

Epidemiologic studies on human enteric viruses in Cameroon

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Pathogens are regularly detected in persons suffering from a certain disease, but the disease-causing properties of these agents are often not well understood. Screening healthy persons for such pathogens might reveal new insights in their pathogenesis.

To my family

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ABSTRACT

Enteric viruses are a diverse group of viruses that include those that multiply in the intestinal epithelium and cause gastroenteritis, and those that first multiply in the intestine and then spread to extra-intestinal target organs where they cause other diseases. Noroviruses and enteroviruses fall within the former and the latter category respectively. Noroviruses are considered to be the most common cause of acute non-bacterial gastroenteritis in both children and adults in industrialized countries. Enteroviruses may induce diseases of varying severity ranging from conjunctivitis, acute flaccid paralysis, encephalitis, aseptic meningitis, and hand, foot and mouth disease. The risk of infection by enteric viruses is higher in sub-Saharan Africa compared to developed countries probably due to sub-optimal sanitation and hygienic conditions as well as to low quality of drinking water especially in rural areas. In Cameroon, very limited studies have been carried out on the epidemiology of these viruses. Globally, comprehensive prospective data on the dynamics of circulation of noro- and enteroviruses are scarce. There are no vaccines or therapeutics for norovirus infections, therefore advanced knowledge of the epidemiology is necessary for adequate prevention and control of the disease. Both viruses are transmitted by the fecal-oral-route, and an important feature of these viruses is the silent shedding in asymptomatic persons which may facilitate the transmission to susceptible persons. This thesis is focused on the seasonality, genetic diversity and dynamics of circulation of noro- and enteroviruses in Cameroon.

Fecal samples were collected from participants and enteric viruses were detected by an in-house multiplex real-time PCR assay. Norovirus genotyping was achieved by RdRp-N/S genomic region sequencing, while enterovirus identification and typing was done by amplification of the 5'UTR-VP4 and partial VP1 regions of the genome respectively. To

investigate the sequence diversity and strain circulation, all the sequences were subjected to phylogenetic analysis.

A high prevalence and diversity of enteric viruses among children and adults was observed which was associated with a high frequency of infections with different strains. Up to five different enteric viruses were detected in a healthy child at a single observation. In a longitudinal follow-up, 1-5 (mean 2.0) different enterovirus infections occurred in children within a year. The study reveals the first description of norovirus in Cameroon and the discovery of a novel enterovirus type designated EV-A119. There was no statistical difference in the detection of norovirus in persons with diarrhea and in those without diarrhea suggesting that, although noroviruses are highly prevalent, they may not be a major cause of diarrhea in the study population. The observed shift in the predominant enterovirus species or norovirus genogroup from one period to the other, and prolonged viral excretion of up to 10 months for enterovirus in asymptomatic persons has great public health implications in the control of diseases caused by these viruses. This study also revealed a strong association between rainfall and the prevalence of noro- and enteroviruses.

Taken together, the high degree of circulation of diverse noro- and enteroviruses in a healthy population in Cameroon suggests silent shedding of these viruses into the environment and eventual contamination of sources for drinking water. These naturally circulating noro- and enteroviruses may induce strong innate and adaptive immune responses and raise question whether these viruses may constitute components of the intestinal 'virobiota'. Conversely, the detection of naturally circulating rotaviruses was low. This may explain why rotavirus detection in feces has been strongly associated with diarrheal disease.

The obtained insights into the dynamics of circulation of these viruses can hopefully be used to develop adequate preventive and control strategies in order to eliminate symptomatic infections caused by these viruses.

Keywords: Norovirus, Enterovirus, Molecular epidemiology, seasonality

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SAMMANFATTNING PÅ SVENSKA

Enteriska virus är virus som antingen replikerar i tarmkanalen och orsakar gastroenterit eller förökar sig i tarmen för att sedan sprida sig till andra organ för att orsaka andra former av sjukdomar. Norovirus hör till den förra kategorin och enterovirus till den senare.

Norovirus anses vara den vanligaste orsaken till akut icke-bakteriell gastroenterit hos både barn och vuxna i industriländerna. Enterovirus kan orsaka sjukdomar av varierande svårighetsgrad, från konjunktivit, akut slapp pares, encefalit, serös meningit, hand-, fot- och munsjuka och ibland övergående gastroenterit. Risken att infekteras med enteriska virus är högre i afrikanska länder söder om Sahara Afrika jämfört med utvecklade länder, förmodligen på grund av dålig sanitet och hygieniska förhållanden, samt låg kvalitet på dricksvattnet. I Kamerun har endast begränsade epidemiologiska studier utförts avseende dessa virus, och även dynamiken i den globala cirkulationen är ofullständigt känd. Bägge virus överförs via fekal-oral smitta, och utsöndring och spridning från asymtomatiska personer är vanligt förekommande. Denna avhandling fokuserar på virusförekomst, säsongvariation, genetisk mångfald och cirkulationsdynamik av norovirus och enterovirus i Kamerun.

Genom epidemiologiska studier baserade på molekylära tekniker påvisades norovirus för första gången i Kamerun. Vidare identifierades ett nytt enterovirus som betecknades som EV-A119. I en undersökning av HIV-positiva patienter med diarré fann vi en liknande prevalens av norovirus som hos friska kontroller vilket talade emot norovirus som viktig orsak till gastroenterit i studiepopulationen. Intressant nog cirkulerade en mångfald av norovirusstammar i Kamerun med betydande genetisk likhet i förhållande till de som beskrivits från västvärlden, vilket talar för en snabb global spridning av dessa virus.

I en prospektiv studie med månatlig faecesprovtagning hos friska barn och vuxna påvisades en hög prevalens av både norovirus och enterovirus. Barn under 5 år uppvisade högre risk för virusinfektion. Multipla infektioner var vanligt förekommande, och upp till fem olika enteriska virus kunde påvisas samtidigt hos en individ. I en longitudinell uppföljning av friska barn fann vi att 1-5 olika enterovirusinfektioner (medelvärde 2,0) inträffade under ett år. Vi fann också en dynamisk förändring av vilka enterovirusstammar som dominerande under året; likaså vilken genogrupp av norovirus som var vanligast från en period till en annan. Denna ständiga cirkulation av enteriska virus inducerar troligen en kortlivad men konstant immunitet, vilket kan ha

betydelse för att infektion med t.ex. norovirus vanligtvis var asymptomatisk i studiepopulationen. Denna höga omsättning av olika norovirus och enterovirus i en frisk befolkning i Kamerun indikerade en tyst utsöndring av dessa virus till miljön, med åtföljande förorening av källor för dricksvatten. I linje med detta påvisades ökad förekomst av både enterovirus och norovirus av genogrupp I i samband med regnperioden. Omvänt var förekomsten av naturligt cirkulerande rotavirus låg, och påvisande av rotavirus i avföringen var starkt förknippad med diarrésjukdom.

De erhållna insikterna i den virala dynamiken under naturlig infektion med enteriska virus kan förhoppningsvis användas för att utveckla förebyggande strategier i syfte att eliminera symtomgivande infektioner orsakade av dessa virus.

