably <3-5 cycles) in comparison with the wide range of Ct values that is observed in clinical samples (from Ct 15 to Ct 40).

The take-home messages from this comparison of patients and controls were that real-time PCR has a high sensitivity that yields high detection rates, and that (at least for some agents) accurate interpretation requires that quantitative information be taken into account. Thus, when Ct values were considered, this method identified rotavirus, ETEC-estA, norovirus GII, and Shigella as the main pathogens, which is in accordance with a recent large-scale global study (GEMS, (Kotloff *et al.*, 2013)

Paper III relates detection rates and concentrations of pathogens (Ct values) to epidemiological and clinical data of 880 patients (including the

544 patients in Paper II). This study did not include healthy controls, but instead explored pathogenicity by comparing clinical parameters with detection rates or Ct values. It also investigated use of antibiotics, and epidemiologic differences between microbial agents. The main findings were that (i) comparison of detection rates identified rotavirus and ETEC-estA as associated with more severe vomiting and dehydration than other agents; (ii) higher microbial load (lower Ct) of rotavirus, ETEC-estA and Shigella was associated with more pronounced clinical symptoms; (iii) antibiotic treatment was frequently prescribed, also for viral infections, and mainly guided by presence of fever and dehydration, and (iv) that the assay was useful for revealing epidemiological differences between diarrhoeagenic agents.

Below the findings in paper II and III are presented in detail for each pathogen.

## **Rotavirus**

Rotavirus was detected in 43% (223/544) of patients and 3.1% (5/162) of the healthy controls in Paper II (OR=23.52,  $P<0.0001$ ), and in 37% of all the patients in Paper III (325/880), which is comparable to previous reports. There was no difference in Ct values between sick and healthy children, but on the other hand only 5 of the healthy children were rotavirus positive. The rate was strongly associated with age ( $P<0.0001$ ), as rotavirus was detected in 50% of patients below 12 months as compared with 18% above 24 months of age, in agreement with previous studies (Kotloff *et al.*, 2013; Moyo *et al.*, 2011; Nyambat *et al.*, 2009). The 325 rotavirus positive patients in Paper III more often had a history of vomiting (OR=2.80,  $P<0.0001$ ), and the frequency of diarrhoea was higher for children positive for rotavirus than those that were negative (mean 6.06 vs. 5.78 stools per day,  $P=0.013$ ). As a result the rotavirus-infected children had more severe dehydration (OR=2.60,  $P<0.0001$ ), were more often hospitalised (OR=3.94,  $P=0.0001$ ), and more often given intravenous rehydration (OR=3.78,  $P=0.0001$ ), as compared with gastroenteritis caused by other agents. These associations with more severe disease remained also when age was taken into account in



multiple regression analysis, showing that the more severe symptoms were not only related to the lower age of children infected by rotavirus.

There were also significant associations between rotavirus levels in faeces (Ct values) and vomiting, ( $P=0.035$ ), frequency of diarrhoea, degree of dehydration ( $P=0.0085$ ) and need for intravenous rehydration ( $P=0.0005$ ). Our results agree with previous reports that have shown that rotavirus is the most frequent cause of diarrhoea. In a compilation of African studies, rotavirus frequencies ranged from 14% to 55% with a mean at 24% (Cunliffe *et al.*, 1998), and a recent study from Burkina-Faso found rotavirus in 30% in children with diarrhoea (Bonkougou *et al.*, 2010). In the GEMS study the rotavirus rates were in the range 15-25% at different sites in Africa and Asia (Kotloff *et al.*, 2013), whereas studies in East Asia have reported rates between 25% and 50% (Chen *et al.*, 2013; Meng *et al.*, 2011; Nyambat *et al.*, 2009).

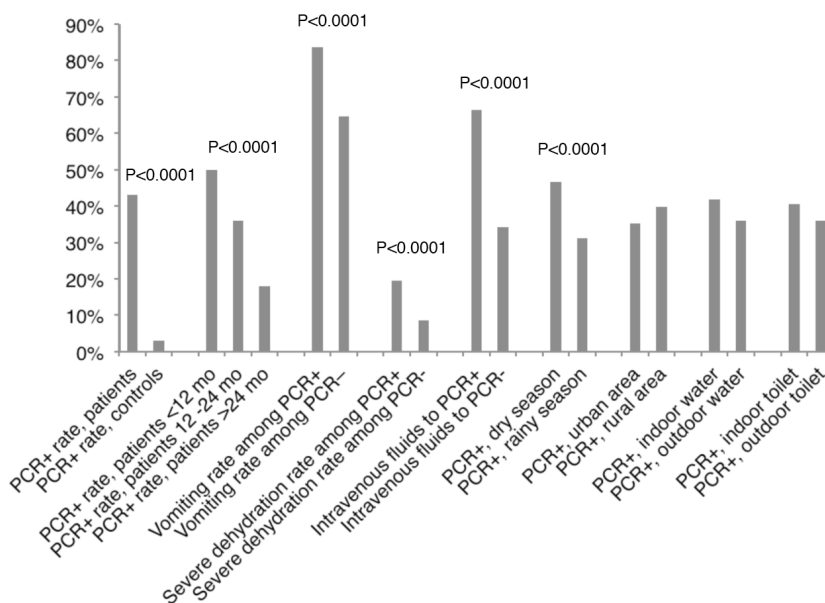


Figure 9. PCR detection rates for rotavirus by study group, age and epidemiology, and rates of selected clinical parameters in patients positive or negative by rotavirus PCR

It is well known that rotavirus may cause severe dehydration, and some studies have reported that rotavirus causes more severe disease than other diarrhoeagenic agents (Aloun *et al.*, 2009). The importance of rotaviral load is not well known. One study reported that rotaviral loads were higher in symptomatic than in asymptomatic infections (Dung *et al.*, 2013), and another that rotaviral load was higher in children with more frequent diarrhoea (Kang *et al.*, 2004).

