Rotavirus and polymicrobial enteric infections and their short-term course in East African children

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To my family

"Thousands have lived without love, not one without water"
W.H Auden

Abstract

Diarrhoeal diseases in children under five years are the second leading cause of deaths in children worldwide, and especially in low-income countries in sub-Saharan Africa and in southern Asia where about 450,000 children die every year as a result of diarrhoea. The main cause of diarrhoeal diseases is acute gastroenteritis that is due to infection with viruses, bacteria or protozoa, most often acquired by ingestion of contaminated water or food, or through contact between persons. Studies of acute gastroenteritis in children in low-income countries have identified rotavirus, norovirus, *Cryptosporidium*, enterotoxigenic *Escherichia coli* and *Shigella* as the most frequent aetiologies to diarrhoea. Rotavirus has been the cause of more than half of all deaths caused by diarrhoea in children, but its impact is declining due to increased use of the rotavirus vaccines Rotarix and RotaTeq.

Enteric infections are frequent in small children in low-income countries, both in those with diarrhoea and in healthy controls, and often two or more pathogens are present at the same time. How co-infecting pathogens are associated, and if multiple infections aggravate symptoms, is not well known. We investigated polymicrobial infections among 1318 children in Rwanda and Zanzibar and found negative associations between the agents that alone are capable of causing diarrhoea. Positive associations between agents only in the patient group were unusual and rarely aggravated the symptoms. Positive associations in both patients and controls were found between two pairs of targets, and these results were useful for estimating the proportion of *Escherichia coli* that carried both or only either of some important virulence factors (ST or LT; *eae* or *bfpA*).

Clearance and acquisition of enteric pathogens were studied in 127 children in Zanzibar with diarrhoea. Faeces samples were collected on admission and at a follow-up 14 days later. The majority of the pathogens detected at baseline had been eradicated or decreased in amount on follow-up, but in parallel new infections occurred at a high rate. The clearance rates were independent of the children's nutritional status. The findings suggest that the high rates of enteric infections in children in low-income settings depend on living conditions with high exposure

rather than failure to eradicate pathogens because of malnutrition and poor immune responses.

Rotavirus vaccines were introduced in Rwanda in May 2012. Analysis of samples from children with diarrhoea during the pre- and post-vaccine period showed a significantly lower rate of rotavirus in vaccinated children less than one year of age compared with unvaccinated children in the same age group, as presented in Paper IV. In children aged 1–5 years the rate of rotavirus was independent on vaccination status. Severe dehydration was more rare in vaccinated children, independently of age.

To allow simple distinction between rotavirus genotypes in large numbers of samples, we developed a multiple real-time PCR method. This assay was used for genotyping of rotavirus in samples from Sweden (n = 775) and Rwanda (n = 549). In Sweden, where vaccination has not yet been implemented, the predominant rotavirus genotype in patients with diarrhoea changed significantly over time during 2010–2014, and these shifts differed also between age groups. Likewise, in Rwanda there were significant genotype shifts during 2009–2015, i.e. both before and after the introduction of vaccination. These results indicate that changes in genotype frequencies observed after the start of vaccination most likely were part of natural fluctuations rather than reflecting that the vaccine induced poorer protection against certain genotypes.

In summary, this work provides new knowledge on the importance of enteric co-infections and shows that children in poor settings are heavily exposed to enteric pathogens that they effectively clear. By the introduction of a new and simple rotavirus genotyping method we show how rotavirus genotypes change extensively over time in both Sweden and Rwanda, irrespective of vaccination. Furthermore, the results demonstrate that the introduction of rotavirus vaccination in Rwanda in 2012 has reduced the number of rotavirus infection in children below, but not above, the age of 12 months. Finally, vaccination has reduced the proportion of rotavirus infections that cause severe dehydration, but resulted in a relative increase of other viruses detected in children with diarrhoea.

Keywords: gastroenteritis, diarrhoea, children, co-infections, aetiology, real-time PCR, follow-up, rotavirus, genotypes, vaccine

Sammanfattning

Diarrésjukdomar hos barn under fem år är den näst vanligaste orsaken till dödsfall hos barn världen över, och framför allt i låginkomstländer söder om Sahara i Afrika och i södra Asien där ca 450 000 barn årligen dör till följd av diarré. Majoriteten av diarrésjukdomarna orsakas av akut gastroenterit till följd av infektion av patogener. Akut gastroenterit kan orsakas av virus, bakterier eller protozoer och sprids främst via kontaminerat vatten eller mat samt genom kontaktsmitta mellan personer. Studier av akut gastroenterit hos barn i låginkomstländer har identifierat rotavirus, norovirus, *Cryptosporidium*, ETEC och *Shigella* som de patogener som är starkast associerade med diarré. Rotavirus, som bedömts orsaka närmare hälften av alla dödsfall till följd av diarré, är sannolikt den viktigaste patogena mikroorganismen bland dessa. Det finns idag två godkända vacciner mot rotavirus, Rotarix och RotaTeq.

Enteriska patogener påvisas ofta hos barn i låginkomstländer, både hos dem med diarré och hos friska kontroller, och ofta förekommer två eller flera patogener samtidigt. Hur patogener är associerade till varandra och om multipla infektioner påverkar graden av symptom är relativt okänt. I delarbete I i avhandlingen studerades associationer mellan olika patogener hos barn i Rwanda och på Zanzibar med polymikrobiella infektioner. Vi fann en negativ association mellan de agens som var för sig är starkt associerade till sjukdom. Positiva associationer mellan agens var ovanliga och samverkade sällan till att förvärra symtomen.

I delarbete II studerades utläkning och ny infektion av enteriska patogener hos barn på Zanzibar med diarré. Faecesprov togs vid sjukdomsdebut samt vid ett uppföljningstillfälle 14 dagar senare. Majoriteten av de patogener som detekterades vid sjukdomsdebuten var utläkta eller hade minskat i koncentration vid uppföljning, men nya infektioner förekom med hög frekvens. Fynden tyder på att den höga frekvensen tarmpatogener hos barn i låginkomstländer snarare beror på levnadsförhållanden med hög exponering än att de skulle vara långtidsbärare av

patogener på grund av undernäring och dåligt fungerande immunförsvar.

Rotavirusvaccin introducerades i Rwanda i maj 2012. Analys av prov från barn med diarré under tidsperioden före och efter vaccinintroduktion visade en signifikant lägre frekvens av rotavirus i vaccinerade barn under 1 år jämfört med ovaccinerade barn i samma åldersgrupp, vilket presenteras i delarbete IV. Hos äldre barn (1–5 år) var frekvensen av rotavirus oförändrad. Allvarlig uttorkning var ovanligare hos vaccinerade barn med rotavirusinfektion jämfört med ovaccinerade barnen med rotavirusinfektion.

För att möjliggöra enkel identifiering av olika varianter av rotavirus, s.k. genotyper, i ett stort antal prover utvecklade vi en realtids-PCR metod, som presenteras i Delarbete III. Denna metod användes för genotypning av rotavirus i prov från Sverige (n = 775, Delarbete III) och Rwanda (n = 549, Delarbete IV). I Sverige, där vaccination ännu inte har införts, förändrades den dominerande rotavirusgenotypen hos patienter med diarré betydligt över tiden under 2010–2014, och dessa förändringar skilde sig mellan olika åldersgrupper. Även i Rwanda förändrades genotyperna påtagligt under 2009–2015, alltså både före och efter införandet av vaccination. Dessa resultat indikerar att de förändringar som sågs efter vaccinationsstart i Rwanda sannolikt var en del av naturliga fluktuationer snarare än ett tecken på att vaccinet inducerar ett sämre skydd mot vissa genotyper.

Sammanfattningsvis ger detta arbete ny kunskap om betydelsen av enteriska saminfektioner och visar att barn i socioekonomiskt utsatta områden är starkt exponerade för tarmpatogener som de dock effektivt klarar av att bekämpa. Vidare introduceras en ny och enkel rotavirusgenotypningsmetod som visar hur rotavirusgenotyper påtagligt förändras över tid i både Sverige och Rwanda oberoende av vaccination. Våra resultat visar att introduktionen av rotavirusvaccinering i Rwanda år 2012 har minskat antalet rotavirusinfektioner hos barn under, men inte över, 1 års ålder. Vaccinationen minskade andelen rotavirusinfektioner som orsakar svår uttorkning, men resulterade i en relativ ökning av andra sjukdomsorsakande virus hos barn med diarré.

List of papers

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. **Andersson ME**, Kabayiza J-C, Elfving K, Nilsson S, Msellem MI, Mårtensson A, Björkman A, Bergström T, Lindh M.
Co-infection with enteric pathogens in East African children with acute gastroenteritis – associations and interpretations.
Manuscript

II. **Andersson ME**, Elfving K, Shakely D, Nilsson S, Msellem MI**,** Trollfors B, Mårtensson A, Björkman A, Lindh M.
Rapid clearance and frequent reinfection with enteric pathogens among children with acute diarrhea in Zanzibar.
Clinical Infectious Diseases 2017; 15;65(8):1371-1377.

III. **Andersson M**, Lindh M.
Rotavirus genotype shifts among Swedish children and adults – application of a real-time PCR genotyping.
Journal of Clinical Virology 2017; 96:1-6

IV. **Andersson ME**, Kabayiza J-C, Elfving K, Nilsson S, Bergström T, Lindh M.
Rotavirus infections and their genotype distribution in Rwanda before and after the introduction of rotavirus vaccination.
Manuscript

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Abbreviation

AF	Attributable Fraction
BL	Baseline
CF	Colonization factor
Ct	Threshold cycle
DNA	Deoxyribonucleic acid
EPEC	Enteropathogenic *Escherichia coli*
EPEC-*eae*	Enteropathogenic *Escherichia coli* with gene coding for intimin
EPEC-*bfpa*	Enteropathogenic *Escherichia coli* with gene coding for bundle forming pilus
ETEC	Enterotoxigenic *Escherichia coli*
ETEC-*estA*	Enterotoxigenic *Escherichia coli* producing heat-stable toxin
ETEC-*eltB*	Enterotoxigenic *Escherichia coli* producing label-stable toxin
FU	Follow-up
GI	Genogroup I
GII	Genogroup II
GBD	Global Burden of Disease collaboration
GEMS	Global Enteric Multicenter Study
LT	Heat labile toxin
MAL-ED	Multisite birth cohort study
NSP	Non-structural protein
nt	nucleotide
OR	Odds Ratio
PCR	Polymerase Chain Reaction
RNA	Ribonucleic acid
ST	Heat stabile toxin
VP	Structural protein
WHO	World Health Organization
z score	Standard deviation score

1. Introduction

Despite a considerable decline, diarrheal disease in children younger than 5 years still causes an estimated 690 million cases of illness and 500,000 deaths every year worldwide. Sub-Saharan Africa and South Asia are most affected and about 90% of deaths occur there (Figure 1) [1,2]. The leading risk factors for diarrhoea are unsafe water, inadequate sanitation and malnutrition [3,4] and the main cause of diarrheal disease is acute gastroenteritis caused by enteric pathogens. This thesis investigates enteric pathogens in samples collected from children less than 5 years in two East African sites, Rwanda and Zanzibar.

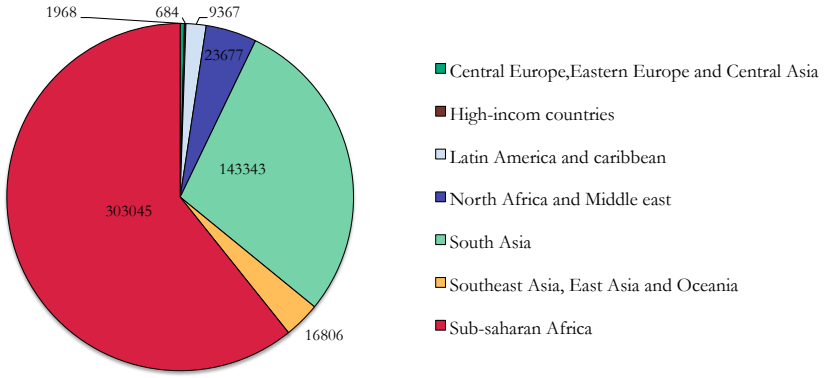

Figure 1. *Worldwide distribution of diarrhoea associated deaths in children less than 5 years of age [5].*

Rwanda is a small country, with a young population; 43% of 11.8 million citizens are below 15 years of age. The access to improved drinking water sources, meaning protected springs, public taps/standpipes or running water in dwelling and to improved sanitation, defined as unshared toilet facility or pit latrine with a slab, reaches 73% respectively 54% of the inhabitants. Severe malnutrition is rare (0.7%) and the

proportion of children under five with underweight is 9.3%. In the last decade the mortality rate due to diarrhoea has been reduced with 48%, but 50 out of 1000 children still die before 5 years of age, and 9% of these deaths are caused by diarrhoea [5,6].