LIST OF PAPERS

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

- I. Ayukekbong J, Lindh M, Nenonen N, Tah F, Nkuo-Akenji T, Bergström T. **Enteric viruses in healthy children in Cameroon; viral load and genotyping of norovirus strains.** *J Med Virol* 2011; 83:2135-41.
- II. Ayukekbong J, Kabayiza J, Lindh M, Nkuo-Akenji T, Tah F, Bergström T, Norder H. **Shift of Enterovirus Species among Children in Cameroon - Identification of a new enterovirus EV-A119.** *J Clin Virol* 2013; 58:227-32.
- III. Ayukekbong J, Andersson ME, Vansarla G, Tah F, Nkuo-Akenji T, Lindh M, Bergström T. **Monitoring of seasonality of norovirus and other enteric viruses in Cameroon by real-time PCR; an exploratory study.** *Epidemiol Infect* 2013. 1-10.
- IV. Ayukekbong J, Fobisong C, Lindh M, Nkuo-Akenji T, Bergström T, Norder H. **Molecular analysis of enterovirus in Cameroon by partial 5'UTR-VP4 gene sequencing reveals a high genetic diversity and frequency of infections.** *Submitted.*

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ABBREVIATIONS

aa	Amino acid
AFP	Acute flaccid paralysis
AGE	Acute gastroenteritis
bp	Base pair
CAR	Coxsackievirus-adenovirus receptor
CD	Cluster of differentiation
CDC	Centers for disease control and prevention
cDNA	Complementary DNA
Ct-value	Cycle threshold value
DNA	Deoxyribonucleic acid
ds	Double stranded
EV	Enterovirus
FUT-2	Fucosyltransferase 2
GI	Genogroup I
GII	Genogroup II
HBGA	Histo-blood group antigen
ICAM	Intracellular adhesion molecule
ML	Maximum likelihood
MP	Maximum parsimony
NCR	Non-coding region
NJ	Neighbor joining
NoV	Norovirus
nt	Nucleotide
ORF	open reading frame
PCR	Polymerase chain reaction
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RT	Reverse transcriptase
RV	Rotavirus
ss	Single stranded
UTR	Untranslated region
UPGMA	Unweighed pair-group method with arithmetic mean
VLP	Virus-like particle
VP	Viral protein
WHO	World health organization

1 INTRODUCTION

Enteric viruses are a diverse group of viruses that either infect and replicate in the gastrointestinal tract causing gastroenteritis, or replicate in the intestinal tract and then spread to extra-intestinal target organs where they cause more complicated and life threatening manifestations such as hepatitis, myocarditis, meningitis, and neurological paralysis [1]. The most common human enteric viruses are caliciviruses, rotaviruses, enteroviruses, adenoviruses, astroviruses, and hepatitis A and E viruses [2].

Table 1. Important human enteric viruses and associated diseases

Virus	Family	Nucleic acid	Disease
Noroviruses	Caliciviridae	ssRNA	Gastroenteritis
Sapoviruses	Caliciviridae	ssRNA	Gastroenteritis
Rotaviruses	Reoviridae	dsRNA	Gastroenteritis
Astroviruses	Astroviridae	ssRNA	Gastroenteritis
Adenoviruses	Adenoviridae	dsDNA	Gastroenteritis, conjunctivitis, respiratory disease
Hepatitis A viruses	Picornaviridae	ssRNA	Hepatitis
Hepatitis E viruses	Hepeviridae	ssRNA	Hepatitis
Enteroviruses	Picornaviridae	ssRNA	Conjunctivitis, respiratory disease, hand-foot-mouth disease, herpangina, pleurodynia, meningitis, paralysis, myocarditis, suspected involvement in the pathogenesis of type 1 diabetes

As shown in Table 1, gastroenteritis is the major disease manifestation caused by most enteric viruses. The disease is usually self-limiting in otherwise healthy persons but can be life-threatening in children, the elderly, malnourished and in those with an impaired immune system [3-5]. It is estimated that more than 1 billion episodes of diarrhea and approximately 1.8 million deaths occur annually particularly in children less than 5 years of age. More than 40% of these deaths occur in Africa [6-8]. The risk of contracting diarrheal disease has been estimated to be 5-fold higher in sub-Saharan Africa compared to developed countries partly due to unsafe water supply and sub-optimal sanitation and hygienic condition especially in rural areas [9, 10]. In Cameroon, as in many low and middle income countries, enteric viruses are highly prevalent [11, 12]. Yet, very limited studies have been carried out on the epidemiology of these viruses in Cameroon. Globally, data on the dynamics of natural circulation of noroviruses are scarce, and most

information has been generated from challenge studies and outbreak investigations. Since there are no vaccines or antiviral therapeutics against noroviruses, advanced knowledge of the epidemiology is necessary for adequate prevention and control of such infections. This thesis focuses on the clinically most important human enteric viruses, namely: noroviruses, rotaviruses, and enteroviruses [13]. Rotaviruses are the main etiological agents of infantile diarrhea worldwide and are estimated to cause over 800,000 deaths annually in children aged less than 5 years in developing countries [14, 15]. Noroviruses are considered to be the most common cause of acute non-bacterial gastroenteritis in both children and adults in industrialized countries [16-18]. In developing countries, these viruses are estimated to cause about 200,000 deaths among children less than 5 years of age [19], but actual studies supporting such figures are lacking. Recent work suggests that with the introduction of rotavirus vaccines, noroviruses may emerge as important etiologic agents of acute diarrhea in children [20, 21]. Enteroviruses are highly prevalent enteric pathogens traditionally associated with viable symptoms ranging from mild febrile illness [22] to hand, foot and mouth disease, myocarditis, aseptic meningitis, encephalitis and acute flaccid paralysis (AFP) [23-27]. In United States, an estimated 10-15 million symptomatic enterovirus infections occur each year [28].

Table 2. *Properties of noroviruses and enteroviruses*

Properties	Noroviruses	Enteroviruses
Size (nm)	30-38	22-30
Envelope	Absent	Absent
Capsid polypeptides	VP1 and VP2	VP1, VP2, VP3, VP4
Size of genome	~7.5 kb	~7.5 kb
Attachment ligands	HBGAs	CD55, CD155, CAR, ICAM, 3 integrins ($\alpha_2\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$)
Transmission	Fecal-oral, contaminated food and water, aerosol	Fecal-oral, contaminated food and water, aerosol

Numerous studies have shown that asymptomatic enteric virus infections are common within different populations [29-31]. These enteric viruses are also detected at high levels in the environment, which may constitute a reservoir of infection to susceptible persons [32-34]. Sources of contamination include

shellfish grown in contaminated waters, food crops grown in land irrigated with wastewater or fertilized with sewage, sewage contaminated drinking or recreational waters [10, 35, 36]. Transmission is predominantly fecal-oral and humans are constantly exposed to these viruses through various routes, principally through contaminated food, water, fomites, utensils and person-person contact [37-39]. Noroviruses and enteroviruses belong to the families of *Caliciviridae* and *Picornaviridae* respectively (Table 1). Although noroviruses and enteroviruses are viruses of different families, they share several characteristics in common.

- Both are RNA viruses with a genome of ~7.5 kb.
- Both replicate primarily within the gastrointestinal tract.
- Both display high viral diversity.
- Asymptomatic infections are common
- Both have peaks of infection in particular seasons in temperate regions.

In temperate regions, outbreaks of norovirus disease are common in the winter season, while outbreaks of enteroviral diseases are common in late summer and fall [40, 41]. A large diversity of norovirus strains has been described from an outbreak linked to transmission by drinking water, suggestive of contamination of the water source by human feces [42]. In tropical regions the seasonality is incompletely studied, and factors which influence the transmission of these viruses in such areas are unclear. Owing to this current gap in knowledge, the present studies were undertaken to increase understanding of the dynamics of circulation of noro- and enteroviruses in Cameroon. Such an understanding may help in suggesting novel strategies for prevention and control of these infections, and for elucidation of their pathogenesis.

1.1 Noroviruses

1.1.1 History, classification and genomic organization of norovirus

In 1929, Zahorsky first described ‘winter vomiting disease’, but it took until 1972 for a norovirus strain to be firmly implicated as the causative agent of an outbreak of acute gastroenteritis in an elementary school in Norwalk, Ohio [43]. The virus was initially named Norwalk agent after this locality.