Rotavirus infections were associated with dry season ( $P=1.92$ ,  $P<0.0001$ ). Although observed also in numerous previous studies in low-income countries (Aloun *et al.*, 2009; Cunliffe *et al.*, 1998; Nitiema *et al.*, 2011), the explanation to this association is not known. There was no association with socioeconomic markers such as type of water source or type of toilet, indicating that vaccination rather than improved water supply or sewage is required to reduce rotavirus infections.

## **Norovirus GII**

Norovirus GII was detected in 8.1% (44/544) of patients and 4.3% (7/162) of the healthy controls in Paper II (OR=1.95,  $P=0.12$ ), and in 8.6% of the patients in Paper III (76/880). In multivariate analysis including other agents and age, norovirus GII was associated with symptomatic infection (OR=2.79, 0.0094).

Ct values tended to be lower in sick as compared with healthy children ( $P=0.09$ ), and by applying a Ct value cut-off at 29, sick children were identified with an OR of 10.4 ( $P=0.003$ ). These associations indicate that norovirus GII was an important cause of gastroenteritis. However, symptoms such as vomiting or degree of dehydration were not more frequent among the 86 patients with norovirus detection (or with lower Ct value) than the children with diarrhoea of other cause (i.e. that were negative by norovirus GII PCR, Paper III). This suggests that even if norovirus GII was a frequent cause of diarrhoea, the symptoms were milder than in rotavirus infections and rarely caused severe dehydration. Our detection rates agree relatively well with previous observations, although some studies have reported higher infections rates (11%-17% for GII, (Bucardo *et al.*, 2008; Chen *et al.*, 2013; My *et al.*, 2013).

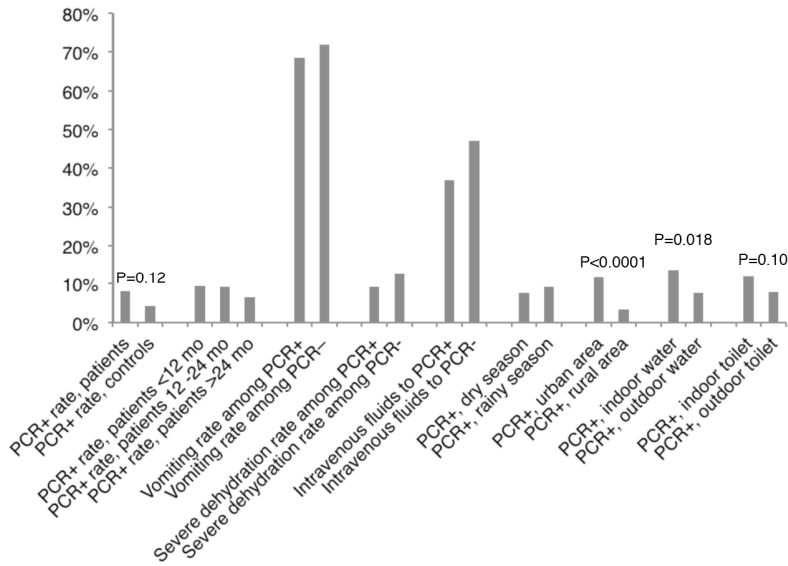


Figure 10. PCR detection rates for norovirus GII by study group, age and epidemiology, and rates of selected clinical parameters in patients positive or negative by norovirus GII PCR

Norovirus G2 infections were not associated with age or season (dry vs. rainy), but were significantly more common among children living in an urban setting ( $P<0.0001$ ) or with indoor water ( $P=0.018$ ).

## Shigella

Shigella was detected in 13% (72/544) of patients and 11% (18/162) of the healthy controls in Paper II ( $OR=1.22$ ,  $P=0.59$ ), and in 17% of all the patients (154/880, Paper III).

Thus, there was no difference between patients and controls in the rate of Shigella infections. Age was however strongly associated with Shigella infections, which were seen in 5.1% of patients younger than 12 months of age as compared with 24% in children above 24 months of age, and in multivariate analysis including age, Shigella detection was associated

with diarrhoea (Paper II, OR=1.79, P=0.0042). These findings agree with previous reports (Bodhidatta *et al.*, 2010; Hien *et al.*, 2008).

Ct values for *Shigella* tended to be lower among sick than healthy children (30.3 vs. 34.0, P=0.10), and lower Ct values were associated with more severe dehydration and need for intravenous fluids (Paper III). A Ct value cut-off at 28 separated sick and healthy with an OR of 6.5 (P=0.04), and identified 3.9% of all patients as probably sick from *Shigella* infection (Paper II). These findings demonstrate that *Shigella* was a cause of symptomatic infection, in particular when the Ct value was below 28, and mainly among children above 24 months of age.