In Zanzibar, a Tanzanian island with 1.3 million citizens, 98% have access to improved drinking water and 59% have improved, not shared sanitation facilities. On the other hand, 17% on the rural Zanzibar population have no facility at all available, the highest percentage in Tanzania. Underweight is present in 14% and malnutrition in 5% of children below five years of age. The mortality is 56 per 1000 children under five in Tanzania, of which 6% is due to diarrhoea, as compared with 29% 10 years ago [5,7].

A wide range of pathogens 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 high-income countries, viruses are the major cause of acute infectious diseases, whereas in addition bacteria, in particular *Escherichia coli* and *Shigella*, are common in low-income countries.

We have previously reported causes of gastroenteritis among children in Rwanda and Zanzibar [8-10]. Pathogens that may cause diarrhoea were identified in a large proportion of children with diarrhoea (>90%), but also among those without diarrhoea (>70%). The importance of the pathogens was investigated by comparing sick and healthy children, and by comparing children with mild or severe diarrhoea. By these comparisons rotavirus, enterotoxigenic *Escherichia coli* producing heat-stable toxin (ETEC-*estA*), *Shigella*, *Cryptosporidium* and norovirus genogroup II (GII) were identified as the most important causes of diarrhoea.

These results agree well with a global enteric multi-center study (GEMS) conducted in 2007–2011 in sub-Saharan Africa and in south Asia, which showed that rotavirus, ETEC-*estA* and *Shigella* were the major causes of

childhood diarrhoea [11]. Additional analyses showed that also adenovirus 40/41 and *Cryptosporidium* were important causative agents [12]. New data presented from the Global Burden of Disease collaboration (GBD) emphasizes the importance of rotavirus, *Cryptosporidium* and *Shigella* as responsible for death in children under five years of age [5], and in addition The Global Rotavirus Surveillance Network identified Norovirus GII, ETEC-*estA* and adenovirus 40/41 to be a major cause of acute watery diarrhoea worldwide [13].

1.1 Rotavirus

Rotavirus is a non-enveloped double stranded ribonucleic acid (RNA) virus in the reoviridae family. The virus is transmitted by faecal-oral route and after a short incubation (1-3 days), symptoms start, typically as nausea and vomiting, often with low-grade fever, followed by diarrhoea [14]. Dehydration is a frequent complication of the infection, especially in low-income countries where severe dehydration in small children can be fatal. Globally, death caused by rotavirus has decreased markedly the last decade, but still approximately 215,000 children die every year, the majority of them in low-income countries [15]. In high-income countries, deaths due to rotavirus are rare and instead rotavirus infections are a socioeconomical problem.

1.1.1 Rotavirus classification

The genome of rotavirus is divided in 11 segments, each encoding for at least one structural protein (VP1, VP2, VP3, VP4 (VP5+VP8), VP6 and VP7) (Figure 2) or a non-structural protein (NSP1, NSP2, NSP3, NSP4, and NSP5). Rotaviruses are classified according to their antigenic specificities into serogroups and serotypes. 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 the infections.

The VP4 and VP7 genes are important because their sequence variability defines the serotypes of rotavirus A. The surface protein VP4, protrudes as a spike (Figure 2), which binds to receptors on cells in the upper part of the small intestine and drives the entry into the cell. The virus becomes infectious when the endogenous enzyme trypsin, modifies VP4 to VP5 and VP8. The glycoprotein VP7 forms the outer surface and is, along with VP4, critical for inducing immunity to the infection. VP7 defines G-types, and the VP4 protein defines P-types of the virus [14]. Although all 11 genomic segments need to be taken in to account for complete classification, the genotype of the virus is usually defined by a combination of G-and P-types. There are 11 known G-types and 13 P-types that infects humans [16-18].

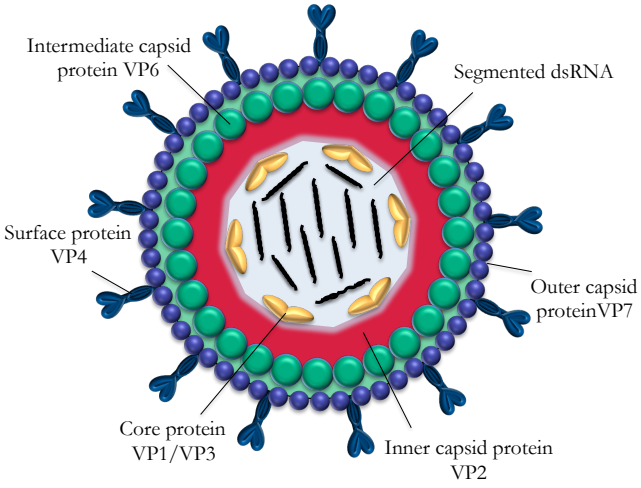

Figure 2. *Rotavirus structural proteins and double-stranded (ds) RNA.*

Because the genes coding for proteins G and P are present on different segments, reassortment may occur when two rotavirus strains within the same serogroup infect the same cell, creating strains with new P-G combinations. Today, at least 73 G/P genotype combinations of rotavirus serogroup A have been described to infect humans [19]. The five P-G combinations G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] are considered to cause more than 75 % of rotavirus diarrhoea among children worldwide [17,18,20]. Additionally, G12P[8] and also G12P[6], has become frequently detected in recent years, flagged as emerging in in several countries, and proposed to be grouped among the most common genotypes [21-23].

1.1.2 Pathogenesis and Immunity

Rotavirus infects mature enterocytes in the mid and upper part of the villi of the small intestine. Viral replication leads to increased intracellular Ca^{2+} level, increased secretion of Cl^- and shut-off of the host cell protein synthesis. The viral protein NSP4 has endotoxin effects and activates the enteric nervous system, leading to the induction of intestinal water and electrolyte secretion. Impaired hydrolysis of carbohydrates may also contribute to excessive fluid loss from the intestine, and destruction of epithelium and villus ischemia may aggravate symptoms [14,24]. Rotavirus also has the ability to infect enterochromaffine cells in the gut and activates vagal afferent nerves, which through release of serotonin can stimulate brain stem structures and cause vomiting production[25].

The human immune response to rotavirus is not completely understood, partly because much of knowledge about protection against rotavirus is based on animal models, with a gut physiology that may not be representative for humans [26]. Primary rotavirus infection results almost exclusively in acute gastroenteritis, and induces immunity that protects against subsequent rotavirus infections. In otherwise healthy children, severe disease normally doesn't occur after two obtained rotavirus infections [27,28]. The acquired immune response is represented by T cells recognizing epitopes on the surface of the infected cell and B

cells producing antibodies against virus specific proteins, including neutralizing antibodies directed against the outer layer proteins VP7 and VP4 [29]. Non-neutralizing antibodies against the structural proteins VP2 and VP6, as well as against NSP2 and NSP4, have also been found in serum from convalescing individuals. The clinical importance of these antibodies and whether they are protective is not known [26].

1.1.3 Rotavirus vaccine

Two vaccines have been available since 2006, Rotarix (GlaxoSmithKline Biologicals, Rixensart, Belgium) and RotaTeq (Merck and Co, Inc, Pennsylvania, USA), and licenced in over 100 countries. Rotarix is a live attenuated vaccine based on a human G1P1A[8] rotavirus strain, and is given in two oral doses. RotaTeq is also a live vaccine taken orally at three occasions, but 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[5] from bovine rotavirus. The fifth virus expresses VP7 of serotype G6 from a bovine rotavirus and VP4 of type P1A[8] from human rotavirus. The two vaccines have been shown to have an equal protective effect. In 2009, World Health Organization (WHO) recommended all countries to include rotavirus vaccination in their national immunisation programs, especially those countries with high diarrhoea mortality rates in children [30,31]. In January 2017, 92 countries globally had introduced rotavirus vaccine, 85 in their national immunisation programs.

In Latin America and Caribbean, several countries had an early introduction of rotavirus vaccination, already in 2006 and 2007, which had a dramatic impact on rotavirus infections. Several studies as well as meta-analyses have shown a vaccine efficacy of 71-85% against severe rotavirus diarrhoea and 73-90% against hospitalization due to rotavirus infection [32-35].

These vaccines also seem to be effective in African populations, but there were some concern that the protection might be inferior because

early case-control studies in different countries showed a variable vaccine efficacy, ranging between 18% and 77% against severe rotavirus diarrhoea in different countries [36,37]. Concern was also based on observed mismatches between the subtype of the vaccine and the subtype of rotaviruses circulating in this region [38-40]. In studies performed in high and middle income countries Rotarix and RotaTeq seem to induce similar broad protection against homotypic strains that matched the G-types and P-type included in the vaccines, and heterotypic strains that did not match any serotypes in the vaccines [41-43]. In total, 32 African countries have introduced rotavirus vaccination, of which 26 uses Rotarix and 6 uses RotaTeq. Rotavirus vaccination was introduced in the general immunization program in May 2012 in Rwanda (RotaTeq) and in February 2013 in Zanzibar (Rotarix). New published data, some years after vaccine introduction, from several African countries have documented a positive effect of rotavirus vaccination, but the methods to measure and report the efficacy are somewhat inconsistent, which makes data difficult to compare. A 23%-52% reduced hospitalization due to rotavirus infection has been observed among children less than 1 year of age, whereas the effect on children aged 1-4 years varies from a modest reduction to even more cases in some regions [44-48]. A compilation of the reduction in hospital admission in different age groups and different countries is presented in Table 1.

In high and middle income countries the vaccine effectiveness against rotavirus hospitalization is 81%-93% [35,49-56]. In Sweden, deaths due to rotavirus in children under 5 years of age are very rare, but the number of hospital admissions is estimated to be 2,100 and visits to an emergency room 3,700 every year. The ministry of health in Sweden estimates further that 14,000 children with rotavirus gastroenteritis visit primary care and that 30,000 children are treated at home each year because of rotavirus infection [57]. The cost caused by rotavirus infection was estimated one decade ago in several European countries, among them Sweden. The cost per episode of confirmed rotavirus gastroenteritis in Sweden was estimated to 2,101 euro [58]. Finland, where rotavirus vaccination was included in the national immunisation program already in 2009, has reported a 91% reduction of rotavirus infec-

tion in out-patients less than 5 years of age [59]. In Sweden, rotavirus vaccination has yet only been introduced in some regions, but is planned to soon be included in the national immunisation program.

Table 1. Reduction in hospital admission due to rotavirus in African countries.

Country	Pre vaccine	Post vaccine	Age (years)	Difference in hospitalization between pre- and post-vaccine	Ref.
Togo	2008-June 2014	July 2014-June 2015	<1 1-4	-23% -4%	[48]
Botswana	2009-2012	2013-2014	<1 1-2 2-5	-43% -20% +14%	[47]
Ghana	2009-Mars 2012	April 2012- 2014	<1 1-2 2-5	-24% -15% no reduction	[45]
Tanzania (Zanzibar)	2010-2012	2013-2014	<1 1 2-4	-52% -30% -25%	[46]
Malawi	Jan 2012-June 2012	2013-2015	<1 1-4	-48% +38,5%	[44]
Rwanda	2011	2013-2014	< 5	-29%	[60]

1.1.4 Genotype circulation

As mentioned, G1P[8], G2P[4], G3P[8], G4P[8] and G9P[8] are considered to be the most common circulating genotypes worldwide. The predominance of certain genotypes fluctuates over time, and may vary at the same time in different areas. Rare genotypes, for instance G6P[6] and G8, often of bovine origin seem to be more common in Africa [39,61-64]. The fluctuation of genotypes in East African countries over a few years, prior to the introduction of rotavirus vaccination [65-69], is presented in Figure 3. After vaccine introduction, genotype fluctuation has continued, and a markedly increased incidence of G12P[8] has been observed in Australia, Europe and Latin America [21,70,71].