Noroviruses belong to the family of *Caliciviridae*, and are small non-enveloped, icosahedral viruses with a diameter of about 38nm [44]. The genome consists of a single stranded positive sense RNA of about 7.5 kb, organized into 3 open reading frames (ORFs) [45, 46] (Table 2, and Figure 1). ORF1 constitutes the first two thirds of the genome (~5 kb) and encodes a 200 kDa polyprotein that is cleaved by the viral 3C-like protease (3CL^{pro}) into at least six proteins. The coding order in the ORF1 proceeds from N to C terminus to express p48, NTPase, p22, VPg, 3CL^{pro}, and RdRp. The non-structural proteins encoded by the ORF1 are involved in replication of the virus [47]. ORF2 is ~1.8 kb in length and encodes the 57 kDa major capsid protein, VP1 while ORF3 is ~0.6 kb and encodes a 33 kDa minor structural protein, VP2 [46]. The VP1 is suggested to be involved in the recognition of the host receptor [48] while VP2 is thought to be involved in the stability of the capsid but also seems to be vital for viral assembly [49]. Recent studies have shown that murine norovirus has an additional ORF, ORF4, which overlaps ORF2 in an alternate reading frame [50]. It is yet to be established whether this ORF codes for a functional protein.

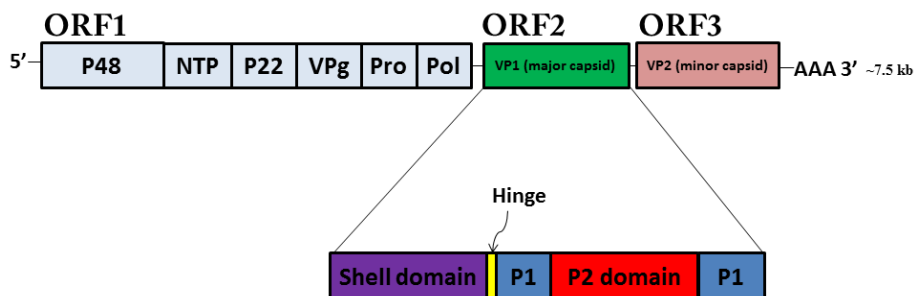


Figure 1. Schematic presentation of the norovirus genome. The genome encodes for three open reading frames. ORF 1 encodes the nonstructural proteins (light blue). ORF2 encodes VP1, the major capsid protein (green); and ORF 3 encodes VP2, the minor capsid protein (brown). VP1 is further divided into the shell (purple), the P1 subdomain (blue) and the P2 subdomain (red). A flexible hinge region occurs between the shell and P1 (yellow).

The viral protein, VPg is covalently attached to the 5' end of the RNA and it is speculated that this protein plays a role in transporting the genome to sites of negative strand synthesis. The 3' end of the genome contains a polyA tail [44].

1.1.2 Genogroups and genotypes of noroviruses

The genus Norovirus is composed of at least 35 genotypes, which are divided into five genogroups (GI-GV) on the basis of sequence similarity [51-53]. Genogroups GI, GII, and GIV primarily infect humans [52]. Multiple porcine noroviruses belong to GII [54-56] while bovine and murine noroviruses are classified into genogroup GIII and GV, respectively [57, 58]. A sixth genogroup has been proposed after the discovery of a new canine norovirus [59]. Each genogroup is further divided into genotypes or genoclusters on the basis of pairwise distribution [52, 60]. In all, the majority of known norovirus genotypes infect humans. The GI genogroup is currently divided into 8 genotypes, GIV contains two genotypes and GII contains at least 21 different genotypes [49, 61]. Genogroups GIII and GV contain three and one genotypes respectively [61]. The various norovirus genogroups and genotypes are designated numerically; with the genogroup indicated first as a roman numeral followed by the genotype in Arabic numeral (Figure 2). For example, the prototype norovirus which is genogroup GI and genotype 1 is designated GI.1. Through analysis of full-length genomic sequences, it has been shown that noroviral strains within a genogroup share 69-97% nucleotide similarity, while strains in different genogroups are only 51-56% similar [62]. Although GI.1 is the prototypical norovirus strain, GII.4 noroviruses are responsible for more than 70% of norovirus outbreaks worldwide [63-66].

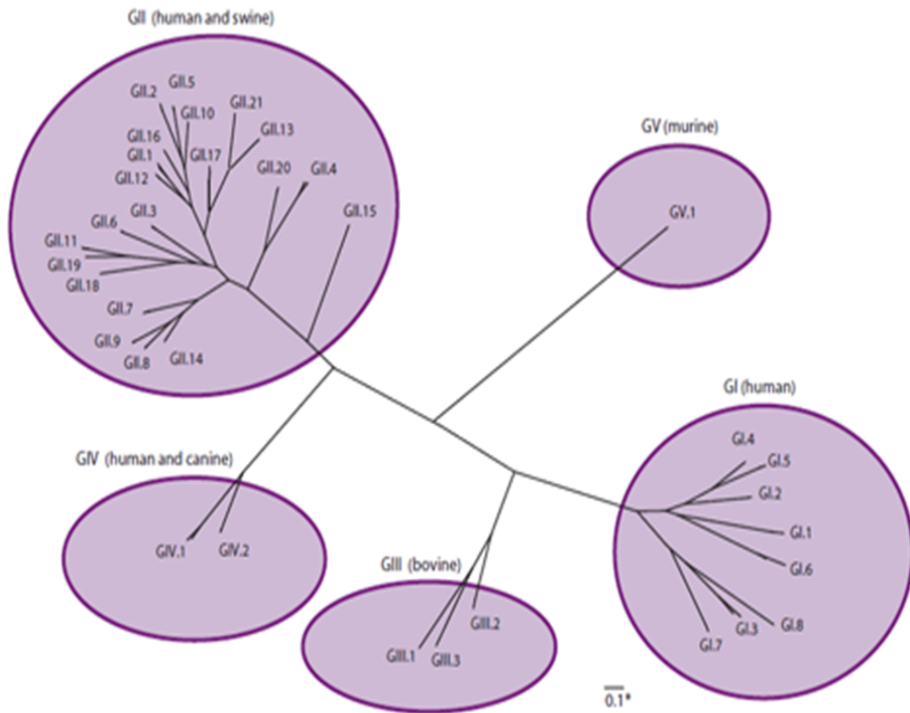


Figure 2. Phylogenetic relationship of the 5 known genogroups (GI to GV) of noroviruses based on sequence diversity in the complete capsid protein. Human strains cluster within GI, GII, and GIV.* The scale bar of 0.1 indicates the number of amino acid substitutions per sites. Adapted with permission from [61]. Initial classification was based on data by [52] and [56].

1.1.3 Transmission, pathophysiology and shedding of norovirus

Norovirus infection is the main cause of non-bacterial gastroenteritis in developed countries [67]. The CDC has estimated that noroviruses may be responsible for about 23 million cases of foodborne disease each year in the United States [68]. As shown in Figure 3A, due to the low infectious dose and high stability in the environment, outbreaks typically occur in places with close living conditions such as hospitals wards, day-care centers, schools, cruise ships, restaurants and homes for the elderly [60, 69-72]. Transmission is primarily via the fecal-oral route, contaminated food or water and person-person transmission via airborne droplets [73-75] (Figure 3B). Waterborne transmission may occur through contamination of drinking or recreational water by waste water [76, 77] and constitutes a significant mode of

transmission of norovirus genogroup GI while norovirus GII is mostly foodborne [17, 78].