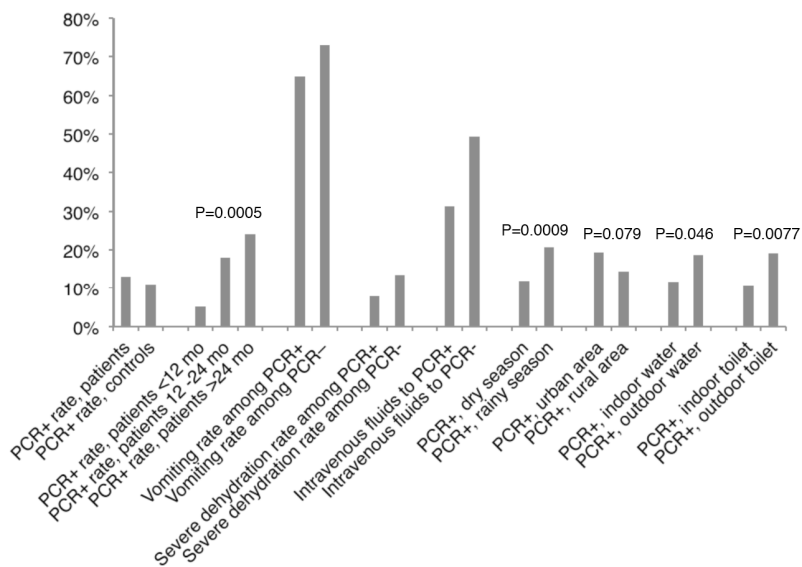


Figure 11. PCR detection rates for *Shigella* (ipaH) by study group, age and epidemiology, and rates of selected clinical parameters in patients positive or negative by *Shigella*.

This agrees with a recent study (also targeting the ipaH gene), reporting that *Shigella* concentrations below a cut-off identified symptomatic infections with an OR $\approx$ 5 (as compared with healthy controls) (Lindsay *et al.*, 2013). That study showed a good agreement between *Shigella* detection by culture and Ct value below cut-off, but that the latter doubled the number of cases attributed to *Shigella*, and also showed that

a Ct below cut-off was associated with bloody diarrhoea. In our analysis of all 880 patients (Paper III), *Shigella* was the only agent associated with bloody diarrhoea and elevated CRP, findings that agree with the knowledge that *Shigella* may cause dysentery and invasive infection (Brooks *et al.*, 2003)

A limitation of the study was that *Shigella* species was not identified, but the low rate of severe bloody diarrhoea as well as earlier data (Mandomando *et al.*, 2007) suggest that most cases probably were caused by *S. flexneri* or *S. sonnei* (i.e. not by *S. dysenteriae*). Part of the cases may also have been caused by enteroinvasive *E. coli*, because they may carry the gene coding for the virulence factor *ipaH*, which was used as target in the PCR (Sethabutr *et al.*, 2000). Epidemiological factors associated with *Shigella* infections were lack of indoor toilet (OR=1.97, P=0.0077) and rainy season (OR=1.93, P=0.0009). The latter is in contrast with a study in Tanzania showing inverse proportions (Vargas *et al.*, 2004).

### **ETEC-estA**

ETEC-estA was detected in 21% (114/544) of patients and 10% (16/162) of the healthy controls in Paper II (OR=2.42, P=0.001), and in 19% of all the patients (167/880, Paper III). The rate was age related: ETEC-estA was detected in 23% in children below 12 months as compared with 12% above 24 months of age. In multivariate analysis including other agents and age, ETEC-estA was associated with symptomatic infection with OR=2.74 (P=0.0014, Paper II). This finding agree with the recent GEMS study, which showed that ETEC producing heat stable toxin was among the five most important causative diarrhoeagenic agents (Kotloff *et al.*, 2013). A similar stronger association with diarrhoea has been observed for ST as compared with LT also by others (Qadri *et al.*, 2005; Steinsland *et al.*, 2002).

The 167 patients with ETEC-estA infection had more severe dehydration (OR=2.03, P=0.0003), were more often hospitalised (OR=1.53, P=0.016), and more often given intravenous rehydration (OR=1.71, P=0.0025), as compared with gastroenteritis caused by other agents

(Paper III). To some extent these associations were related to the younger age of children infected with ETEC-estA, and were weaker when age was taken into account in multiple regression analysis (OR=1.89, P=0.0077 for dehydration, OR=1.54, P=0.018 for intravenous rehydration).

Ct values for ETEC-estA were lower in sick than healthy children (24.7 vs. 34.4, P=0.0008), and a Ct value cut-off at 28 identified 13% of all children as sick from ETEC-estA infection with an OR=8.0 (P<0.0001, Paper II). The impact of bacterial load was also supported by the associations between ETEC-estA Ct values and presence of vomiting (median Ct 24.7 vs. 33.3, P=0.0087, or need for intravenous fluids (median Ct 24.7 vs. 31.6, P=0.032, Paper III).

ETEC-estA infections were more common during dry season (OR=1.75, P=0.018), in agreement with a previous study (Vargas *et al.*, 2004), and more common in rural living areas (OR=1.79, P=0.012).

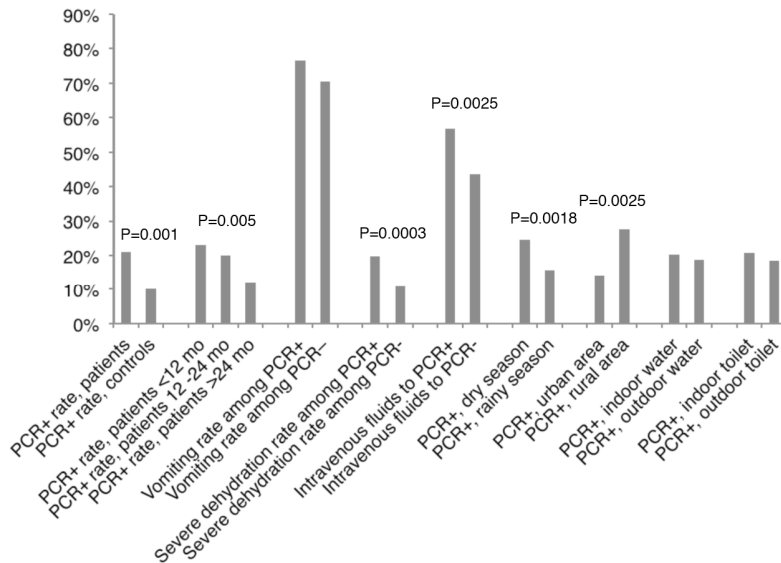


Figure 12. PCR detection rates for ETEC-estA by study group, age and epidemiology, and rates of selected clinical parameters in patients positive or negative by ETEC-estA.