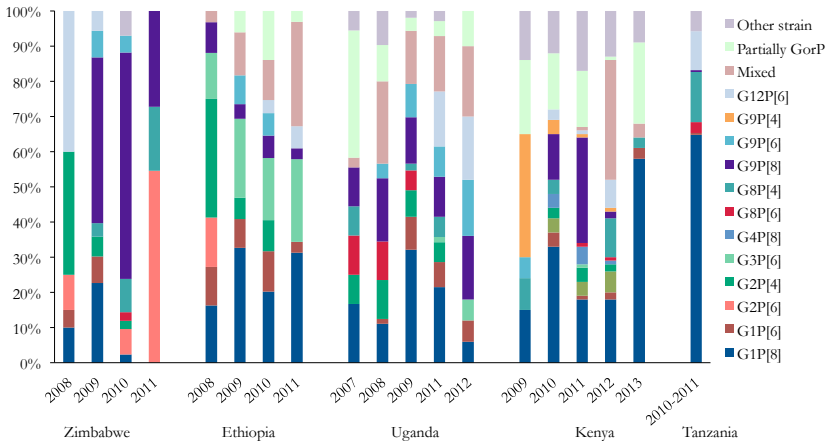

Figure 3. *Genotype distribution of rotavirus in some East African countries before vaccine introduction.*

1.2 ETEC and Shigella

Despite improvements of water quality and sanitation, and the introduction of rotavirus vaccination, the incidence of acute diarrhoea remains high among children less than five years of age in the developing world. ETEC and Shigella are the two most important bacterial pathogens for which there are no currently licensed vaccines.

ETEC is usually acquired by ingestion of contaminated food or water and may cause watery diarrhoea. ETEC strains are characterized by the production of binding proteins called colonization factors (CF) and at least one of two enterotoxins: heat labile toxin (LT) and heat stabile toxin (ST). The gene coding for LT is called eltB and the gene coding for ST is called estA [72]. Approximately one-third of the ETEC strains isolated from diarrheic patients express only LT, one third only ST, and another third both toxin types [73]. ETEC strains infect the host epithe-

lial cells in small intestine using CF antigens to adhere, and production of LT and ST may lead to over secretion of fluids and electrolytes, leading to diarrhoea [72]. CF and LT induce immunity. The immunity provides only short-term protection for LT, and because there are approximately 30 genetically different CF, the immunity towards this antigen is often insufficient. The ST is a short peptide that is poorly immunogenic [74].

Shigella, like ETEC is transmitted by infected food or water, and since the infectious dose is low, person-person transmission is also possible. *Shigella* is genetically very similar to *E. coli*, and may be considered as an *E. coli* with certain phenotypic characteristics. *Shigella* is still classified as a separate genus and divided into four species; *S. dysentariae, S. flexneri, S. sonnei* and *S. boydi* [75,76]. The most important species in Africa is *S. dysenteriae* and *S. flexneri*, because they are more frequent and have a great clinical impact. They cause invasive infection of the colon, and may induce watery diarrhoea as well as bloody diarrhoea, i.e. dysentery. *S dysenteriae* may produce Shigatoxin which can cause additional complications, including life-threatening kidney damage, besides severe diarrhoea [77].

Overall, the majority of studies indicate that ETEC producing ST alone or in combination with LT, are more strongly associated with diarrhoea than ETEC producing only LT. The frequency of ETEC producing ST among children in developing countries with diarrhoea is 4%-19% [9-12,78] and globally ST strains are responsible for an estimated 5% of deaths due to diarrhoea [5]. LT is frequently detected among asymptomatic controls [8,10,11,79], probably reflecting that acquired immunity prevents disease but not infection. ETEC producing ST infections are most important in the second year of life [11,78] and considered to be less frequent with higher age. However, in sub-Saharan Africa and South Asia, ETEC and *Shigella* infections in older children, adolescents and adults represent an underestimated problem that contributes to lost productivity and school absence, especially among children 5–14 years of age [80].

Shigella is one of the most important causes of diarrhoeal disease among children in developing countries, causing an estimated 11% of deaths due to diarrhoea according to GBD [5]. The frequency of *Shigella* in children less than 5 years of age, living in developing countries, is 4%–13%. Among children older than 2 years, an adjusted attributable fraction (AF), i.e. contribution of a risk factor or pathogen to a disease, of 35% has been shown. [8-10,12,78,81,82].

1.2.1 Vaccines

Vaccination against ETEC and *Shigella,* especially by a combined vaccine against both pathogens, would be extremely valuable for saving lives and promoting the health of infants and children in the developing world [83,84]. There has been a range of vaccine candidates and research on potential vaccine components for ETEC is constantly on going, supported among others by WHO, but today there is no licensed vaccine available. Ducoral, vaccine against the *Vibrio cholerae* enterotoxin, is used to prevent ETEC associated travellers diarrhoea, probably protective by LT being similar in structure and function to cholera enterotoxin [85]. The main obstacles to designing a vaccine against ETEC are the great diversity of strains, their virulence repertoire and the poor immunogenicity of ST [86,87]. Humans being the only reservoir for *Shigella* complicate vaccine development since there is no animal models that successfully replicate Shigellosis. The main candidates for *Shigella* vaccine are whole-cell and conjugated vaccines [86].

Oral vaccination. www.alamy.com F4785Y

1.3 Other enteric pathogens of clinical importance

The importance of pathogens infecting humans varies in different populations. The most important enteric pathogens that, besides rotavirus, ETEC and *Shigella,* may cause acute gastroenteritis in children are presented below.

1.3.1 Adenovirus

Adenovirus is non-enveloped DNA virus, divided in 7 species (A-G) and classified in more than 50 subtypes or serotypes. Adenoviruses are frequently detected in children, among whom acute respiratory infection is the most common clinical presentation, but subtypes 40-41 and 52 belonging to species F respectively G mainly cause diarrhoea [88,89]. Other subtypes are commonly detected in faecal samples but whether they cause diarrhoea is insufficiently known.

In our previous studies, related to this thesis, adenovirus of any type was detected in 40% of patients and 42% of the healthy controls. Adenovirus of types 40/41 was detected in 7.0% of patients and 6.8% of controls and there was no association with diarrhoea [8,9]. A study performed in Tanzania show lower frequencies of adenovirus infection, 3.5% in patients and 2.4% in healthy controls, possibly explained by the lower sensitivity in detection method (enzyme-linked immunosorbent assay vs. polymerase chain reaction (PCR)). They reported that adenovirus was significantly associated with diarrhoea in children less than one year [90]. Similarly, a reanalysis of data from GEMS showed that adenovirus 40/41 was one of the most important causes of diarrhoea, estimated to cause 11% of cases in children less than one year [12].

1.3.2 Astrovirus

Astrovirus is a non-enveloped virus with a positive sense, single-stranded RNA, belonging to the Astroviridae family. Astrovirus has

been identified as a common viral aetiology of acute gastroenteritis in children, but causes outbreaks also in adults and elderly [91,92]. In African studies astrovirus has been detected in 4.5%-6% of children with diarrhoea, significantly more often than in non- diarrhoea controls, especially in children aged 1-5 years [9,93-95].

1.3.3 Norovirus

Noroviruses are non-enveloped, single-stranded RNA viruses belonging to Caliciviridae family. Noroviruses are classified in five genogroups (GI–GV), of which GI, GII, and GIV infect humans [96]. GII, and in particular genotype GII.4, has been most strongly associated with diarrhoea, causing >80% of gastroenteritis outbreaks [97]. Noroviruses are highly contagious pathogens and they affect individuals of all age groups in high- and low-income countries. They are transmitted by faecal contaminated food and water, by person-to-person contact, or through aerosol of the virus [98-100]. Norovirus often induces cascade vomiting and intense diarrhoea after an incubation period of 24-48 h, with symptoms normally lasting a few days [101]. It is not well known whether human norovirus infections induce any lasting protective immunity or to what extent immunity protects against exposure to different strains [102]. This is important because noroviruses are highly genetically diverse, making the development of an efficient norovirus vaccine a challenge.

Both longitudinal [103] and case-control studies [104] have shown that norovirus infections in children in low-income countries often are asymptomatic, but also that they are causing a significant part of diarrhoea in children under five years of age [9,13,78].

1.3.4 Sapovirus

Sapovirus, like noroviruses, belongs to the Caliciviridae family and are divided in to five genotypes, GI-GV, of which all but GIII infect humans. Sapovirus infect both human of all ages and animals, causes both sporadic cases and outbreaks of acute gastroenteritis worldwide.

The clinical importance of sapovirus in African children with diarrhoea is not well documented, but sub-Saharan studies show a detection frequencies between 8% and 18% in children < 5 years with diarrhoea [10,81,94,95,105], and 4%-11% in non-diarrhoea controls [9,10,95].

1.3.5 *Campylobacter*

Campylobacter are gram-negative bacteria belonging to the Campylobacteriaceae family. There are several species that infect humans, with *C. jejuni* and *C. coli,* being the clinically most important. *Campylobacter* is the most common cause of bacterial diarrhoea in industrialized countries. It is an important aetiology also in and low-income settings [106], and is estimated to cause 6.2% of deaths due to diarrhoea in sub-Saharan African children less than 5 years of age [5]. In industrialized countries, both children and adults are affected by *Campylobacter* through traveling or outbreaks related to consumption of poultry products or water, often present with abdominal pain, fever and bloody diarrhoea reflecting invasive colitis that may last for 7 days or more. *Campylobacter* is endemic in many low-income countries causing diarrhoea especially in children under 1 year of age, with asymptomatic infection increasing with age [107], suggesting that repeated exposure in early life leads to the development of protective immunity [108].

1.3.6 Enteropathogenic *Escherichia coli*

Enteropathogenic *Escherichia coli* (EPEC) do not produce toxins or have invasive properties, but may cause diarrhoea by other mechanisms. The only known reservoir for EPEC is humans. EPEC are usually classified by molecular techniques that include identification of genes coding for intimin (*eae* gene) and bundle forming pilus (*bfpA* gene). Typical EPEC carry both the *eae* and *bfpA* genes, whereas atypical EPEC code only for *eae* [109]. Whether the atypical EPEC cause any disease in humans is debated. Diarrhoea due to EPEC decreases with age and studies conducted worldwide have shown that typical EPEC are mainly associated with diarrhoea in children <1 year of age, especially in low-income countries [11,110-112] . Infections in adults or older children

are rarely reported. This apparent resistance in adults has been attributed to the loss of specific receptors with age or development of immunity [113].

1.3.7 *Salmonellae*

Salmonellae are gram-negative bacteria of the Enterobacteriaceae family, classified in two species, *S. enterica* and *S.bongori. S.enterica*, a common cause of infectious disease in humans and animals throughout the world, is further divided in subspecies and nearly 1,500 serological variants (serovars). Human *Salmonellae* infections are classically divided into diseases caused by typhoidal (infection by servoar Typhi or Paratyphi) or non-typhoidal *salmonellae* [114]. The former category causes the systemic disease typhoid, while non-typhoidal salmonella is comprised of the majority of other serovars that predominantly cause uncomplicated gastroenteritis in high-income countries [115], but frequently causes invasive bacterial disease in sub-Saharan Africa [116,117]. Its epidemiology among children in low-income countries is insufficiently studied, but it appears to be an important cause of gastroenteritis, detected in about 5% of children and less often in healthy controls [10,81]. The rate of antibiotic resistance, both against typhoidal and non-typhoidal strains, is alarming [118] and WHO recommends use of the two available licenced vaccines against typhoid fever [119].

1.3.8 *Cryptosporidium*

Cryptosporidium is a protozoa that forms oocysts, which after ingestion releases sporozoites that infect enterocytes. In acute infections, diarrhoea is often accompanied by fever and vomiting, sometimes causing dehydration that requires hospitalisation. There are several species, which infect different hosts. The two main species are *C. hominis*, which infects only humans, and *C. parvum*, which infects humans as well as animals. *Cryptosporidium* infections appear with seasonal variation, occurring more frequently at higher temperature and more rainfall. Animal exposure, particularly cats and cattle, is also associated with

increased risk of infection [120]. *Cryptosporidium* has been detected in between 4% and 30% of children in low-income countries, and is strongly associated with diarrhoea. It is in particular a major cause of acute gastroenteritis among children below 5 years of age [10,11,78]. Prolonged infections also appear to be common, and have been associated with malnutrition, in particular with stunting [121,122].