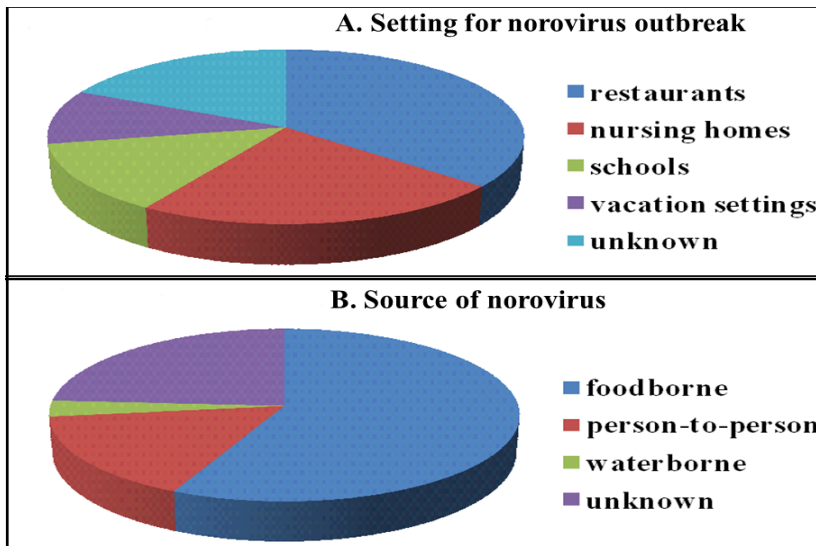


Figure 3. Norovirus outbreak settings (A) and source (B) based on data collected by the Centers for Disease Control and Prevention on 232 norovirus outbreaks from July 1997 through June 2000. The figure is reproduced from [79].

Usually, the infection is self-limiting and symptoms subside within 2-3 days [16, 49]. However, severe forms of the disease may occur in young children, the elderly, malnourished and immune-compromised individuals [80, 81]. Due to lack of cell culture system or small animal model for norovirus research [82], current knowledge on norovirus pathophysiology is limited, with just a few volunteers challenge studies performed to date. Histological analysis of proximal intestinal biopsy samples from volunteers who become ill after administration of either GI.1 (Norwalk) or GII.1 (Hawaii) showed Crypt cell hyperplasia [83] and mild inflammatory infiltration into the lamina propria [84]. Another study also suggests that noroviruses may cause apoptosis of enterocytes in humans [85]. Symptoms include vomiting, diarrhea, nausea, and abdominal cramps. Low grade fever and malaise can also develop [86]. While norovirus infection typically causes an acute bout of gastroenteritis that resolve within days of onset of disease, the course of infection and viral shedding is more complex. There is a report in which norovirus RNA was detected in an individual the day before the onset of symptoms [87]. Viral shedding has been detected for up to 6 weeks post-resolution of symptoms [81, 88]. Furthermore, norovirus can be demonstrated

in infected individuals with no symptoms at all [11, 89, 90]. These properties provide efficient means for transmission of the virus to large populations. Other factors such as extreme stability of the virus particle outside the body, resistance to common disinfectants, low infectious dose, infectivity of all age groups, efficient spread from human-to-human have all contributed to their classification as Category B biodefense agents [32, 80].

1.1.4 Immunity to norovirus infection

Most of the information regarding immunity to norovirus infections has been based on volunteer challenge studies and outbreak investigations. The nature of the immunity constitutes a key determinant in efforts towards designing and delivery of a vaccine [91]. An early study on immunity to norovirus infection suggested that individuals with high serum or fecal antibody titers to norovirus before challenge were more likely to become infected with the virus than individuals with low pre-existing antibody titers, suggesting that antibodies against norovirus may not confer protection [92]. Most of the volunteers were resistant to subsequent infection with the same virus 6 months later; and less than half of the subjects maintained high antibody titers 6 months after secondary challenge [92]. However, conclusions on studies regarding long-term immunity have been difficult to establish. One study demonstrated that individuals infected with GI.1 Norwalk virus were all symptomatically re-infected 27-42 months later [93]. Through modeling, Simmons et al. have suggested a mean duration of immunity of between 4-8 years [94]. The absence of long-term immunity may be confounded by pre-exposure to a large number of circulating norovirus strains. However, a major concern with most challenge studies is that the virus dose given to volunteers was several fold greater than the small infectious dose (estimated to be ~18-1,000 virus particles) [95]. Dose exposure in the community is likely to be smaller and the evoked immune response might be more robust and broadly protective as demonstrated by Teunis et al. [95]. Results from a cross-challenge study of volunteers infected with GI.1 (Norwalk) who were still susceptible to subsequent infection with GII.1 (Hawaii) suggests that immunity to one strain does not confer cross-genogroup protection against infection with another strain [96]. On the other hand, the role of antibodies in preventing norovirus infection has been difficult to discern. Cross reactive studies have shown that antibodies can recognize heterologous norovirus antigens within a genogroup [97]. However, while homotypic antibodies from human antisera following infection can block virus-like particle (VLP) binding to synthetic histo-blood group antigen (HBGA) receptor, heterotypic antisera to strains within the same genogroup are less likely to block this binding [98].

1.1.5 Susceptibility to norovirus infection

In several studies worldwide, norovirus infection has been detected in a substantial proportion of individuals lacking gastroenteritis symptoms [30, 31, 99]. Asymptomatic norovirus infection has been estimated to account for 15-40% of norovirus infections among participants in several studies [11, 31, 89, 100]. An early norovirus challenge study showed that 6 of 12 volunteers did not develop symptoms of gastroenteritis after norovirus challenge. These 6 individuals still remained asymptomatic when rechallenged 31 to 34 months later with the same Norwalk inoculum [93]. It is not entirely clear why a subset of individuals remain asymptomatic despite norovirus infection. However, it is presently well established that histo-blood group antigens (HBGAs) and in particular secretor status, controlled by the α 1,2-fucosyltransferase *FUT2* gene located on chromosome 19q, determine the susceptibility to most norovirus infections [101-104]. Secretors are therefore individuals carrying a functional *FUT2* allele who can express the A and B blood group antigens, as well as the H type 1 and Lewis b (Le^b) antigens on mucosal surfaces and in secretions while those with an inactive *FUT2* gene are termed non-secretors (Figure 4) [105, 106]. Saliva binding studies have shown that different norovirus genotypes exhibit different binding patterns [107, 108]. Most GII.4 variants were previously thought to bind saliva from all secretors but not from non-secretors [109] while other genotypes such as GI.3 (Kashiwa 645), GI.8 (Boxer) have been found to bind saliva of non-secretors as well [107, 108]. On the other hand, although some volunteer challenge studies and outbreak investigations have suggested a secretor-dependent susceptibility pattern of norovirus [102, 110], there is increasing evidence that non-secretors can be infected as well with recent variants of the predominant GII.4 genotype [111, 112] and also GI.3 genotype [113]. More authentic studies are warranted to determine other susceptibility factors of norovirus infection taking into account strain specificity for HBGAs.

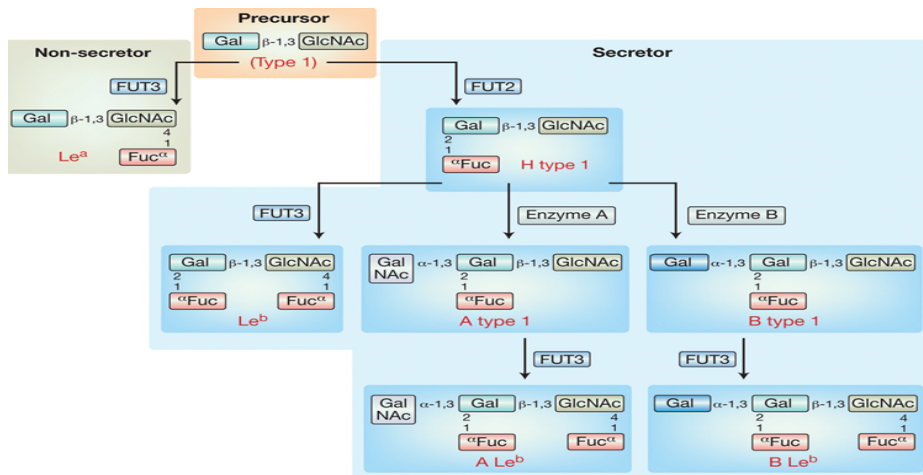


Figure 4. The biosynthesis pathways of the human ABO and Lewis histo-blood group antigens (HBGAs). Copied with permission from [114].