## **ETEC-eltB**

ETEC-eltB was detected in 29% (159/544) of patients and 33% (54/162) of the healthy controls (Paper II, OR=0.83, P=0.33), and in 31% of all the patients (275/880, Paper III). Thus, there was no association between ETEC-eltB and symptomatic infection, and no association between Ct values for ETEC-eltB and symptomatic infection (Ct 33.9 vs. 34.1, Paper II) or between Ct values and degree of symptoms (Paper III).

Our results, as well as previous reports, indicate that ETEC-eltB is less pathogenic than ETEC-estA (Qadri *et al.*, 2005), a conclusion that agrees with the recent GEMS study, in which the same was found when diarrhoeagenic agents in more than 9000 patients and 12000 controls were investigated (Kotloff *et al.*, 2013). These findings suggest that a vaccine towards ETEC-estA would be more important for reducing childhood diarrhoea than one against ETEC-eltB.

There was no association between ETEC-eltB and age. ETEC-eltB infections were more frequent in patients who lacked indoor water (OR=1.74, P=0.0073), suggesting that these infections might reflect exposure to contaminated drinking water.

Out of the 272 children infected with either ETEC-estA or ETEC-eltB only 90 carried both, and in these 90 cases Ct values showed a relatively weak correlation  $R^2=0.08$  (Figure 13), indicating that the ETEC-estA and ETEC-eltB genes usually were present in different bacterial strains.

## **EPEC eae and bfpA**

These targets are considered to be markers for enteropathogenic *E. coli* (EPEC)(Ochoa & Contreras, 2011). In Paper II EPEC eae was detected in 22% (120/544) of patients and 29% (47/162) of the healthy controls (OR=0.69, P=0.07), and in 26% of the patients in paper III (222/880). EPEC bfpA was detected in 10% (53/544) of patients and 8.0% (13/162) of the healthy controls in Paper II (OR=1.24, P=0.64), and in 14% of the patients in Paper III (125/880). Thus, an association with diarrhoea was lacking for detection of eae or bfpA, and such an association was lacking also when only those that were positive for both targets were compared. This is in contrast to studies reporting EPEC to be more frequent among

sick than healthy children, indicating a causal role, in particular for children younger than 12 months (Albert *et al.*, 1999; Meng *et al.*, 2011; Nguyen *et al.*, 2005).

There was no association between symptoms and Ct values for *eae* or *bfpA* either. This is in contrast to a report showing that Ct values for *eae* were lower in children with diarrhoea than in controls (Barletta *et al.*, 2011). In Paper III, both *eae* and *bfpA* were detected in 75 cases (i.e. in 32% of *eae* and 44% of *bfpA* infections). In these cases Ct values for EPEC *eae* and *bfpA* correlated strongly ( $R^2=0.90$  when 3 outliers were excluded,  $P<0.0001$ ) as shown in Figure 13, indicating that in most of these cases the *eae* and *bfpA* genes were present in the same strain. The results show that *eae* and *bfpA* had no impact on symptoms in the children in this study, and even if these factors may promote mucosal adherence of *E. coli*, our findings suggest that EPEC of this type is a pathogen of limited importance for paediatric gastroenteritis, at least in children more than 12 months of age.

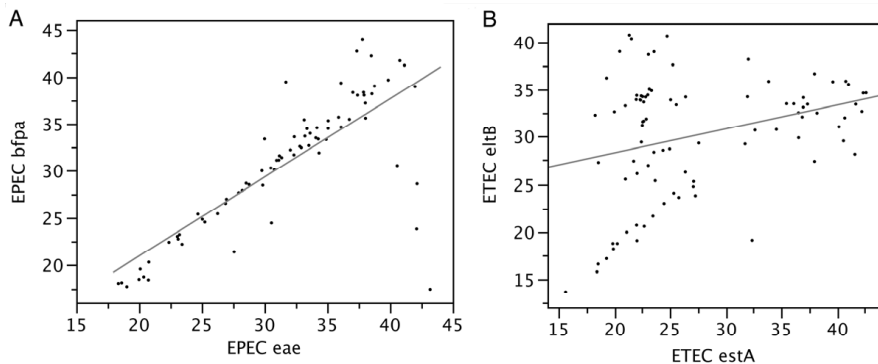


Figure 13. Ct values for patients that were positive for both ETEC *estA* and *eltB* (A), or for both EPEC *eae* and *bfpA* (B).



## **Campylobacter**

Campylobacter was detected in 15% (81/544) of patients and 19% (30/162) of the healthy controls (Paper II, OR=0.77, P=0.27), and in 16.7% of all the patients (147/880, Paper III).

Thus, detection of Campylobacter was not associated with diarrhoea. Campylobacter Ct values were however significantly lower in sick children (29.8 vs. 33.0, P=0.007), indicating a causative role. This association was weaker when age was included in multiple regression analysis (P=0.038). A Ct value cut-off at 26 identified 4% of all patients as sick from Campylobacter with OR=5.5, but with P=0.056.