1.4 Co-infections

In the studies of diarrhoea aetiology, sensitive molecular methods targeting virus, bacteria and parasites, have revealed that several pathogens often are present in the same faecal sample [9,11,12,123,124], in particular in low-income countries. The observed rates of polymicrobial infections in patients with diarrhoea vary between 20-76%, depending on geographic area, the number of targeted pathogens and detection method. Infections with several pathogens are more rare in controls without diarrhoea, as shown in Table 2.

The importance of co-infections, and whether more than one agent contributes to the symptoms in patients in whom multiple pathogens are detected, is not well known. Studies on co-infections are rare, their results inconsistent, and the interpretations are not always justified. One study from Ecuador reported that co-infections with rotavirus and *Giardia* as well as with rotavirus and *E.coli/Shigella* had synergistic effect on symptoms [125]. Another study, conducted in China, reported that co-infections with rotavirus and norovirus GII increased the severity of diarrhoea [126].

Pathogens that independently can cause diarrhoea should of statistical reasons show negative associations among patients (unless acting synergistically on symptoms) but not among healthy controls. Negative associations were recently reported for *Vibro cholerae* and any of rotavirus, adenovirus, *Cryptosporidium*, *Shigella* and ETEC, but possible interpretations of this finding were not discussed [127].

Table 2. Proportion of samples with more than one pathogen detected.

Patients	Controls	Country	Methodology	Pathogen panel	Ref
45%	31%	GEMS[a]	Culture, ELISA, PCR	Broad[c]	[11]
41%	29%	MAL-ED[b]	Culture, ELISA, PCR	Broad[c]	[78]
21%	4%	Ecuador	Culture, PCR, immunochromatograpy	Limited (6 pathogens)	[125]
20%	5%	China	Culture, PCR	Limited (7 pathogens)	[126]
76%	60%	Zanzibar	Real-time PCR	Broad[c]	[10]
63%	57%	Rwanda	Real-time PCR	Broad[c]	[8]
35%	8%	Jordan	Culture, PCR	Broad[c] (except viruses, only rotavirus)	[128]
27%	15%	Ghana	Culture, Microscopy, PCR	Broad[c]	[129]

[a] Kenya, Mali, Mozambique, The Gambia, Bangladesh, India, and Pakistan.

[b] Bangladesh, India, Nepal, Pakistan, South Africa, Tanzania, Brazil and Peru.

[c] Including a wide range of pathogens; bacteria, viruses and protozoa that causes diarrhoea.

To further evaluate the importance of co-infections, especially rare combinations, further studies, fulfilling the following requirements, are needed:

- Large number of both patients and controls are needed to obtain sufficient number of each pathogen combination to allow accurate statistics.
- Documentation of relevant symptoms.
- The analytical methods should target a wide range of pathogens – virus, bacteria and protozoa – and should have high and equal sensitivities.
- The studies should be performed in different geographic areas and during different time periods since the importance of co-infections may be influenced by season, climate and socio-economic factors.

1.4.1 Concepts to interpret and present polymicrobial infections

Association tells whether two variables are related to each other, positively or negatively. Regarding pathogens, positive and negative association describe if they occur together more or less often than expected from the frequency with which they are detected alone. In addition, the pathogen concentration or quantitative parameters can correlate.

Thus, enteric pathogens that can cause diarrhoea should show negative associations among patients (unless acting synergistically on symptoms), but not among healthy controls.

A positive association may be observed if pathogens act synergistically on symptoms, i.e. if they cause more severe symptoms together than would be expected from the effect of each agent alone. Such synergy may counteract the anticipated negative association within the patient group mentioned above. Presence of a synergistic effect on diarrhoea can be evaluated by calculating the odds ratio (OR) of a pathogen or a pathogen combination to occur, in patients compared to healthy controls. Synergistic interaction is presented if the ratio of $OR_{co\text{-}infection}/(OR_{single\ infection\ 1} \times OR_{single\ infection\ 2})$ exceeds 1.

Whether a certain co-infection aggravates symptoms in persons who are sick can also be studied by comparing the severity of symptoms, for example the degree of dehydration, in patients with co-infection and those with pathogen alone.

Positive associations between pathogens can also be found in both patients and controls. Such associations can reflect that pathogens depend on the same environmental or host factors for their transmission or ability to infect. Positive associations can also be observed if the target genes used for detection are present in the same virus particle or bacteria.

1.5 Asymptomatic infections

Beyond higher frequency of co-infection among patients with diarrhoea, the use of more sensitive molecular methods have also revealed that enteric infection is very common among asymptomatic controls. The most likely explanation to acquired immunity after a previous infection abrogates symptoms but does not prevent infection. In addition, there are other possible explanations for finding pathogens in asymptomatic controls. In some cases an infection might seem to be asymptomatic, if sampling was performed during a shedding period after recovery from diarrhoea [130-132]. Asymptomatic infections might also be the result of a low infectious dose in patients that do not have acquired immunity, or be caused by bovine pathogens incapable of causing human disease. The presence of maternal secretory immunoglobulin A antibodies from breast milk, or protective host factors such as variants of blood group antigen (demonstrated for norovirus and cholera) [133], can also prevent symptomatic infection.

1.6 Important risk factors for diarrhoea

1.6.1 Unsafe Water, Unimproved Sanitation and Hygiene

The WHO/UNICEF Joint Monitoring Program working with the access to safe drinking water and basic sanitation reported in 2015 that 91 per cent of the global population uses an improved drinking water source. In sub-Saharan Africa 68% of the population have access to improved drinking water, but still 100 million people use surface water. Improved sanitation facilities are available for 68% of the global population but reach only 30% of the sub-Saharan population [134].

Unimproved drinking water is defined as unprotected springs and dug wells, surface water and water stored in a tank.

Improved drinking water is protected springs, public taps/standpipes or running water in dwelling.

Improved sanitation facilities is defined as flush toilets and pit latrines using the flush/pour flush method that are connected to either a sewer or a septic system, ventilated improved pit latrines, and pit latrines with slab and composting toilet. Sanitation facilities that are shared by two or more households, even if improved, are classified as unimproved because shared sanitation facilities tend to be less hygienic and less accessible than private sanitation facilities used by a single household. Sanitation also includes distribution of disposal of garbage on a hygienic basis [135].

The frequency of diarrhoea is related to water quality and facilities, and there is a potential to reduce diarrhoeal disease through improvements of both water supply and sanitation in low- and middle-income settings. It has been shown that pathogens, like *E. coli*, detected in the drinking water are associated with an increased prevalence of child diarrhoea [136]. The most effective measure to improve water for individual households is the use of filter, boil or chlorinate at the point of consumption in combination with safe water storage. At the community level, introduction of high-quality piped water of good microbial quality, supplied continuously (minimize the use of unsafe water) to the household is the most effective improvement [137-139].

The impact of improved sanitation on diarrhoea has not been studied to the same extent as the impact of water quality, but reviews based on available data indicate that a 30% reduction of diarrhoea could be achieved [139,140]. Miserable conditions in the slum, sanitation facility shared by six or more households, presence of faeces on the floor around sanitation facility as well as uncollected garbage indoors were significantly associated with acute diarrhoea in a study performed in Ethiopia [141]. Introduction of sewage sanitation in urban areas in low-income settings would be expected to have a positive impact on health with decreased frequency of diarrhoea even if improved latrines would alleviate the sanitation problem [137]. Simple improvements like intro-

duction of coverage for latrines were shown to significantly reduce the prevalence of diarrhoea in children under 4 years of age living in Congo [142].

1.6.2 Malnutrition

Malnutrition is divided in wasting (low weight-for-height), stunting (low height-for-age), underweight (low weight-for-age) and deficiencies in vitamins and minerals. To measure nutrition status, *Z* scores (standard deviation scores) for anthropometric data can be calculated, and typically reported using a cut-off value, with <–2 defining moderate to severe malnutrition, <–3 defining severe malnutrition, and >+2 defining overweight [143,144]. Malnutrition is both consequence of and a risk factor for diarrhoeal disease. For example, *Cryptosporidium parvum* impairs nutrient absorption and has been shown to have a lasting adverse effect on height growth, especially when acquired during infancy [121,122]. Both symptomatic and asymptomatic *Campylobacter* infections in Peruvian children were associated with poorer weight gain while symptomatic infections additionally were marginally associated with poorer height growth [145]. In a study performed in Bangladesh, infection with *Cryptosporidium*, LT producing ETEC, *Shigella*, norovirus GII, and Giardia were more commonly detected in malnourished cases than controls [146]. Children who die from diarrhoea often suffer from underlying malnutrition, which makes them more vulnerable to diarrhoea and dehydration. Each diarrhoeal episode, in turn, makes their malnutrition worse [147]. A decrease in the gut permeability and the amount of inflammatory cells in intestine, as well as increase of gastric acid production and vaccination titre response are immune parameters among others that have been shown to be affected by malnutrition, but the mechanisms for immune dysfunction in malnourished children is still poorly understood [148].

1.7 Persistence and clearance of enteric pathogens

Persistent diarrhoea in infants and young children living in low-income countries is associated with a greater risk of subsequent growth faltering and high mortality [149]. It is however not well known to what extent persistent diarrhoea is due to persistent infection. In order to elucidate this question longitudinal studies that both document symptoms and analyse pathogens are required. However, such studies are rare and usually focus on one or a few pathogens. Longitudinal studies are also required to clarify to what extent persistent infections explain the high prevalence of enteric pathogens in children in poor living conditions. An alternative explanation could be a heavy environmental exposure.

Three longitudinal studies with repeated sampling of children in Brazil showed that the mean number of diarrhoea episodes was on average 5 per child and year [150-152]. Persistent diarrhoea (last $\geq$ 14 days) was observed in 5-8% in these studies but whether enteric pathogens persisted in children without diarrhoea was not well studied. A study from Bangladesh in children less than one year showed a mean incidence rate of infectious diarrhoeal events of 4.7 per year with a mean duration of these episodes of 5.5 days [152]. The association between persistent diarrhoea and specific pathogens is not well known, but long-lasting protozoa infections are considered to be of particular importance [153,154].

A longitudinal study from Cameroon, including monthly sampling during one year reported that 6% of norovirus infections lasted longer than 1 month [155]. In Guinea-Bissau, longitudinal sampling with focus on ETEC showed that the majority (81%) of ETEC infections were cleared within 2 weeks [156].

2. Aims

The overall aim of this thesis was to investigate enteric infections in children in Rwanda and Zanzibar below five years of age, with focus on co-infections, short-term course and impact of rotavirus vaccination.

The specific aims were:

Paper I
To characterize associations between different enteric pathogens or virulence genes in children with or without diarrhoea.

Paper II
To determine to what extent enteric infections among these children were cleared or acquired two weeks after acute gastroenteritis.

Paper III
To develop a new rotavirus genotyping method and apply it to study rotavirus genotypes in Sweden.

Paper IV
To investigate how rotavirus vaccination in Rwanda has influenced the number of rotavirus infections, their genotype distribution and clinical presentation.

3. Materials and Methods

3.1 Patients

The number of included patients and healthy controls from each location, sampling year and in which paper they are a part of is presented in Figure 4.

Location	*Number*	*Sampling year*	*Paper*
Rwanda	829 patients	2009-2012	I, IV
	159 healthy controls	2009-2012	I
	818 patients	2014-2015	IV
Zanzibar	165 patients	2011	I, II
	127 Follow-up	2011	II
	165 healthy controls	2011	I
Sweden	775 patients	2010-2014	III

Figure 4. *Number of included samples from Rwanda, Zanzibar and Sweden.*

3.1.1 Rwanda

Children seeking care in 5 health centres, 3 district hospitals and 2 university hospitals were included during both the main raining season (from March to May) and the main dry season (July to August) in January 2009 – April 2012. After vaccine introduction in May 2012, additional patients from 6 district hospitals and 2 university hospitals were included between Junes 2014 to December 2015. The localisation of hospitals and health care centres are presented in Figure 5. The inclusion criteria was age ≤5.0 years and diarrhoea with a duration of <96 hours (with or without vomiting or fever). Diarrhoea was defined as

passage of 3 or more loose or watery stools per day, but in breast-feeding infants, diarrhoea was considered when they had more than 6 stools per day.