1.1.6 Detection and molecular characterization of noroviruses

Diagnosis of noroviruses is hampered by the inability to cultivate these viruses on cell lines [82]. Routine diagnosis is instead based on detection of norovirus RNA by reverse transcriptase polymerase chain reaction (RT-PCR) or of norovirus antigen by enzyme immunoassays [115, 116]. It is possible to distinguish between the genogroups without sequencing using either of the approaches [62, 117]. The R-Biopharm RIDASCREEN and IDEIA from OXOID are used for preliminary identification of noroviruses when testing multiple specimens during outbreaks [118]. However, due to their low sensitivity (30-85%), the US Food and Drug Administration recommends that samples that test negative should be confirmed by a second technique, such as RT-PCR during outbreak investigations. The RT-PCR method is gaining recognition as the preferred laboratory method of norovirus detection. Advantages of the RT-PCR technique are the ability to quantify the viral load and to detect noroviruses in a broad range of specimens such as stool, vomitus, water, food and environmental samples [16, 17, 119]. Further genotyping of norovirus strains can be achieved by sequencing parts of the genome. Typically sequencing of a partial region of the capsid gene called the N/S region is used by most laboratories [62, 120]. Genetic characterization of this region has a relatively high resolution and broad detection range [121], and the cut-off limits for differentiating between genotypes have been suggested to 15-45% amino acid difference. That means if there is < 15% amino acid difference of a strain compared to references, the strain belongs to an existing genotype [52].

1.1.7 Epidemiology and seasonality of norovirus infection

Noroviruses are considered as the most common cause of acute non-bacterial gastroenteritis among all age groups [13, 122]. Outbreaks occasionally occur in facilities such as restaurants, hospitals, nursing homes, cruise ships, schools, and day-care centers [60, 73]. Factors such as the stability of the virus in the environment, low infectious dose, multiple routes of transmission, and lack of long-lasting immunity contribute to the high impact of norovirus outbreaks [123]. It is estimated that each year, norovirus causes 900,000 clinic visits among children in industrialized countries [19]. In developing countries, norovirus is estimated to cause a striking number of over 200,000 deaths among children aged less than 5 years [19]. These data, which need to be validated, highlight a possible impact of norovirus infection in the population and the need of effective control strategies.

In temperate regions of the northern hemisphere, outbreaks are particularly common during the winter seasons [40, 124], although summer time peaks have been observed as well [125]. The reason for the increase in norovirus outbreaks in the winter is unclear, but several factors have been suggested such as biological, environmental, and behavioral factors. Virulence and persistence of the virions in host populations is also plausible [126]. In the tropics, increase in norovirus activity has been observed in the rainy season [127] and high rainfall has been associated with increase transmission of noroviruses [128].

In recent years, GII.4 strains have emerged as the predominant norovirus genotype, causing up to 70% of reported norovirus outbreaks [129-131]. A major public health question on the epidemiology of norovirus is why GII.4 strains have been so predominant over the last two decades. The reason why GII.4 strains dominate is still unclear, but it is suggested that it could be due to one or a combination of the following reasons: high viral shedding in infected individuals [127], a more diverse receptor specificity than any other norovirus genotype [108], high mutation frequency, genetic drift [132], and absence of long term immunity which is present for other norovirus genotypes like GII.3 [131, 133]. The appearance of new variants in cycles of 2-7 years with mutations that result in modification of antigenic epitopes have resulted in increased affinity of novel strains of the genotype to recognize supplementary glycan patterns. This novel properties allowed new GII.4 variants to spread easily within the population [112].

1.2 Enteroviruses

1.2.1 Genomic organization of enterovirus

Like noroviruses, enteroviruses are small (~30 nm), single stranded, and positive sense RNA viruses of ~7.5 kb. They are non-enveloped viruses having an icosahedral capsid. As shown in Figure 5, their genome consists of a single ORF flanked by two non-coding regions [134]. The first ~750 nucleotides constitute the 5' non-coding region which is covalently linked to a protein called VPg which is cleaved off before translation. There is an internal ribosome entry site which is essential for cap-independent viral RNA translation and also for replication [135, 136].

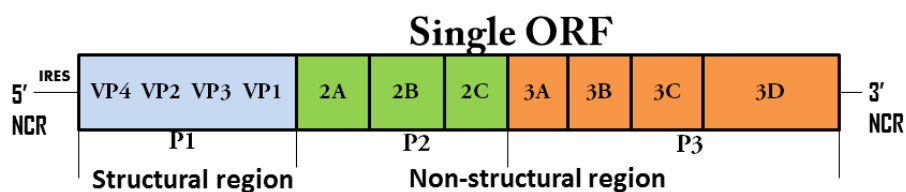


Figure 5. Genome organization of human enteroviruses. Abbreviations: IRES; Internal Ribosome Entry Site, NCR; Non Coding Region.

The coding region of the viral genome is divided into three parts (P1, P2, and P3) encoding the four structural (VP1-VP4) derived from P1 and seven nonstructural viral proteins (2A-2C and 3A-3D) derived from P2 and P3 respectively [137]. The non-structural proteins take part in RNA synthesis and protein processing. The 3' non-coding region is short (70-100 nt) and is important in the initiation of minus-strand RNA synthesis [138]. At the end of the 3' non-coding region is a poly(A) tail [139].

1.2.2 Classification of enterovirus

Human enteroviruses were previously classified as Coxsackievirus A (CVA), Coxsackievirus B (CVB) and Poliovirus on the basis of their pathogenicity in humans and newborn mice [140, 141]. The introduction of cell culture techniques enabled the detection and classification of enteroviruses that did not cause disease in experimental animals. This technique led to the discovery of several enteroviruses that were referred to as Enteric Cytopathogenic Human Orphan Viruses (echoviruses). The association of these viruses with human disease was unknown at the time (1955) [142]. Neutralization assays using serotype specific antibodies were used to identify

serotypes [140]. Since 1962, with the discovery of enterovirus 68, all new enteroviruses are designated EV and a subsequent number [143-146]. Since serotypes previously assigned to the same group of viruses, such as the Coxsackie virus A on the basis of pathogenicity, were later found to be genetically distantly related, the current typing scheme was improved based on sequence divergence within the VP1 protein [147, 148]. Typing in this region has been shown to correlate with serotype designation of poliovirus isolates [149] and other enteroviruses [150]. To date, there are 113 different enterovirus types grouped into four species designated enterovirus A (EV-A), EV-B, EV-C including poliovirus and EV-D [145]. There are 24 types belonging to EV-A, 61 types to EV-B, 23 types to EV-C and 5 types to EV-D [151].

Table 3. Classification of human enterovirus serotypes/types within species enterovirus A-D

	Species enterovirus-A *	Species enterovirus-B	Species enterovirus-C	Species enterovirus-D
Polioviruses			1-3	
Coxsackie virus A	2-8, 10, 12, 14, 16	9	1, 11, 13, 15, 17-22, 24	
Coxsackie virus B		1-6		
Echoviruses		1-9, 11-21, 24-27, 29-33		
Enteroviruses	71, 76, 89-92, 114, 119, 120, SV19, SV43, SV46, BA13	69, 73-75, 77-88, 93, 97-98, 101, 106-107, 110, 111, SA5	95-96, 99, 102, 104-105, 109, 113, 116, 117, 118	68, 70, 94, 111, 120

* Enterovirus A-119 was discovered in the current study.

1.2.3 Transmission, pathogenesis and shedding of enteroviruses

Enteroviruses are transmitted mainly via the fecal-oral or respiratory route. They are among the most common viruses infecting humans and could cause a wide spectrum of diseases, ranging from relatively mild symptoms like transient fever and skin rash to severe conditions such as aseptic meningitis, encephalitis, pleurodynia, acute hemorrhagic conjunctivitis and acute flaccid paralysis (Figure 6) [152, 153]. Enteroviruses have also been implicated in the pathogenesis of type 1 diabetes mellitus [154], myocarditis [155], and neuromuscular disease [156]. The incubation period is usually 3-10 days and the virus replicates mainly in the oropharyngeal and intestinal mucosa.