Analysis of all 880 patients (Paper III) showed that Campylobacter infection was associated with intravenous rehydration (OR=1.49, P=0.030), and that lower Ct values for Campylobacter were associated with vomiting. These results indicate that Campylobacter detection and Ct values were associated with symptoms, but that the association was weaker than for ETEC-estA and Shigella. The detection rates were higher than in many previous reports, but lower than in a study from Thailand which observed 22% and 25% in sick and healthy children (Bodhidatta *et al.*, 2010).

Campylobacter infections were associated with rural living area, which might reflect exposure to poultry or other animals that carry Campylobacter (Rao *et al.*, 2001).

## **Cryptosporidium**

Cryptosporidium was detected in 3.1% (17/544) of patients and 3.7% (6/162) of the healthy controls (Paper II, OR=0.84, P=0.80), and in 7.8% of all patients. Thus, the rate of Cryptosporidium was similar as in some previous reports (Gatei *et al.*, 2006), but lower than other (Kotloff *et al.*, 2013), and – as opposed to several other studies – there was no association with diarrhoea. The rate was however considerably higher at two of our 10 sites (19%), suggesting that Cryptosporidium might be a frequent cause of acute diarrhoea only under certain circumstances, such as if water sources are contaminated. The latter possibility was supported by our finding that Cryptosporidium infections were more common if

water supply was from river or outdoor taps rather than indoor water (OR=4.92, P=0.0014).

The possibility that *Cryptosporidium* may spread by household water is supported by a strong association with rainfall observed in a previous study (Mølbak *et al.*, 1993). In that study, *Cryptosporidium* infections increased just before start of the rainy season, indicating that use of contaminated water sources at the end of the dry season might be of importance. These findings call for studies of how *Cryptosporidium* infections are acquired in these settings and how they best can be prevented.

Ct values for *Cryptosporidium* were higher than for most other agents and tended to be lower among patients than controls (36.6 vs. 39.8, P=0.12). In the 69 patients in Paper III, Ct values for *Cryptosporidium* tended to be associated with degree of dehydration (P=0.09), and were significantly lower in patients given intravenous rehydration (35.5 v. 38.0, P=0.042).

## **Adenovirus**

Adenovirus of any type was detected in 40% (216/544) of patients and 42% (68/162) of the healthy controls (Paper II, OR=0.91, P=0.64).

Adenovirus of types 40/41 were detected in 7.0% (38/544) of patients and 6.8% (11/162) of controls (P=1.0). There was no association between detection of adenovirus and diarrhoea. Among children younger than 18 months there was an association with diarrhoea, but this was lost in multivariate analysis and probably a result of coinfection with rotavirus. The Ct values of adenovirus were not associated with diarrhoea (36.3 in patients, 35.9 in controls, Paper II), and not with severity of symptoms (Paper III).

Our results are similar to some previous reports. One study from Ghana found adenovirus in 28% of 243 children with diarrhoea and in 31% of controls (Reither *et al.*, 2007). Another study from Nigeria found adenovirus of any type in 23% of children with diarrhoea and 18% of controls, and that 8% of the positive samples were of types 40 or 41 (Aminu *et al.*, 2007).

## **Salmonella**

Salmonella was detected in 5.3% of patients and 10% of the healthy controls (Paper II, OR=0.48, P=0.03), and in 6.6% of all patients. Thus, the detection rate was lower in patients than controls, but this difference was not significant in multivariate analysis including other agents as well as age. The Ct values were high, with median around 41 in both patients and controls. The outer membrane protein C (ompC) was chosen as target for PCR because it is present in all serogroups of Salmonella (Alvarez *et al.*, 2004). The choice of a target that is not specific for diarrhoeagenic Salmonella might explain the lack of association with symptoms. However, rather the low rate of detection in patients argues against Salmonella as important cause of diarrhoea in this population. The relatively high detection rate and low faecal concentration found in controls suggest that the children frequently are exposed to Salmonella, but acquire immunity. If so, the source of infection might be poultry just like it may be for Campylobacter, but this requires specific study.

## **Norovirus GI and sapovirus**

Norovirus GI was detected in 2.8% (15/544) of patients and 7.4% (12/162) of the healthy controls (Paper II, OR=0.35, P=0.02), and in 2.5% of all the patients (22/880, Paper III). Sapovirus was detected in 3.7% (20/544) of patients and 11% (18/162) of the healthy controls (Paper II, OR=0.31, P=0.006), and in 3.8% of all the patients (33/880, Paper III). In multivariate analysis including other agents as well as age, both sapovirus and norovirus GI were significantly less frequent among patients than controls (OR=0.26, P<0.01). This finding agrees with previous observations that norovirus GI infections are common among healthy children (Ayukekbong *et al.*, 2013). It still may seem odd that infections were more frequent in healthy than sick children. One possibility is that these viruses, which were often present as asymptomatic infections, were suppressed when another agent induced diarrhoea and innate immune responses.

## **Vibrio cholerae and Yersinia**

Vibrio cholerae was not detected in any case, Yersinia in only one case.

## Co-infections

Infections with more than one pathogen were frequent in both patients and children without diarrhoea (Figure 15). The number of detected agents was not associated with living area or water supply, or with presence of vomiting, degree of dehydration or need of intravenous rehydration. Younger children had significantly higher number of agents than older (mean 2.34, 2.22 and 1.91 agents in children <12, 12-24 or >24 months of age ( $P=0.0009$ )). This difference might reflect greater exposure for the younger children, or acquisition of some degree of immunity in the older children.