The healthy controls included were children below 5.0 years of age, living in the same geographic area as the patients, without any episode of diarrhoea in 14 days prior to the sampling date. Nurses and community health workers at nursing schools and immunization centres recruited them during the same time period as patients in 2010–2012.

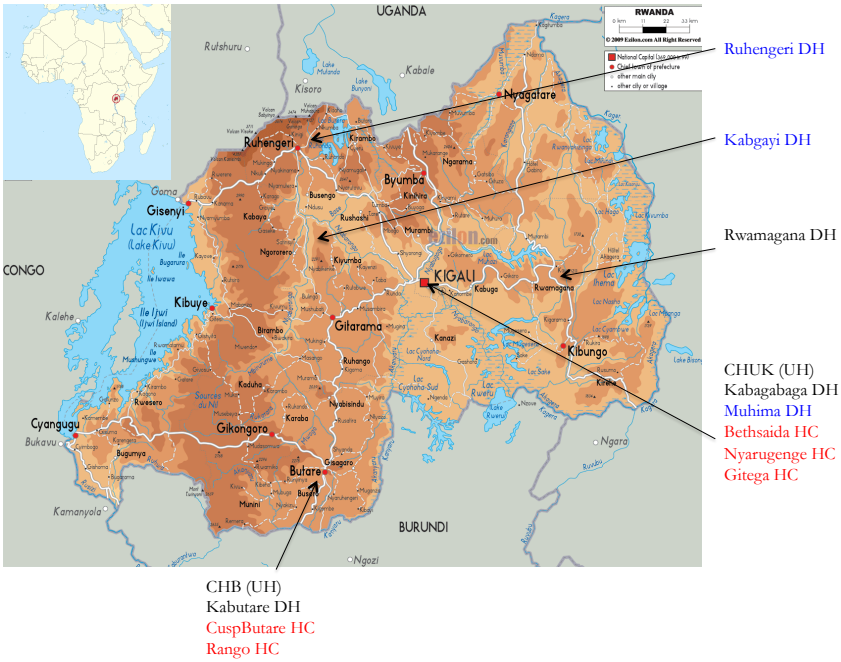

Figure 5. *University Hospitals (UH), District Hospitals (DH) and Health care centres (HC) included in the study conducted in Rwanda. Health cares facilities written; in red were included in 2009–2012, in blue 2014–2015 and in black both periods.*

3.1.2 Zanzibar

Children (n=165) with diarrhoea who participated in a larger study of fever aetiologies, carried out during April-July 2011, were included in this work. Sampling was performed during the end of the rainy season and beginning of the dry season. The patients were aged 2-59 months and attended Kivunge Primary Health Care Centre in rural Zanzibar (North A district) with fever (measured axillary temperature of ≥37.5˚C or a history of fever during the preceding 24 hours according to the accompanying guardian) and diarrhoea (history of loose stools during the preceding 24 hours). A part of them (n=127) revisited the health care centre 14 days after initial sampling, when a follow-up sample was collected.

This study also included healthy controls that were children aged 2-59 months, matched for living area and sampling time period, and having no history of diarrhoea, cough, running nose or fever in the preceding 10 days.

3.1.3 Sweden

During January 2010 to December 2014, 18,996 clinical samples, the majority from patients with acute gastroenteritis, were received at the molecular virology unit of the department of Clinical Microbiology, Sahlgrenska University Hospital in Gothenburg. Rotavirus was identified in 4.9% and these samples were analysed with a real-time PCR genotyping method that we developed (Chapter 3.4).

3.1.4 Classification of dehydration

A thirsty, restless and irritable child with sunken eyes and a skin that returns slowly to normal structure after pinch is classified as having a moderate dehydration, while a child who is lethargic or unconscious, not able to drink, with very sunken eyes and a skin that normalizes very slowly after pinch is severely dehydrated, according to WHO guidelines.

3.1.5 Anthropometric data

For assessment of nutritional status, weight, height, and upper arm mid circumference were recorded in participating patients in Zanzibar. From these data we calculated z scores of height for age, weight for height, and mid upper arm circumference for age, using the World Health Organization Anthro for personal computers, version 3.2.2, 2011.

3.2 Sample material and nucleic acid extraction

Stool samples from Rwanda and Sweden were collected with a rectal swab (Copan Regular Flocked Swab 502CS01, Copan Italia Spa, Brescia, Italy) in a tube with 1 mL of sterile saline, or as faeces. The samples from Zanzibar were all collected as rectal swabs. The samples from Rwanda and Zanzibar were stored in a local laboratory at -80 ºC until transport to Sweden.

Approximately 250 µL of faeces were dissolved in 4.5 mL of saline and centrifuged 5 min at 750xg. Then, 250 µL of dissolved faeces or 250 µL 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 µL. These procedures correspond to an approximate dilution of faeces to 1:10 and the dilution of rectal swab samples depends on the specimen volume contained in the swab, but estimated to be between 1:10 and 1:100.

3.3 Pathogen panel

All samples from patients and healthy controls that were collected in Rwanda 2009-2012 were analysed by an in house multiplex real-time PCR panel as previously described [10,157]. The enteric panel targeted adenovirus, astrovirus, norovirus GI or GII, rotavirus, sapovirus, *Campylobacter jejuni*, *Cryptosporidium parvum/hominis*, ETEC-*eltB*, ETEC-*estA*, EPEC-*eae*, EPEC-*bfpA*, *Salmonellae*, *Shigella*, *Vibrio cholera* and

Yersinia enterocolitica. In the analyses of the second set of samples from Rwanda (2014-2015) adenovirus, *Vibrio cholera* and *Yersinia enterocolitica* were excluded. In the analyses of samples from Zanzibar, EPEC-*eae* and EPEC-*bfpA* were excluded from the panel. Due to the low numbers detected, norovirus GI, *Vibrio cholera* and *Yersinia enterocolitica,* these agents were excluded from the analyses of co-infections and cause of infection in *papers I* and *II*, and likewise *Salmonellae* was excluded from the analysis of the cause of infection in *paper II*.

3.4 Method development of multiplex real-time PCR for genotyping of rotavirus

We developed a new real-time PCR-based method for genotyping of rotavirus, which was applied in *paper III* and *IV*. The development of the method is described below.

3.4.1 Primers and probes

Nucleotide sequences representing each VP7 (G1, G2, G3, G4, G9 and G12) and VP4 type (P[4], P[6] and P[8]) were downloaded and aligned. Primers and probes were designed to discriminate between genotypes. They were located to segments that were conserved within the targeted genotype, but differed to other genotypes either at the 3' ends of either primer or at one or several positions in the probe. These matches and mismatches were presented as heat maps in *paper III*. In Figure 6, heat maps show the reactivity for the systems against the sequences from strains included in Rotarix and RotaTeq vaccines respectively.

A	VP 7 Number of mismatches					
	Rotarix	RotaTeq				
	G1	G1	G2	G3	G4	G6
G1 Forward Primer	1	1	3	6	5	4
G1 Reverse Primer	0	2	8	5	2	6
G1 Probe	0	0	4	5	3	6
G2 Forward Primer	10	10	1	14	15	13
G2 Reverse Primer	3	3	1	5	5	4
G2 Probe	4	5	1	7	7	1
G3 Forward Primer	3	3	3	1	3	5
G3 Reverse Primer	11	12	4	3	9	4
G3 Probe	9	11	10	4	9	6
G4 Forward Primer	5	4	4	5	1	7
G4 Reverse Primer	7	7	6	5	3	8
G4 Probe	3	5	4	5	0	3
G9 Forward Primer	7	9	10	7	8	9
G9 Reverse Primer	7	7	9	5	5	7
G9 Probe	3	4	6	1	6	7
G12 Forward Primer	9	8	6	9	10	12
G12 Reverse Primer	8	8	5	3	9	5
G12 Probe	8	9	9	5	8	7

B	VP4 Number of mismatches		
	Rotarix	RotaTeq	
	P[8]	P[8]	P[5]
P4 Forward Primer	3	3	8
P4 Reverse Primer	3	2	15
P4 Probe	2	1	9
P6 Forward Primer	6	4	6
P6 Reverse Primer	6	5	7
P6 Probe	4	4	5
P8 Forward Primer	3	4	6
P8 Reverse Primer	3	4	16
P8 Probe	1	2	7

Figure 6. *Heat map showing the degree of fit between primers and probes for G types (A) or P types (B) and Genbank sequences representing the monovalent Rotarix and pentavalent RotaTeq vaccines. Green colour indicates very good fit (0-1 mismatch), yellow indicates 2-4 mismatches in primers and 1-2 mismatches in probes, and red indicates presence of either a mismatch at the 3' position of the primer or more than 4 mismatching positions in primer or probe. The matching was based on comparison with sequences with accession numbers HG917354 (G1) and HG917355 (P[8]) for Rotarix, and GU565057 (G1), GU565068 (G2), GU565079 (G3), GU565090 (G4), GU565046 (G6), GU565044 (P[8]), and GU565055 /GU565066 /GU565077 /GU565088 (P[5]) for RotaTeq. Based on the matching one can predict that genotyping of a sample containing a Rotarix strain would be positive by the G1 PCR and probably also by the P[8] PCR (likely with a higher Ct value than for G1), and that a RotaTeq strain would be positive by the G1, G2, G4 and P[4] PCR, but negative by the P[8] PCR.*

3.4.2 Multiplexing

The evaluation of the real-time method for genotyping of rotavirus comprised the following steps:

1. Firstly the performance of each real-time PCR was evaluated by analysis of serial dilution in five steps of a pUC57 plasmid containing the target regions for all real-time PCR systems (GenScript, Piscataway, NJ). This either confirmed sufficient amplification efficiencies or indicated that adjustments needed to be carried out.

2. Optimization of primer concentration, annealing temperature and adjustment of the instrument temperature ramp rate.

3. When all systems had a sufficient efficiency with a standard curve slope as close to -3.32 (efficiency 100%) as possible (Figure 7), every duplex combination was tested. This step showed the duplex combinations that performed well.

4. Triplex combinations were tested and accepted if the Ct value did not increase by more than 1 cycle as compared with each component PCR (Figure 7) and if analyses of serial dilutions indicated that the amplification efficiencies were good.

5. In the last step of evaluation, patient samples with rotavirus detected in the clinical diagnostics (using a real-time PCR targeting NSP3 gene) and 4 different Equalis panels from 2011–2014 including rotavirus samples with known genotype were analysed by both single and multiplex real-time PCR.

6. At least three samples of each genotype were confirmed by Sanger sequencing [158].

The genotyping method with three triplex reactions, presented in *paper III*, was then applied on rotavirus positive samples detected in Clinical Microbiology, Sahlgrenska University Hospital, Gothenburg, during 2010–2014.

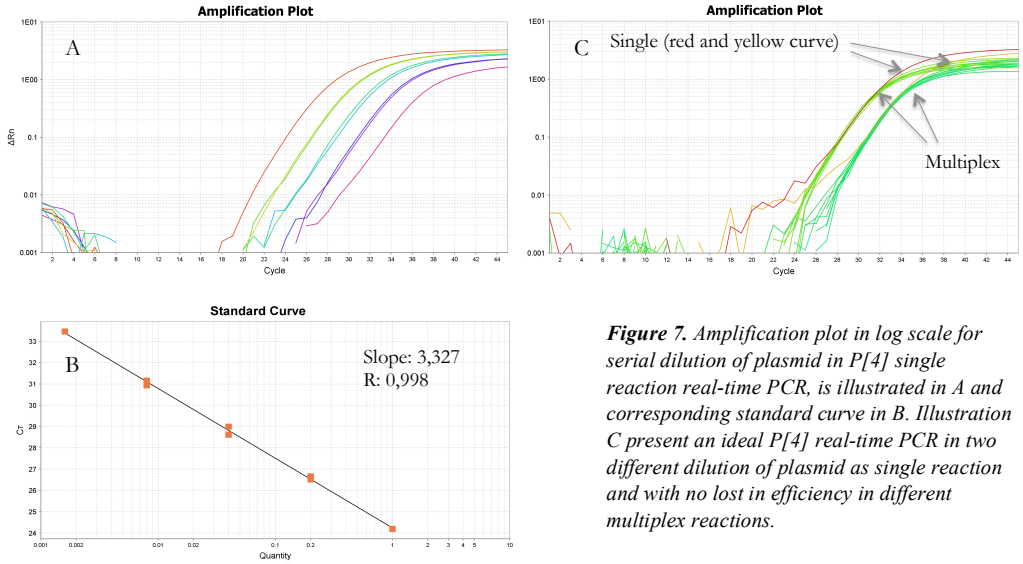

Figure 7. *Amplification plot in log scale for serial dilution of plasmid in P[4] single reaction real-time PCR, is illustrated in A and corresponding standard curve in B. Illustration C present an ideal P[4] real-time PCR in two different dilution of plasmid as single reaction and with no lost in efficiency in different multiplex reactions.*

3.5 Statistical analyses

Statistical analyses were performed using JMP® Statistical Software.