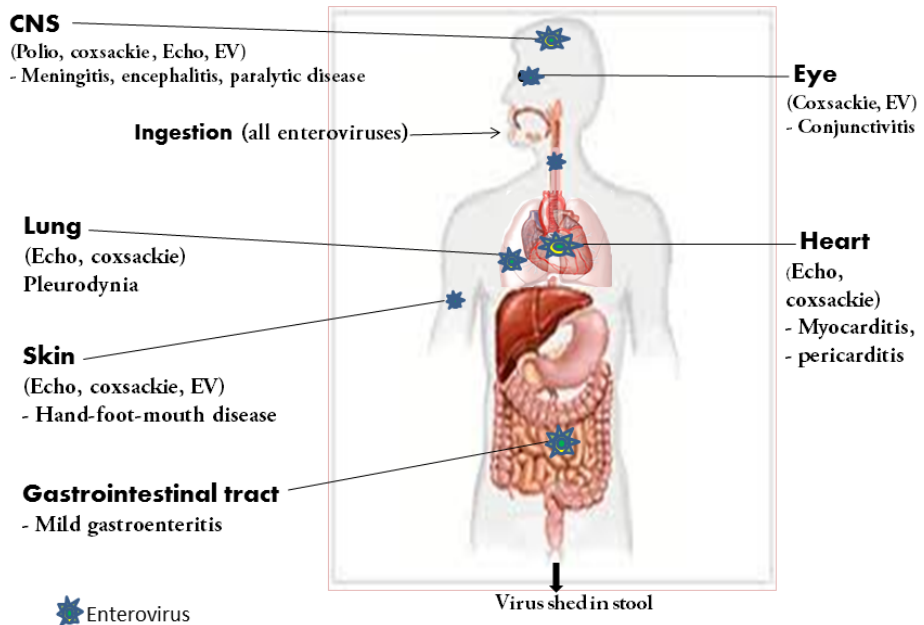


Figure 6. Pathogenesis of enterovirus infections. The target tissue infected by the enterovirus determines the disease outcome of the virus. Echo = echovirus; coxsackie = coxsackievirus; polio = poliovirus; EV = numbered enteroviruses.

The virus then crosses the intestinal barrier and reaches the blood through the lymph nodes, resulting in primary viremia that allows the virus to reach multiple tissues [157]. Enteroviruses can infect most human tissues and the infection largely depends on the presence of specific receptors. At least seven distinct receptors used by different enteroviruses have been characterized to-date; the poliovirus receptor (PVR; CD155), three integrins ($\alpha_2\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_6$), intracellular adhesion molecule 1 (ICAM-1), decay-accelerating factor (DAF; CD55), and coxsackievirus-adenovirus receptor (CAR) [158-160]. The development of infection depends on which organ that is infected (Figure 6). Infection of the central nervous system may result in aseptic meningitis, encephalitis or paralysis [161, 162]. Other tissue-specific infections are also seen, such as herpangina, pleurodynia, acute hemorrhagic conjunctivitis, acute pericarditis, myocarditis, type I diabetes mellitus and rarely gastroenteritis [157]. Excretion of enterovirus may continue for 11 weeks after an enterovirus infection in children [163].

1.2.4 Immunity to enterovirus infection

Enteroviral infections are common during infancy, and immunity depends on circulating and mucosal neutralizing antibodies [164, 165]. Neutralizing IgG can be detected a few weeks after the first IgM response, and may persist for decades. In cell culture, it has been shown that neutralizing antibodies are serotype-specific [22]. However, it was later shown that enterovirus infection elicits a strong humoral heterotypic immune response in older children and adults. This means that infection with one serotype induces an immune response that cross-reacts with that to other serotypes [166]. Younger children develop a more homotypic response. The age difference in the specificity of antibody response may be associated to exposure to a greater number of serotypes with increasing age.

1.2.5 Detection and molecular characterization of enteroviruses

Laboratory diagnosis of enterovirus infections is important in the surveillance of outbreaks of enteroviral diseases, and in order to distinguish poliovirus from the other enteroviruses in cases with AFP, in line with the WHO global eradication programme of poliomyelitis. The traditional method of enterovirus diagnosis was based on virus isolation in cell culture, and typing of the isolates with serotype-specific neutralizing antisera [149]. This approach is however labor-intensive and time consuming since it requires prior isolation of the virus in cell cultures, and a pool of serotype specific antibodies. Reference antisera are not available for the newly identified types. In addition, there are Coxsackie A viruses which do not grow well in cell lines. Moreover, the discovery of new types or presence of multiple viruses in the specimen being tested renders the use of neutralization test limited. Serological diagnosis for typing is further complicated by the possibility of cross-reacting heterotypic antibody response between closely related enterovirus serotypes [166]. Advances in molecular techniques have improved the identification and characterization of enteroviruses. Reverse transcriptase polymerase chain reaction (RT-PCR) is currently used by most laboratories for the identification of enteroviruses. Primers targeting the well conserved 5'-untranslated region of the virus genome are used to detect enteroviruses either directly from clinical samples or from cell cultures [144, 167]. Further, typing of enteroviruses is achieved by sequencing of complete or partial VP1 capsid region and genotypes have been shown to correspond with serotypes obtained by testing with neutralizing antibodies [149, 150, 168]. This molecular technique for typing of enteroviruses has led to the discovery of several new enteroviruses [169, 170]. Based on this approach, it is proposed that enteroviruses should be classified as the same type if they have $\geq 75\%$ nucleotide similarity in the VP1 coding sequence ($\geq 85\%$ amino

acid similarity), and into a new type if the nucleotide similarity is < 75% with existing types [147, 150].

1.2.6 Epidemiology of enterovirus infection and the post-polio era

Enteroviruses are among the most common human viruses causing a broad spectrum of diseases. A Finnish study estimates that 50-80% of children experience at least one enterovirus infection by the age of 1 year [164]. An estimated 10-15 million symptomatic enterovirus infections occur each year in the United States resulting in 30,000 to 50,000 yearly hospitalizations [28]. Infection occurs in all age groups, although most primary infections, with the highest amount of virus shedding, take place during infancy (paper IV). Enteroviruses are endemic but epidemics causing meningitis and hand foot and mouth disease are frequently reported [23, 171]. In temperate regions an increased incidence is observed during summer and early fall [172] while in the tropical areas enteroviruses circulate throughout the year [41, 128]. The number of cases of acute flaccid paralysis (AFP) due to polioviruses has been reduced globally following WHO efforts to eradicate poliomyelitis, and just a few polio endemic countries worldwide remain (Nigeria, Pakistan and Afghanistan). It is estimated that more cases of non-polio enterovirus mimicking acute paralytic poliomyelitis may emerge [25]. Enterovirus 71 has been regarded as the second most important neurotropic enterovirus, causing frequent outbreaks of paralytic disease and hand foot and mouth disease [173-175]. A crude mortality rate of 16% among children aged <14 years was observed during an EV-A71 outbreak between 1998 to 2005 in Taiwan [176]. Recombination events between vaccine derived polioviruses and other species enterovirus C viruses may also results in the emergence of viruses causing similar paralytic diseases [177]. Over the last few decades, several non-polio enteroviruses causing diverse disease conditions have been discovered [157]. Therefore, as polio eradication is near attainment, future controlling efforts would probably be needed for focus on the surveillance of non-polio enteroviruses.

2 AIMS

The general aim of this work was to investigate the genetic diversity and dynamics of circulation of human enteric viruses in Cameroon. The specific objectives were:

To investigate the etiological role of norovirus and other enteric viruses in diarrhea disease in HIV infected persons and to determine the genetic relatedness of norovirus genotypes from Cameroon to that from other geographical regions.

To determine the variability of enterovirus types circulating among healthy children in a small population in Cameroon who were sampled at two occasions two years apart.

To prospectively investigate the epidemiology and seasonality of noroviruses and enteroviruses in a cohort of children and adults in Limbe, Cameroon, and to analyze the impact of weather variables on the prevalence of infection.