Multiple logistic regression analyses were performed with each agent as dependent variable and all other agents as well as age and gender as independent variables. These analyses demonstrated that co-infections between certain pathogens were more frequent, while other combinations were more rare, as shown in Table 9. Many positive associations involved pairs of enterobacteria, the strongest being those between EPEC bfpA and eae, and between ETEC-estA and -eltB. However, co-infections with rotavirus and ETEC-estA, main causes of diarrhoea in the younger children, were also positively associated, independently of age. Rotavirus was otherwise involved in negative associations with norovirus GII, Shigella, EPEC bfpA and Cryptosporidium. Both positive and negative associations are interesting, and challenging to understand (Grimprel *et al.*, 2008; Lindsay *et al.*, 2011). The explanation might be related to the microbes, to the host, or perhaps more likely to both. For example, some microbial combinations might be positively selected because certain hosts have poor immune response against both agents. A negative association is an expected finding for agents that independently may cause diarrhoea (for example rotavirus and Shigella). For other agents, there may be various explanations. For example, one microbe may elicit immune responses that suppress other agents.

Table 9. Multiple regression analysis of co-infections

	t ratio	P value
<i>Positive association</i>		
EPEC eae and EPEC bfpA	6.90	<0.0001
ETEC-eltB and ETEC-estA	6.23	<0.0001
ETEC-estA and sapovirus	3.65	0.0003
ETEC-eltB and Campylobacter	3.26	0.0012
Norovirus GII and EPEC eae	3.10	0.002
ETEC-estA and norovirus GI	2.87	0.0043
EPEC bfpA and Campylobacter	2.72	0.0067
ETEC-eltB and EPEC eae	2.57	0.010
Rotavirus and ETEC-estA	2.33	0.020
Shigella and ETEC-eltB	2.25	0.025
Shigella and EPEC bfpA	2.25	0.025
ETEC-estA and astrovirus	2.20	0.028
ETEC-estA and adenovirus	2.05	0.041
<i>Negative association</i>		
Rotavirus and Norovirus GII	-3.57	0.0003
Rotavirus and Shigella	-3.37	0.0008
Rotavirus and EPEC bfpA	-2.95	0.0034
ETEC-estA and Salmonella	-2.56	0.011
Rotavirus and Cryptosporidium	-2.38	0.018
EPEC eae and Campylobacter	-2.35	0.019

Each agent was evaluated as dependent variable, with other all agents, season, age and gender as independent variables.

Even if multiple infections do not influence severity of disease they are clinically important because they make interpretation regarding cause of diarrhoea difficult. As mentioned earlier, Ct value cut-offs that distinguished symptomatic infections with relatively high odds ratios were identified for ETEC-estA, Shigella and norovirus GII, whereas for rotavirus the OR was high for mere detection. By application of these Ct values, causative agents may be identified with greater specificity. The number of patients with Ct values below these cut-offs of all the 880

patients, was 325 for rotavirus (37%), 98 for ETEC-estA (11%), 65 for Shigella (7.4%), and 42 for norovirus GII (4.9%) (Paper III).

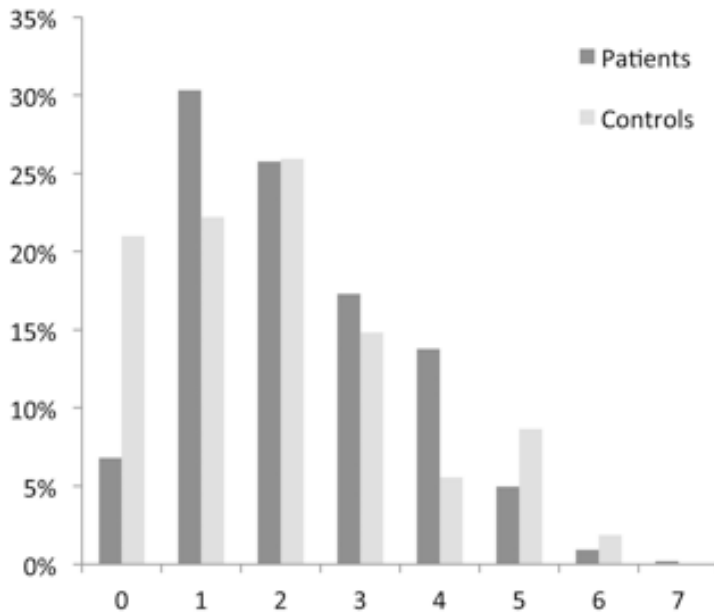


Figure 14. Proportion of patients with no, one or several pathogens detected in faeces by PCR.

## Age

Age was strongly associated with detection of some of the pathogens. Rotavirus and ETEC-estA infections were more common in smaller children, but for ETEC-estA this was restricted to patients. Shigella was more frequent in older children, both in patients and controls, whereas the higher rate of adenovirus among older children was restricted to those without diarrhoea. Associations between age, pathogen detection and symptoms are shown in Figures 15 and 16.