3.5.1 Fisher's exact test

Fisher's exact test is used to test if two categorical variables are associated. Often it is used to compare the proportions of a feature between two groups. This test was used in *paper I* to find potential pathogenic importance of co-infection. Presence of vomiting or dehydration in co-infections and mono-infections were compared, for each pair of pathogens. In *paper II* Fisher's exact test was used for comparison of detection frequencies between baseline and follow-up sample and to find possible association between z scores < -2 for height or weight and clearance, persistence or new infections. Furthermore, the test was used to analyse for each antibiotic-bacterial combination if prescription of antibiotics at baseline was associated with clearance of bacterial pathogens. Fisher's exact test was further used in paper IV to compare groups as regards categorical data such as pathogen frequencies, rotavirus genotype, sampling time point, dehydration and age group.

3.5.2 Mann-Whitney U test (rank sum test)

Mann-Whitney U test is a non-parametric test that is used to compare two sample medians, assuming that the shape of the two distributions is the same. In *paper I*, severity of certain symptoms; body temperature, frequency of diarrhoea or degree of dehydration, were compared between co-infection and mono-infection with Mann-Whitney U test. This test was also used in *paper II* when grouped Ct-values for each pathogen were compared between baseline sample and follow-up sample. Mann-Whitney U test was used in *paper IV* to compare groups as regards numerical data such as Ct values and age.

3.5.3 McNemar test

The McNemar test is used with paired nominal data to test if there is a systematic difference between the two conditions. This test was applied in *paper II* to analyse if presence of a pathogen changed between baseline and follow-up, i.e. the combined effect of cleared and new infections.

3.5.4 Paired t test

The paired sample *t*-test is used for paired numeric data, assuming the differences are normally distributed, to data to test if their mean differs from zero and this was applied in *paper II* to compare Ct values between baseline and follow-up in cases with persistent infection.

3.5.5 Logistic regression

Logistic regression is used to model the relationship between a binary response and one or more predictor variables. In *paper I*, associations between co-infection and symptoms, which might indicate synergistic interactions, were evaluated by logistic regression analysis for all pairwise pathogen combinations with presence of symptoms (i.e. patient vs. control) as dependent variable and detection (yes or no) of the two pathogens as independent variables, and with an interaction term of the two. Synergy was considered to be present if the odds ratio (OR) for co-infection was greater than the product of the OR for each of the two compared pathogens.

4. Results and Discussion

4.1 Paper I

In *paper I,* associations between co-infecting pathogens were studied in samples from 994 children with diarrhoea and 324 healthy controls without diarrhoea, which originated from studies in Rwanda and Zanzibar. Among the patients, 65% had more than one pathogen detected, as compared with 58% in healthy controls. Table 3 summarises the positive and negative associations that were statistically significant.

4.1.1 Positive associations only in patients and association with symptoms

Positive associations that were found in patients only might indicate synergistic impact on symptoms. As shown in Table 3, such associations were observed for several pathogen combinations. Three of them had p values ≤ 0.001 and OR>2: *Campylobacter* + ETEC-*eltB*, *Campylobacter* + *Cryptosporidium* and *Shigella* + EPEC-*eae*.

To evaluate potential synergy further, logistic regression comparing detection rates in patients and controls was performed. This analysis showed that some co-infections were more frequent in patients than in controls also when their frequencies alone were taken into account. These co-infections were *Shigella* and EPEC-*eae* (OR = 4.85, (95% CI: 2.51, 12.50), p = 0.01), and norovirus GII and EPEC-*eae* (OR = 5.57, (95% CI: 3.98, 5.86), p = 0.006).

Shigella and EPEC-*eae* was the only co-infection of pathogens that aggravated symptoms as compared with infection with each pathogen alone. This combination was significantly) associated with an increased frequency of diarrhoea ($p<0.001$).

Table 3. Associations between enteric pathogens.

	No. co-infections	OR	P-value[a]
Positive association in patients			
Campylobacter + ETEC-*eltB*	56	2.03	0.0005
Campylobacter + ETEC-*estA*	32	1.67	0.026
Campylobacter + *Cryptosporidium*	37	2.11	0.001
Astrovirus + ETEC-*eltB*	18	2.24	0.026
Norovirus GII + EPEC-*eae*	28	1.78	0.027
Salmonellae + ETEC-*eltB*	31	1.84	0.021
Shigella + EPEC-*eae*	59	2.14	0.0001
Shigella + EPEC-*bfpA*	34	1.86	0.0081
Shigella+ Sapovirus	16	2.01	0.041
Positive association in patients and healthy controls			
ETEC-*estA*+ ETEC-*eltB*			
Patients	112	3.85	<0.0001
Healthy controls	41	6.02	<0.0001
ETEC-*estA*+ Sapovirus			
Patients	21	3.67	<0.0001
Healthy controls	10	3.81	0.0035
EPEC-*eae* + EPEC-*bfpA*			
Patients	75	6.09	<0.0001
Healthy controls	9	6.39	0.0024
Negative associations in patients			
Rotavirus + Norovirus GII	9	0.19	<0.0001
Rotavirus + *Campylobacter*	15	0.31	<0.0001
Rotavirus + ETEC-*eltB*	83	0.73	0.046
Rotavirus+ EPEC-*eae*	54	0.59	0.0025
Rotavirus+*Shigella*	23	0.23	<0.0001
Shigella + Astrovirus	2	0.22	0.020

[a] P value by Fisher´s exact test

Taken together, these findings suggest that *Shigella* and EPEC-*eae* co-infection might have a synergistic effect to cause aggravated diarrhoea. We do not have any mechanistic explanation to this putative synergy. A possible explanation might be that, although *Shigella* and EPEC infections have different pathogenesis, together they might induce a more intense inflammatory response [159], which could result in a higher rate of diarrhoea than expected from the rate observed in patients with only either pathogen.

Similar calculations to determine multiplicative interaction (potential synergy) between co-infecting pathogens was used by Bhavnani et al., whose findings indicated synergy between rotavirus and Giardia [125].

4.1.2 Positive correlation in both patients and controls

Positive correlation in both patients and controls were found both for the mere detection of, and for Ct values for ETEC-*estA* and ETEC-*eltB* and for EPEC-*eae* and EPEC-*bfpA*. In addition, the Ct values were similar, differing by less than 3.3 cycles (corresponding to $< 1 \log_{10}$) in many samples, suggesting that the target genes were likely present in the same bacterial strains. In samples with a greater difference in Ct the target genes were probably present in separate strains, each carrying only one of them. This use of Ct values to estimate the proportion of samples with typical EPEC, or with ETEC producing both toxins, is to our knowledge novel and represents a strategy to avoid that the transition from traditional to molecular methods leads to loss of important information [160].

4.1.3 Negative associations

As presented in table 3 and in *paper I*, strong negative associations ($p<0.0001$) were found only in patients, and only for rotavirus in combination with *Shigella*, norovirus GII or *Campylobacter*. These

negative associations were thus only found for pathogens that previously have been strongly associated with symptomatic infection [8-11,13,78], and were from a statistical point expected.

In a large study from India, negative associations were seen between rotavirus and Shigella, and between *Vibrio cholerae* and rotavirus, *Shigella* and ETEC [127]. In Chinese patients with diarrhoea, negative associations were observed between rotavirus and norovirus GII or diarrhoeagenic *E. coli* [126]. The interpretation of negatively associated pathogens differs, but could point out that these are reflections of the capacity of these agents to cause diarrhoea on their own.

ETEC-*estA* and rotavirus were not negatively associated despite their strong association with diarrhoea. Possibly, this expected negative association was lacking because the two pathogens had a synergistic effect on symptoms. This possibility is supported by a Chinese study in which co-infection between rotavirus and ETEC was significantly more frequent in patients than in controls [161], and by an experimental study on pigs showing that infection with rotavirus and ETEC together caused much more severe histopathological changes than infection with rotavirus alone [162].

4.2 Paper II

This study investigated a subset of children with diarrhoea that were included in a study of aetiologies to gastroenteritis. Out of these 165 children with diarrhoea, aged 1-60 months and living in a rural part of Zanzibar, 127 provided a follow-up sample (FU) 14 days after the first visit, allowing investigation of the short-term course of infection.

4.2.1 Clearance rates

In total, 289 agents were detected at baseline (BL) in the 127 patients samples and 271 agents were detected in FU samples, meaning that numerous polymicrobial infections were present at both time points. Fifty-two percent of the agents detected at BL were no longer detected at FU, and 49% of pathogens detected at FU were new infections. Detailed information about presence of each pathogen at BL and FU is presented in Table 1, *paper II*. The clearance rates for each pathogen detected at BL ranged between 34% and 100% (Figure 8).

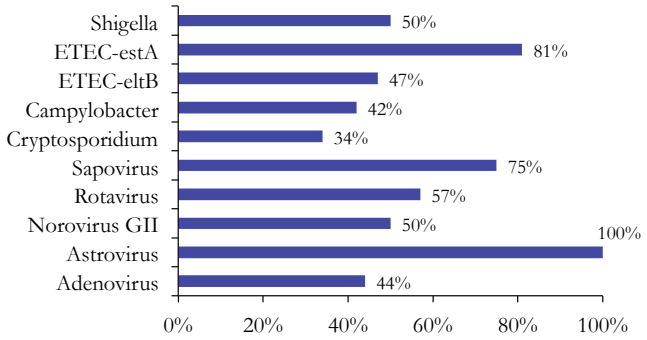

Figure 8. *Clearing rates for each individual pathogen between BL and FU.*

Data on short-term clearance of enteric pathogens have rarely been reported. A study from Guinea-Bissau in agreement with our findings showed that 81% of ETEC infections were cleared within 2 weeks. Another study [156], from Bangladesh performed in 1992, with sampling at BL and at FU 15-17 days later, showed an overall pathogen clearance rate of 90% and presence of 89% new infections at FU [163]. In our study, *Cryptosporidium* had the lowest clearance rates (34%) among the pathogens, which is in line with other studies showing that *cryptosporidium* often is associated with extended diarrhoea [78,153,154,164].

4.2.2 Persistent infections and sequencing

For all pathogens detected at both BL and FU, with the exception of *Campylobacter*, the mean Ct value increased, corresponding to a decline of the microbial concentration (Table 4). For *Campylobacter* the microbial concentration increased, and sequencing showed that a different strain than at baseline was present at follow-up in 75% of cases (Table 4). Sequencing was also performed for norovirus GII, rotavirus and *Shigella* in samples from BL and FU that had sufficient concentration for sequencing (Table 5). In norovirus GII persistent infections occurred in 13 cases, of which 11 were sequenced. One patient had norovirus GII.4 at BL and a new infection with norovirus GII.16 at FU. In the other cases sequencing data indicated that the same norovirus GII strain was present at both occasions. The persistent norovirus GII infections indicate that long-time shedding was frequent, as previously observed in immunocompetent [131,165] and immunosuppressed children [166-169].

Most children with putative persistent infection did not have persistent diarrhoea. Exceptions – with the same pathogens detected at BL and FU and with diarrhoea at follow-up – were one child with norovirus GII and one with *Shigella* and ETEC-*eltB*. In addition to these two cases, 3 more children had recorded diarrhoea at follow-up. One child had

received a new infection with *Shigella* and another a new infection with astrovirus while the last child had no pathogen detected.

Table 4. Number of persistent infections and sequencing results.