To investigate the incidence and genetic diversity of enteroviruses and the duration of viral shedding in asymptomatic persons of the same cohort.

3 METHODOLOGICAL CONSIDERATIONS

3.1 Study localization and demographic data

This study focuses on Cameroon, a middle income country located in West Central Africa covering a surface area of 475,440 km² with a population of about 20.5 million inhabitants.



Figure 7. Map of Cameroon (source Elizon.com, 2009).

As shown on Figure 7, Cameroon is bordered by Equatorial Guinea, Gabon, and Congo to the south; Nigeria to the west; Chad to the northeast; and the Central African Republic to the east. The country is divided into 10 regions; north, south, west, north west, south west, east, littoral, adamawa, far north, and central. Compared to other African countries, Cameroon enjoys relatively better political, financial and social stability. The country is often referred to as ‘Africa in miniature’ as it exhibits all the major climate and vegetation of the continent such as mountains, rainforest, grassland, desert and ocean coastland. The climate varies from tropical along the coast to semiarid and hot in the north. The prospective study was undertaken in Limbe (Figure 8) while other studies included participants from cities around the south west and littoral regions. On the coastline, the rainy season starts in April through November and in this region is Debundscha, which is considered one of the wettest places on earth. Debundscha is located at the base of Mt Cameroon and just 28km from Limbe, and has an average annual rainfall of 10,287mm. The south west and littoral regions are the two main coastline regions with a population of about 1.4 and 2.9 million inhabitants, respectively. The two main cities (Limbe and Douala) are located close to two main rivers; the Limbe River and the Wouri River in Douala. The Limbe River runs through the city of Limbe and serves as the main source of water to the Cameroon water cooperation which is responsible for purification and delivery of pipe borne water to the city. Human actions, such as indiscriminate construction along the river bank, human waste being emptied into the river, and cars being washed in the river impact negatively on the overall quality of the water. Apart from tap water, a substantial proportion of the population of the study region use water from borehole wells. Inhabitants are mainly merchants, civil servants, and students. English and French are the two main official languages spoken.

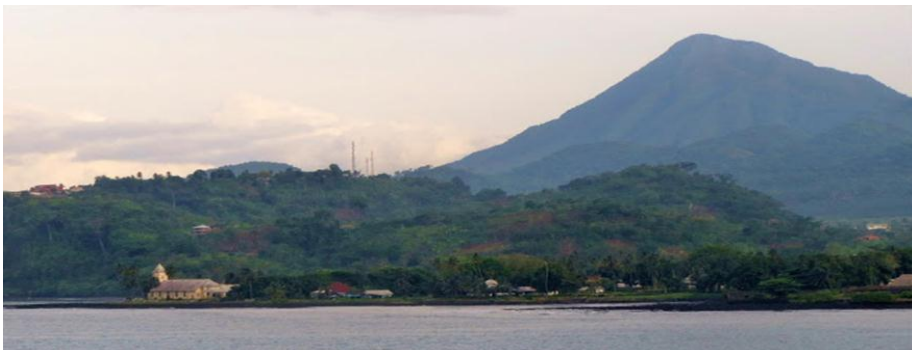


Figure 8. Topography of Limbe located at the foot of Mt Cameroon and close to the Atlantic Ocean. From www.billtrip.blogspot.se/2013/04/cameroon.html

3.2 Participants and samples

Paper I. The study participants included 54 healthy children between 5 and 15 years of age (mean 9.2) and 93 adults infected with HIV within the age range of 16-75 years (mean 42.6). All participants provided fecal samples and were free of gastroenteritis 1 week before and 1 week after sample collection. Another group of 93 HIV infected adults with gastroenteritis was included.

Paper II. The study involved fecal samples from 150 children; the 54 healthy children who previously provided fecal samples in October 2009 (**paper I**) and an additional 96 healthy children aged 2-15 years (mean 6.4) from the same community who provided fecal samples two years after the first population was sampled.

Paper III. The study included 154 children (age 1-17 years) and 146 adults (age 18-69 years). Participants provided monthly fecal samples from September 2011 to August 2012. A total of 2,484 samples were collected; 1,257 from children and 1,227 from adults.

Only the first 100 children and adults who provided ≥ 7 samples were included in the follow-up analysis. However, in the overall analysis of the prevalence of enteric viruses during the dry and rainy season, all 2,484 samples collected during the two seasons were included (paper III, Table 4).

Table 4. Participants involved in the prospective study of healthy children and related adults described in papers III and IV. Subjects were providing monthly fecal samples for up to 12 months

Samples	No of participants		No of samples		Total no of samples
	Children	Adults	Children	Adults	Both
1	8	9	13†	13†	26
2	8	7	16	14	30
3	9	4	27	12	39
4	7	2	28	8	36
5	7	11	35	55	90
6	6	10	36	60	96
7	9	10	63	70	133
8	12	7	96	56	152
9	12	6	108	54	162
10	23	16	230	160	390
11	31	43	341	473	814
12	22	21	264	252	516
Total	154	146	1257	1227	2484

Paper III; Subjects providing 1-12 samples were included, *Paper IV;* only subjects providing 2-12 samples were included. † In the denoted groups; 5 additional samples without labels were obtained from children and 4 samples from adults.

In paper IV, 146 children and 137 adults who provided ≥ 2 fecal samples were included.

All samples were separated into 2 ml aliquots and stored at -20°C in Douala, Cameroon, prior to transportation to the department of Clinical Virology at the University of Gothenburg, Sweden, for analysis.

3.3 Nucleic acid extraction

Prior to real-time PCR, the total nucleic acid content was extracted from fecal samples by the MagNA Pure LC robot (Roche Molecular System, Mannheim, Germany), using the Total Nucleic Acid external lysis protocol. The MagNA Pure LC instrument is an automated system using magnetic beads to isolate nucleic acid. Briefly, stool suspensions were prepared by adding 1 g of stool sample to 5 ml of phosphate-buffered saline containing 1 g of glass beads (Corning Inc., Corning, NY). The mixture was shaken for a few minutes, and centrifuged at 1,500 x g for 25 min at 4°C. Stool supernatants were aspirated and stored in aliquots. Nucleic acid from 130 µl of stool supernatant was extracted into an elution volume of 100 µl.

3.4 Real-time PCR

The principle of real-time PCR is similar to that for conventional PCR described on page 22. The main difference is that the assay is not followed by any gel electrophoresis. Instead the visualization is achieved by means of fluorescent probes. By measuring the amount of amplified product at each stage during the PCR cycle, quantification is possible. Basically, two fluorescent dyes, a reporter (e.g. FAM, VIC) and a quencher (e.g. TAMRA, BHQ), are attached to the 5' and 3' ends respectively of a TaqMan probe. During each extension cycle in the presence of the target sequence, the probe anneals downstream from one of the primer sites, the Taq DNA polymerase cleaves the reporter dye from the probe. Once separated from the quencher, the reporter dye emits its characteristic fluorescence. If the amount of DNA or RNA is abundant in the sample, amplification is observed in earlier cycles; if the amount is low, amplification is observed in later cycles. That is, the increase in fluorescent signal is directly proportional to the amount of PCR product produced in the exponential phase of the reaction. The threshold of the real-time PCR reaction is the level of signal that reflects a statistically significant increase over the calculated baseline signal. It is set to distinguish relevant amplification signal from the background. The cycle threshold (Ct) is the cycle number at which the fluorescent signal of the reaction crosses the threshold. The Ct is used to calculate the initial DNA copy number, because the Ct value is inversely related to the amount of starting template. In these studies, all assays were performed on an ABI 7300 real-time PCR platform (Applied Biosystems, Foster City, California). The target viruses were detected in 25 µl reaction volumes containing 5 µl nucleic acid, 13 µl 2x

reaction mix with ROX (Invitrogen Ltd, Paisley, UK), 0.5 μ M each primer and probe, 20 U RNase OUT and 0.5 μ l Superscript III platinum one-step following the kit protocol. Each reaction well contained primers and probes specific for two enteric viral targets. After a reverse transcription step at 48°C for 25 min and an initial denaturation at 95°C for 10 min, 45 cycles of two-steps (95°C for 15 sec, 60°C for 60 sec) PCR was performed. The Ct-value < 40 indicated that the specimen was positive. The method enables the semi-quantitative detection of target viruses [178]. The limit of detection was approximately 50 copies per reaction as determined by serial dilution of the plasmid containing inserts of the target region. The quality of the assays was ensured with parallel runs of positive and negative controls samples. In paper I, the assay was adapted to detect 8 enteric viruses while in paper II, III, and IV, the assay was adapted to detect 4 different viruses.