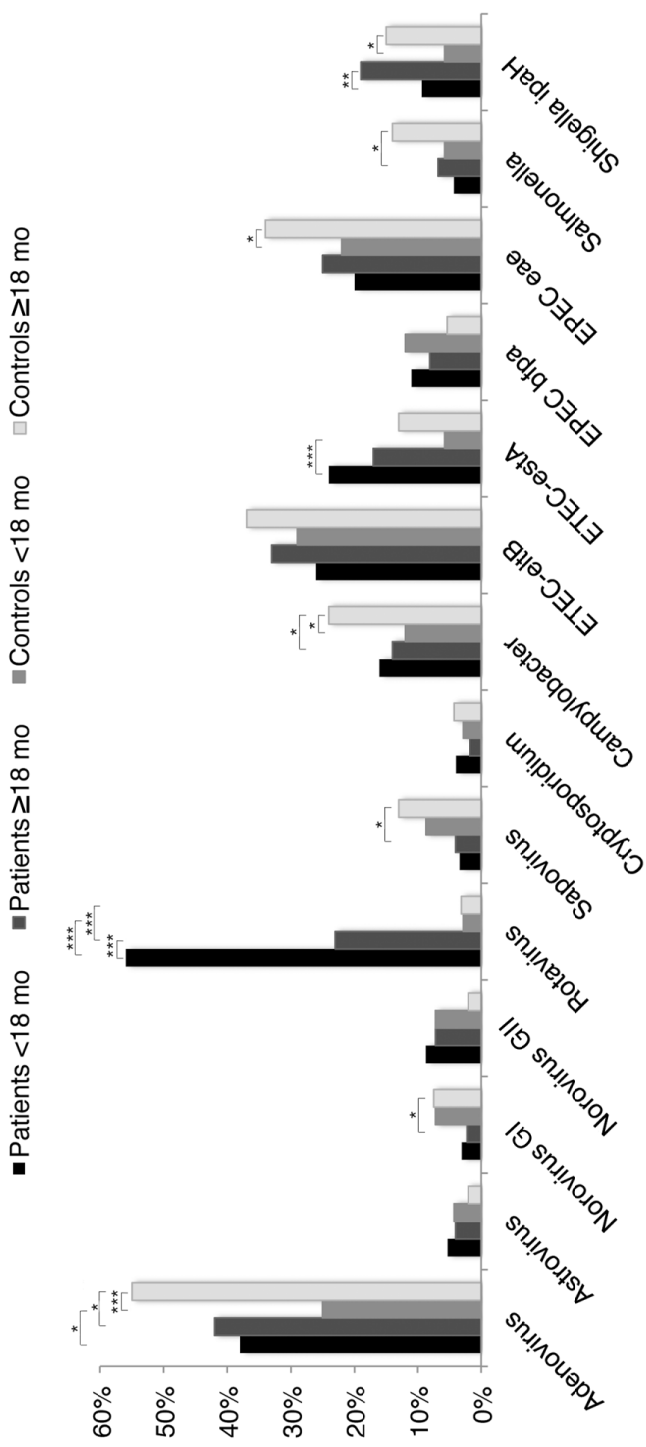


Figure 15. Proportions of 544 patients and 162 controls in Paper II that were positive by PCR for each pathogen and aged below or above 18 months. \* P<0.1; \*\* P<0.01; \*\*\* P<0.001.

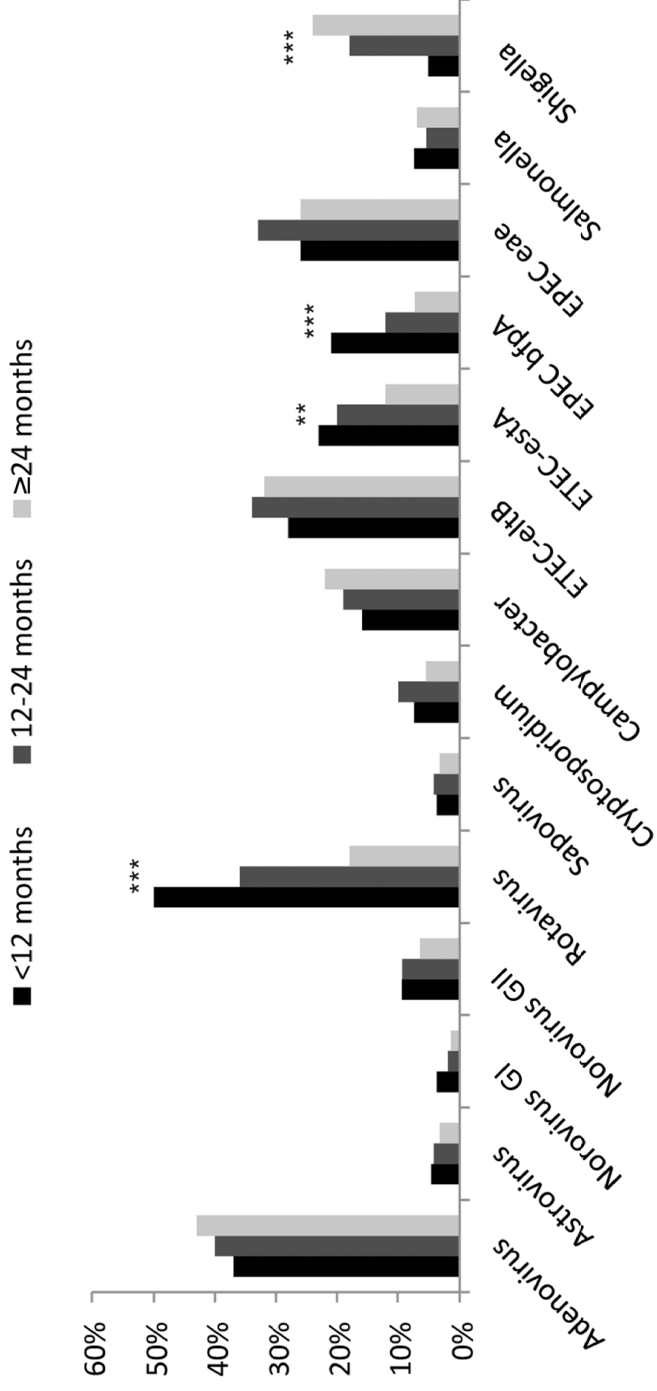


Figure 16. Proportions of the 880 patients in Paper III, who were positive by PCR for each pathogen in the age groups <12 months, 12-24 months and ≥24 months. \*\* P<0.01; \*\*\* P<0.001