Pathogen	No. Persistent	Ct Change Mean	P value[a]	No. Sequenced	BL vs. FU
Adenovirus	14	+4.63	0.008		
Astrovirus	0				
Norovirus	13	+3.86	0.007	11	7 with GII.4 →GII.4
					3 with GII.16 →GII.16
					1 with GII.4 →GII.16
Rotavirus	3	+12.2	0.015	3	3 with G10P[8]→G10P[8]
Sapovirus	2				
Cryptosporidium	23	+3.09	0.0005		
Campylobacter	25	-0.02	0.98	8	2 with 1-3 nt differences
					6 with 50-102 nt differences
ETEC-*eltB*	30	+3.34	0.02		
ETEC-*estA*	8	+3.84	0.084		
Shigella	21	+.4.78	<0.001	6	3 with 2-5 nt differences
					3 with 17-27 nt differences

[a] P value by paired t test, nt = nucleotide

4.2.3 Nutrition status and clearance

Most of the 127 children in this study were living under poor conditions and the nutritional status of 124 children was calculated through anthropometrical data that was recorded at baseline. The median z scores for weight for age, height for age, weight for height and for upper arm circumference for age were –1.15, –1.22, –0.88 and –1.05 respectively. The z scores indicated moderate wasting (z score for weight for height below –2) in 21% and moderate stunting (z score for height for age below –2) in 31%.

Diarrhoea, enteric infections, and malnutrition are interrelated in a complex manner, in which diarrhoea may contribute to malnutrition, and malnutrition leads to more frequent and prolonged enteric infections [148,170,171]. Despite that z score for weight and height being on average 1 standard deviation lower than normal for the age of the studied children, there was no statistically significant association between moderate wasting and detection at baseline or with clearance, persistence, or new infection of any of the pathogens, as shown in detail for weight for height in Table 3, *paper II*. At baseline, the 26 children with moderate wasting had 2.54 agents per child, among which 67% had been cleared at follow-up, compared with the 2.20 per child detected pathogens in 98 children without wasting, among which 48% had been cleared at follow-up. New infections were also acquired at follow-up with the same rate (1.15 vs. 1.01 agents per child).

4.2.4 Polymicrobial infections and new infections

More than one pathogen was detected in 70% of samples at baseline, indicating presence of a large number of asymptomatic pathogens together with causative pathogen. The analysis of follow-up samples showed that all pathogens, both causative and non-causative were rapidly eradicated or reduced in number. At the same time, new infections were acquired at high rates and as shown in Figure 9, 28% of all children had acquired a new ETEC-eltB infection at follow-up.

These findings indicate that the high rates of enteric infections among healthy children in low-income countries are not due to persistence, but probably a result of frequent transient infections, most of which are cleared by effective immune responses.

The high clearance rates were observed in both wasted and well nourished children demonstrating that malnutrition had not markedly impaired immune clearance in these children.

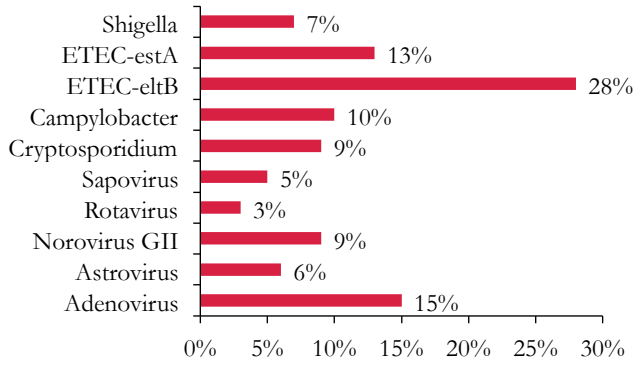

Figure 9. *Frequency of new infections at FU for each individual pathogen.*

In reports compiled by National Bureau of Statistics and Millennium Development goals 2010 in Tanzania, 60%-80% (lower frequency in rural areas) of the total population in Zanzibar had access to improved water sources but only for limited hours of the day and less than 23% treated the water through boiling, bleach or filter before use [172,173]. The lack of access to high quality water together with missing improved facilities, used by less than 40%, are probably the major contributing factors to the high exposure of pathogens showed in this study, and these findings suggest that improving sanitary conditions and safe water supply are essential for reducing diarrhoeal disease among children in low-income parts of the world [137,139].

4.3 Paper III

Rotavirus genotyping has until today mainly been performed with multiplex PCR, often as a nested reaction, followed by gel electrophoresis discrimination based on amplicon length or by Sanger sequencing [20,174,175]. Compared with these methods genotyping by real-time PCR has several advantages, including a lower risk of contamination due to the closed systems, quantification that may help to distinguish mixed infections, and in particular it is much less cumbersome and has a faster turnaround time. A few previous reports have described genotyping methods based on real-time PCR, including one with a separate reverse transcriptase step, one using Luminex-based detection, and another melting point analysis [176-178]. Centre for Disease Control and Prevention (Atlanta, USA) published a multiplex real-time PCR assay in 2016 [179].

Our plan to develop a new genotyping method was initiated as a result of our identification of a large number of rotavirus in the studies in Rwanda in 2009-2012, and our wish to genotype these strains. The method we developed is a triple triplex real-time PCR that is presented in *paper III*. By targeting G1, G2, G3, G4, G9, G12, P[4], P[6] and P[8] the method identifies the vast majority of currently circulating genotypes. It also provides Ct values that allow deduction of probable G/P combinations in the samples that contain mixed infections.

4.3.1 Genotype distribution

The genotyping method was first applied on samples that were rotavirus positive in clinical diagnosis in Western Sweden during 2009-2014. Out of these 775 rotavirus positive samples 97.3% could be genotyped. G1P[8] was the most common genotype, observed in 34.9%, followed by G2P[4] (28.3%), G9P[8] (11.5%), G3P[8] (8.1%) and G4P[8] (7.9%). Rare types (1%) genotyped were four G3P[6], and one of each of G1P[4], G1P[6], G4P[6] and G9P[6]. Mixed infections were found in 4% of samples and 1.5% were partly genotyped. Additional rare genotypes

were found among the untypeable samples with Sanger sequencing, presented in *paper III*, Table 4.

During 2010–2012, G1P[8] was the most prevalent genotype, whereas G2P[4] became the most common genotype in 2013 and 2014. The change over time in genotype distribution was related to changes in the number of rotavirus infections in children and elderly, as shown in Figure 10. The rotavirus detection frequencies within these two age groups varied during the five-year period. Infections in children less than 2 years of age were more often detected 2011-2012, whereas infections in persons above 70 years were more common during 2011 and 2013-2014. In children below 5 years of age G1P[8] was the most common genotype, being observed in 46.6 % of patients, whereas G2P[4] was the most common type, observed in 46.1%, in individuals older than 70 years.

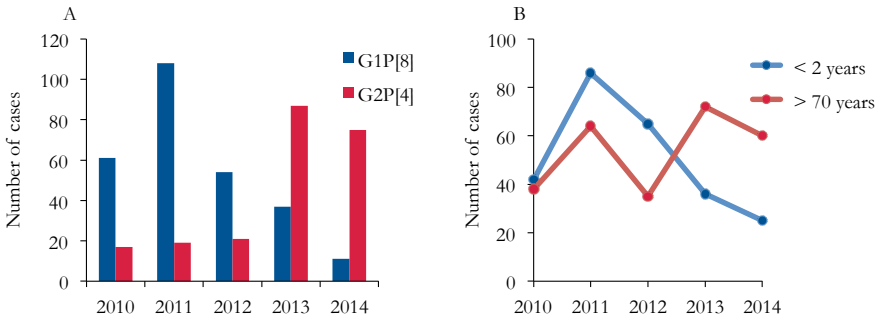

Figure 10. *Distribution of rotavirus A genotype G1P[8] and G2P[4] (A) and the number of rotavirus cases in the age group < 2 and > 70 years (B) in Sweden during 2010-2014.*

The decline in number of rotavirus infections in children in 2013 and 2014 when G2P[4] was more prevalent is interesting but difficult to explain. One possibility is that in 2011 when the number of detected

rotavirus was high in all age groups, and in particular in children below 2 years of age, a large proportion of the population may have acquired immunity, and this might explain the fewer rotavirus infections in children the subsequent years in accordance with a previous report [27]. There was a relative increase of rotavirus infections in elderly during 2013-2014 when G2P[4] was common. This finding agrees with a study from Finland reporting G2P[4] to be commonly detected in elderly between 2013 and 2015 [180]. Outbreaks due to G2P[4] in nursing homes for elderly have been detected in different settings [181,182]. A study from Illinois reported that 17% of the elderly with G2P[4] infection during an outbreak in a retirement community were hospitalized. Rotavirus infections in elderly outside the retirement community were caused by G2P[4] in 89%, while in children this genotype was found in 38% [182].

Rotavirus infection in elderly have been reported to cause 3-18% of adult diarrhoea, and 2-5% of hospitalizations because of gastroenteritis in adults [183,184]. Due to waning immunity and risk of severe dehydration in elderly >70 years, this group has been proposed to be vaccinated [185] Interestingly, elderly have also been shown to indirectly benefit from paediatric vaccination [186].

The overall genotype distribution between years and different age groups are described in detail in *paper III*.

4.3.2 Methodological considerations

The rotavirus concentrations in the clinical samples was high, and >75% had a Ct below 25 (approximately corresponding to >1 million copies/mL). Despite high target concentrations, there was essentially no signs of cross-reactivity in genotyping, and even in the samples with Ct values below 18, the PCR was reactive for only one G type and one P type. The exceptions were 12 samples with a low Ct value for both G2 and P[4]. They were classified as G2P[4], but also showed reactivity for P[8], however with a Ct value at least 10 cycles higher than for P[4]. This

weak cross-reactivity can be understood by the relatively few nucleotide differences between primers and probes for the P[8] and P[4] sequences, as shown in Figure 1, *paper III*). Distinguishing these cross-reactions was relatively easy since they resulted in differences, in both Ct value and shape of amplification curves (Figure 11).

In chapter 3.4, heat maps showing the degree of fit between primers and probes for G types or P types and Genbank sequences representing the monovalent Rotarix and pentavalent RotaTeq vaccines are presented. Based on the matching, a sample containing a Rotarix strain would be positive by the G1 PCR and probably also by the P[8] PCR but likely with a higher Ct value than for G1. Despite that general vaccination against rotavirus has not yet been introduced in Sweden, it is possible that some children had received vaccination. Among the 9 samples with multiple G and/or P types (Table 4, *paper III*), only three were from children in age of vaccination, and none had a the genotyping pattern that one would expect from a vaccine strain. Still, the possibility that vaccine strains can be shed in faeces up to 45 days after the first vaccine dose (shorter after subsequent doses) [187-189], should be kept in mind if the analysis is performed close in time after vaccination.

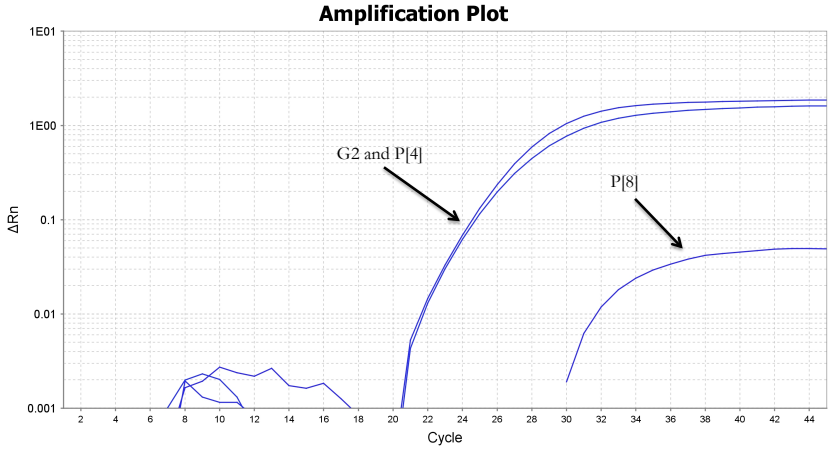

Figure 11. *Amplification plot in log scale showing a rotavirus strain reactive for G2 and P[4] with a low Ct value and cross-reactive P[8] with a much higher Ct value and different curve appearance.*

4.4 Paper IV

4.4.1 Rotavirus frequency

In total 1639 children with diarrhoea were included during two time periods, before and after the introduction of rotavirus vaccination in Rwanda in May 2012. In children younger than 12 months rotavirus infections were significantly less frequent in those that were vaccinated than those that were not (33% vs. 47%, p=0.0003). This difference was however not seen in children that were 12-36 months of age, among whom the rate rather tended to be higher among vaccinated children (35% vs. 30%, p=0.08). These results show that vaccination did not induce an overall reduction of rotavirus in the population, but postponed the infections until after one year of age. This indicates that vaccination did not induce a lasting protection against rotavirus infection. The effect of vaccination was still important because older children, as well as those below 12 months of age, had a lower risk to develop severe dehydration (as discussed below). These findings agree with a report from Zanzibar in which the rotavirus frequency declined from 44% before to 28% after vaccine introduction in children below 1 year of age [46]. Reports from other African countries [45,46,48,60,190], have also shown a more pronounced reduction of hospitalization due to rotavirus after vaccine introduction in younger children (Table 1).

4.4.2 Genotype distribution

The genotyping method presented in *paper III* was applied on 549 out of 552 rotavirus positive samples collected in Rwanda before (n=279) and after vaccine introduction (n=270). The vaccination coverage in Rwanda during 2014 and 2015, when the samples were collected, has been estimated to 98% in a recent study [191]. Accordingly, in our study, 94% of the children with rotavirus infections presenting after vaccine introduction had indeed been vaccinated.

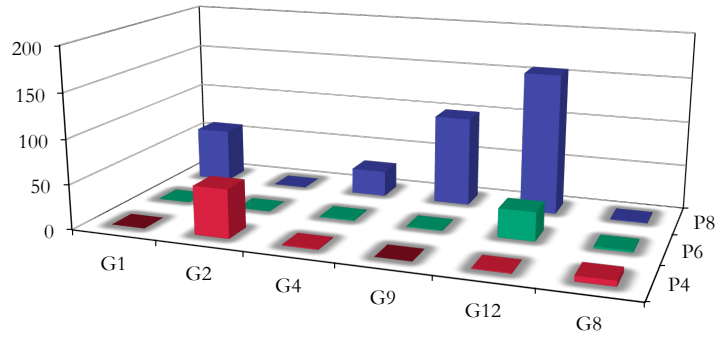

Figure 12. *The total G and P distribution in 2009-2012, 2014 and 2015 among children below5 years of age in Rwanda.*

Genotyping was successful in 91.5% of the rotavirus positive samples. The observed combinations of G and P types in these samples are presented in Figure 12. The prevailing genotypes varied widely over time as shown in Figure 13. The most common genotypes were G2P[4] (50%) during 2009–2010, G9P[8] (51%) during 2011–2012, and G12P[8] (59%) during 2014–2015 after the rotavirus vaccination was introduced.

G12P[8] was frequently detected after vaccine introduction and has been described as an emerging genotype [21,192-195]. Reports about this and other rotavirus genotypes in Sub-Saharan Africa after vaccine introduction are however lacking. An exception is a study from Malawi, which reported an increased frequency of G2P[4] after vaccine introduction [190]. Increased rates of G2P[4] has been observed also in other countries using Rotarix [194,196-199]. Surveillance of rotavirus in Australia has shown that G12P[8] has been more frequent in states using RotaTeq vaccination, whereas G2P[4] and G3[8] has been more frequent in states using Rotarix [70,200-202]. Whether these differences reflect a lower degree of protection towards certain genotypes or merely represents normal fluctuations remains to be elucidated.

Genotype G12P[6] has been observed in several African countries with a modest frequency among circulating rotavirus [69,203-205] In our study it was only found during 2009-2012, before introduction of the vaccine. G1P[8] was relatively common in 2011 (28%) and 2015 (26%), but rare the other years. G4P[8] and G8P[4] were found in 2014 (15% and 3%), but essentially absent the other years.

Rare genotypes, genotype mixtures, or only either a G or a P type, were observed in 7-15% of the samples over the years. The very rare genotypes, detected in only a few cases during the whole period, were G4P[4], G4P[6], G8P[8], G9P[6], G12P[4] and 2 G8P[6]. In total there were 29 mixed infections with several G and/or P types present in the same sample. One sample, taken 13 days after vaccination, had a pattern that might represent the RotaTeq vaccine (G1, G2 and G4), but that sample also contained G12.

In general, a low viral load likely explained failure to detect any type in 47 samples or that only either a P or G type was detected in 28 samples. Exceptions were 3 samples from 2011 and 7 samples from 2014, in which no G type was identified despite detection of P types (6 P[6] and 4 P[4]) with low Ct values, neither by the real-time PCR genotyping, nor by the primers we used in amplification before sequencing. Further analysis with alternative primers might identify a genotype in these cases.

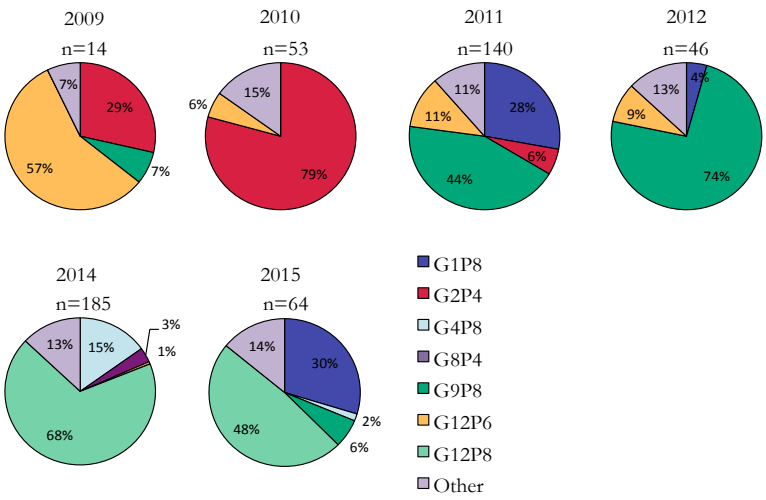

Figure 13. *Rotavirus genotype distribution in subsequent years before vaccine introduction 2009-2012 and after vaccine introduction in 2014-2015 in Rwanda.*

4.4.3 Infections with other pathogens

The introduction of rotavirus vaccination had an impact also on the relative frequency of other pathogens in children with diarrhoea. As shown in Table 6, astrovirus, norovirus GI and GII, sapovirus, ETEC-estA and EPEC-*bfpA* infections were significantly more common while *Cryptosporidium* infections were less frequent in the vaccinated children (Table 5). After vaccine introduction, *Shigella* infections (p=0.0002) were more frequent in unvaccinated children as compared with the rate before vaccination started, but this was likely due to the higher age.

A similar increase of other pathogens after rotavirus vaccine introduction has to our knowledge not been reported earlier, but a few studies from USA, Canada, Finland and Nicaragua have reported norovirus to replace rotavirus as the leading viral cause of acute gastroenteritis in children [55,206-210].

After the introduction of vaccination, we also observed that rotavirus was more often detected as co-infection together with other pathogens (76% vs. 66%, p=0.019) and that the mean number of co-infecting pathogens was higher (mean 1.65 vs. 1.24, p=0.0005), in vaccinated children. These findings suggest that in vaccinated children rotavirus was often not the cause of diarrhoea, or at least not the main cause.

Table 5. Detection frequencies before and after the introduction of rotavirus vaccination in Rwanda.

	Before vaccine introduction	After introduction, vaccinated		After introduction, unvaccinated	
Mean age (months)	18.7	13.9		35.2	
	n=829	n=735	p value[a]	n=75	p value[a]
Rotavirus	34% (279)	34% (251)	0.87	29% (22)	0.52
Astrovirus	4% (31)	10% (77)	**<0.0001**	9% (7)	**0.032**
Norovirus GI	2% (17)	5% (34)	**0.0062**	5% (4)	0.089
Norovirus GII	9% (76)	19% (143)	**<0.0001**	8% (6)	1
Sapovirus	4% (33)	15% (109)	**<0.0001**	7% (5)	0.24
Cryptosporidium	16% (135)	10% (74)	**0.0003**	7% (5)	**0.029**
Campylobacter	8% (69)	8% (57)	0.71	5% (4)	0.51
ETEC-*eltB*	31% (254)	35% (257)	0.075	33% (25)	0.70
ETEC-*estA*	17% (137)	22% (160)	**0.0097**	13% (10)	0.62
EPEC-*bfpA*	15% (125)	23% (172)	**<0.0001**	11% (8)	0.39
EPEC-*eae*	26% (214)	28% (208)	0.28	23% (17)	0.68
Salmonella	7% (58)	5% (36)	0.088	4% (3)	0.47
Shigella	18% (152)	21% (152)	0.25	37% (28)	**0.0002**

[a] By Fisher's exact test comparing with rates before vaccination was introduced.

4.4.4 Symptoms before and after vaccine introduction

Infants and younger children are more likely to become dehydrated due to lower body weight and a large turnover of water and electrolytes, while older children more easily handle minor fluid imbalances. The rate of severe dehydration in children infected with rotavirus was significantly ($p<0.0001$) lower in vaccinated than in unvaccinated children (3.6% vs. 18.3%). These declined rates of sever dehydration was present among children less than 12 month as well as in children between 12 and 36 month. In children over 36 month sever dehydration was not present, neither in unvaccinated or vaccinated. The lower rate of severe dehydration in vaccinated children with rotavirus infections could have two explanations. One is that the diarrhoea was caused by another, less virulent pathogen, the other that diarrhoea was indeed caused by rotavirus but that the vaccine-induced immunity reduced the symptoms.

6. Conclusions

Paper I

- Polymicrobial enteric infections were very common in East African children with or without diarrhoea.
- Negative associations were only found in patients, between pathogens known to have a strong association with diarrhoea on their own
- Positive associations indicating synergistic interaction were rare.
- Co-infection between *Shigella* and EPEC-*eae* was positively associated in patients but not in controls, and was also associated with more pronounced symptoms in children with diarrhoea, suggesting that it might have a synergistic effect on symptoms.
- Ct value for *estA*/*eltB* and *eae*/*bfpA* genes in ETEC or EPEC correlated in both patients and controls, allowing estimation of the proportion of strains that carried both or only either of these virulence genes.

Paper II

- Most symptomatic and asymptomatic infections were cleared within 2 weeks, also in malnourished children.
- Children in poor socioeconomic setting acquire new enteric infections within two weeks after gastroenteritis at a high frequency.
- The findings show that the high frequency of enteric infections in these children was due to living conditions with massive exposure rather than to malnutrition and poor clearance due to defect immune responses.

Paper III

- The new real-time PCR genotyping assay identified a rotavirus genotype in 97% of all rotavirus strains in Swedish clinical samples.
- The genotype distribution of rotavirus in Sweden was age related and changed over time.

Paper IV

- The overall frequency of rotavirus remained high after vaccine introduction in Rwanda.
- Rotavirus infections in children below one year of age were significantly less frequent after vaccine introduction against this virus.
- Infections by astrovirus, norovirus GI and GII and sapovirus were significantly more common after rotavirus vaccine introduction.
- Co-infection between rotavirus and other pathogens appeared more often after than before vaccine introduction.
- The predominant rotavirus genotype changed over time, both before and after vaccine introduction.
- Symptoms in children with rotavirus infections were milder in those that were vaccinated than in unvaccinated.

7. Concluding remarks and future perspective

In agreement with previous studies we found that polymicrobial enteric infections are common in low-income settings. The negative associations between some pathogens and the rare signs of synergistic effects indicate that probably only one pathogen is responsible for the symptoms. Further studies of how enteric pathogens interact and if certain co-infections may aggravate symptoms are however needed. In these studies, it is important that the statistically expected negative associations between pathogens have been considered, which has not been the case in previous studies.

The high frequency of new enteric infections within two weeks after diarrhoea in children less than 5 years, living in poor socioeconomical settings, rather reflect the frequent exposition to enteric pathogens than an inadequate immune response. This is strengthened by the high clearance rates, of both the causative and co-infecting pathogen. This was not affected by malnutrition, which indicates an effective immune response rather than long duration of carriership. Overall, these findings emphasize the importance of continuing the work for improved water quality and sanitation in poor socioeconomical settings in order to decrease the number of diarrhoea incidences.

The introduction of rotavirus vaccination in Rwanda had reduced the number of rotavirus infections in children less than 1 year old and the proportion with severe dehydration, but the number of rotavirus infections in all ages remained high and unchanged. This emphasizes the importance of continued monitoring of both rotavirus incidence and rotavirus genotypes, in order to fully evaluate the effect of the vaccine introduction. In this work, the genotyping method that we have developed should be very useful.

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