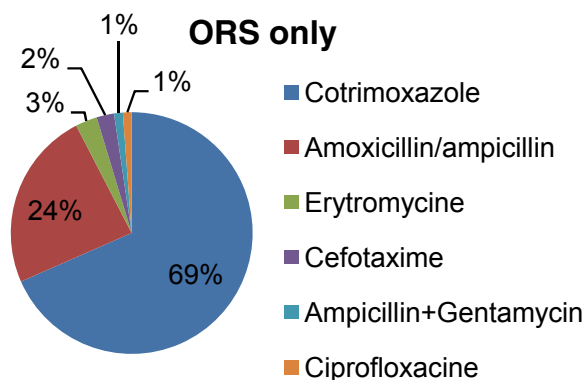


## **CRP**

Elevation of CRP to above 10 mg/L was seen in 50% of the children (385 of 773; CRP results were lacking in 109 cases) with diarrhoea (Paper III). CRP was moderately increased (10-50 mg/L) in most cases (n=248, 32%), and above 50 mg/L in 18% (n=137). CRP was associated with body temperature ( $P<0.0001$ ), but not with age, gender, vomiting, frequency of diarrhoea, Higher CRP levels were associated with fewer number of pathogens, and with presence of Shigella infection. CRP was however of little help for identifying Shigella infections: Shigella was detected in only 23.9% of cases with CRP >10 mg/L, and in 23.4% of cases with CRP >50 mg/L, which was not much above the overall rate of Shigella infection (19.4%). Interestingly, higher CRP levels were also associated with absence of sapovirus ( $P<0.0001$ ), norovirus GII ( $P=0.016$ ) and EPEC bfpA ( $P=0.0021$ ). The latter might reflect that CRP is marker for an innate immune response that may suppress intestinal pathogens in an unspecific manner during gastroenteritis caused by another agent.

## **Antibiotics**

Antibiotics were prescribed to 42% of the 880 children with diarrhoea (Paper III). Antibiotic treatment was associated with fever ( $P<0.0001$ ), degree of dehydration ( $P<0.0001$ ) and CRP level ( $P=0.0059$ ), but not with any specific agent. However, agents significantly associated with dehydration (rotavirus and ETEC-estA) or CRP elevation (Shigella) tended to be given antibiotics more often (45.5%-47.4%). Surprisingly, children with bloody diarrhoea were not given antibiotics more often than those with non-bloody diarrhoea. The choice of antibiotics was influenced by whether intravenous rehydration was considered indicated, as described in the Figure 20.



**Intravenous rehydration**

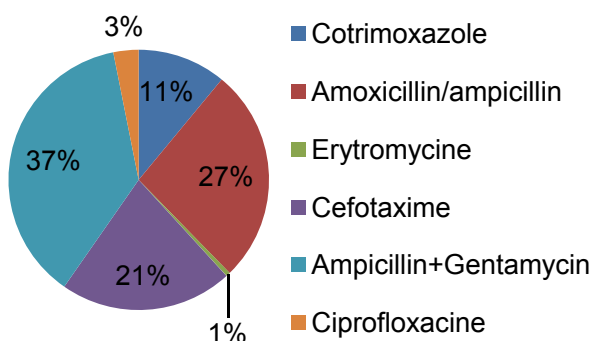


Figure 20. Antibiotics prescribed to 528 children with diarrhoea by type of rehydration.

**SUMMARY AND CONCLUSIONS**

These studies demonstrate the utility of broad molecular testing for investigating causes of acute gastroenteritis. Such an assay can analyse essentially all pathogens that may cause diarrhoea with similar performance and therefore provide results that are not (much) influenced by differences in sensitivity of detection. The results show that infection with several diarrhoeagenic agents are common, thus pointing at the risk of incorrect conclusions regarding aetiology if only one or a few pathogens are investigated. The high rate of multiple infections also demonstrates the difficulty to identify the causative agent in individual patients.

The high detection rate among children without diarrhoea underlines the need to include healthy controls in studies of diarrhoeal aetiologies in developing countries. However, it may be difficult to clarify causes of diarrhoea even if a control group is included, because comparison of detection rates may not be sufficient. For infections that are detected at similar rates in patients and controls, but with higher microbial load in symptomatic infections, the quantitative information provided real-time PCR might be useful for identifying causative agents. Our results indeed indicate that the application of cut-offs regarding the concentration of pathogens in faeces can help to distinguish the cause of diarrhoea, at least at group level and possibly also in diagnostics of individual infections. Before this can be applied our results however have to be confirmed, and quantification of pathogens in faeces should be standardised.

Several important observations were made. Firstly, it was shown that rectal swabs can be used as alternative to faeces for detection of pathogens in faeces. Secondly, the main causative agents, identified by comparing detection rates and Ct values in sick and healthy children and by comparing clinical parameters between patients, were rotavirus, ETEC-estA, Shigella and norovirus GII. In particular, rotavirus stood out as the clearly the most important aetiology, in terms of its high detection rate and the high odds ratio by which its mere detection was associated with disease and severity of symptoms.

Our results agree regarding detection rates and main causative agents agree well with previous reports, including the GEMS study, supporting that the method is accurate and should be useful for future studies. Thirdly, we found that antibiotics were frequently given, and in an irrational manner, treating children with viral as often as those with bacterial infections, guided mainly by fever and dehydration. Finally, we found associations between epidemiology and microbial detection, pointing at the possibility to use broad molecular methods for identifying causes of diarrhoeal disease that could be prevented, for example poor water supply or poor sewage.

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