3.5 Polymerase chain reaction for sequencing

Polymerase chain reaction (PCR) is a molecular biology technique used to amplify a target region of DNA by a repetitive series of cycles involving denaturation of double stranded DNA template, annealing of short oligonucleotide sequences called primers and the extension of annealed primers by Taq DNA polymerase. The primers are designed to span the region of the genome to be amplified. Increasing the PCR yield could be achieved by nesting with sets of inner primers. The PCR product is stained with ethidium bromide to bind DNA and visualized by exposure to UV light after electrophoretic separation on agarose gel alongside a DNA ladder.

3.5.1 Norovirus PCR and sequencing

Noroviral RNA corresponding to the RNA-dependent RNA-polymerase N-terminal shell (RdRp-N/S) region of the genome was amplified by a combine reverse transcriptase PCR (RT-PCR) and nested PCR assay [179, 180]. Total nucleic acid (10 μ l) was added to the first round RT-PCR master mix (40 μ l) and amplified as follows: 43°C for 60 min, denaturation at 94°C for 2 min 30 sec and then 40 cycles of 94°C for 20 sec, 50°C for 20 sec and 72°C for 1 min, followed by a final extension at 72°C for 5 min. An aliquot (5 μ l) of the RT-PCR amplification products was added to 45 μ l nested PCR master mix. The DNA template was initially denatured at 94°C for 2 min 30 sec, followed by 40 cycles of denaturation at 94°C for 20 sec, annealing of primers at 53°C for 20 sec, extension at 72°C for 1 min, and a final extension at the same temperature for 5 min. PCR products were purified on QIAmp columns (Qiagen, Hilden, Germany) and cycle sequencing was performed with nested PCR primers using the ABI BigDye terminator V3.1 Ready reaction cycle sequencing kit (Applied Biosystem).

3.5.2 Enterovirus PCR and sequencing

Two main regions of the enteroviral genome were targeted for PCR and sequencing; the partial 5'UTR-VP4 and the partial VP1. The well conserved and readily amplified 5'UTR-VP4 was used for characterization of enteroviruses into species. For each sample, 5 µl of nucleic acid was mixed with 45 µl master mix containing 5 µl of 10 x PCR buffer (Roche Applied Science), 200 µM each dNTP, 10 pmol of forward and reverse primers, and 2.5 U *Taq* DNA polymerase (Roche Applied Science). Primers 457 and 1087 were used for the first round of PCR, and 574 and 1087 for the semi-nested PCR generating amplicons of 500 bp (paper II, Table 2). The program for both PCR systems included an initial denaturation at 95°C for 5 min, followed by 40 cycles (95°C, 90 sec; 50°C, 60 sec; 72°C, 90 sec). Typing of enteroviruses (paper II) was accomplished by amplification and sequencing of partial VP1. Ten µl cDNA was added to 40 µl master mix. The reaction mix consisted of 5 µl 10 x PCR buffer (Roche Applied Science), 200 µM each dNTP, 50 pmol each of primers 224 and 222 [181], and 2.5 U *Taq* DNA polymerase (Roche Applied Science), and amplified as follows; 40 cycles of amplification (95°C for 30 sec, 42°C for 30 sec, 60°C for 45 sec). One microliter from the first PCR was added to a 49 µl mix containing 40 pmol each of primers AN89 and AN88, 200 µM dNTP, 5 µl 10 x FastStart *Taq* buffer and 2.5 U of FastStart *Taq* DNA polymerase (Roche Applied Science). The thermo cycler was programed as follows; 95°C for 6 min followed by 40 cycles (95°C, 30 sec; 60°C, 20 sec; 72°C, 15 sec). For samples difficult to amplify with primers AN89 and AN88, primers 012, 040 and 011 targeting the VP1-2A-region were used as described by Oberste et al [150]. Complete VP1 was amplified for strains diverging with more than 25% from known enteroviruses in partial VP1 with forward primers 1504, 2376 and reverse primers 2055 and 2823. (Paper II, Table 2). The PCR amplified products were purified using a QIAquick gel extraction kit (QIAGEN) and sequencing was performed in both directions using 1 pmol of each nested PCR primer. Cycle sequencing was performed using the ABI BigDye terminator V3.1 Ready reaction cycle sequencing kit (Applied Biosystem).

3.6 Sequence analysis

All sequences generated in the study were initially compared with sequences available in GenBank using BLAST, which stands for Basic Local Alignment Search Tool. This program finds regions of local similarity between sequences and compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can also be used to infer functional and evolutionary relationships between sequences as well as to identify members of gene families

(<http://www.ncbi.nlm.nih.gov/BLAST/>). Prior to phylogenetic analysis, the sequence has to be aligned. Obtaining a good alignment is one of the most crucial steps towards generating a representative phylogenetic tree. This is usually easier for closely related genes or gene fragments. Several software packages that implement particular algorithms are available. In the current study, enterovirus sequences were aligned with reference types using Clustal X, followed by visual correction. For enterovirus partial VP1 alignment, Hasegawa-Kishino-Yano (HKY) genetic distances with gamma correction ($\alpha = 0.59$; transition/transversion ratio = 1.34) were calculated in the winPAUP-4b programme package [182].

3.7 Phylogenetic analysis

Phylogenetic analysis is typically used to establish the relationships between genes or gene fragments, by inferring the common history of such genetic elements. These relationships are usually presented in bifurcating graphs consisting of nodes and branches referred to as phylogenetic trees. The nodes represent taxonomic units which can be single genes or a population of organisms. Prior to phylogenetic analysis, nucleotide or amino acid sequences have to be properly aligned. Several different algorithms can be applied to construct phylogenetic trees. The most common are based on distance matrices, maximum parsimony and Bayesian inference. However, it is not unusual to obtain slightly different tree topologies using these different tree-building algorithms. The reliability of branches in the trees is evaluated by bootstrap analysis with 1,000 replicates. Phylogenetic trees representing partial VP1 sequences from enteroviruses were constructed by the Neighbor-joining programme in the Phylip package version 3.69 [183]. Maximum likelihood algorithm was also used to construct trees of norovirus RdRp-N/S gene fragment.

3.8 Statistical analysis

Data were analyzed using the paired-sample T-test, Mann-Whitney test, and Fisher's exact test. SPSS package v. 17.0 for Mac (SPSS Inc., Chicago, IL, USA) and Prism (GraphPad, CA) softwares were used for statistical analysis. Alpha was set at 0.05 and all reported p-values are two-sided and those below 0.05 were considered significant. Pearson regression analysis was used to evaluate the effect of weather variables and incidence of infection and a bivariate analysis to determine risk factors of infection.

4 RESULTS AND DISCUSSION

In this study, we have initially screened for noroviruses, sapoviruses, rotaviruses, enteroviruses, astroviruses, hepatitis A viruses (HAV), and adenoviruses. With the exception of HAV, all enteric viruses were detected among healthy children and adults (**paper I**). As shown in Figure 9, in this preliminary study to investigate the etiological role of norovirus in diarrheal disease in HIV infected persons, there was no statistical difference in norovirus detection among HIV infected adults with diarrhea and in those without diarrhea. Only data from subjects without diarrhea were included in paper 1.

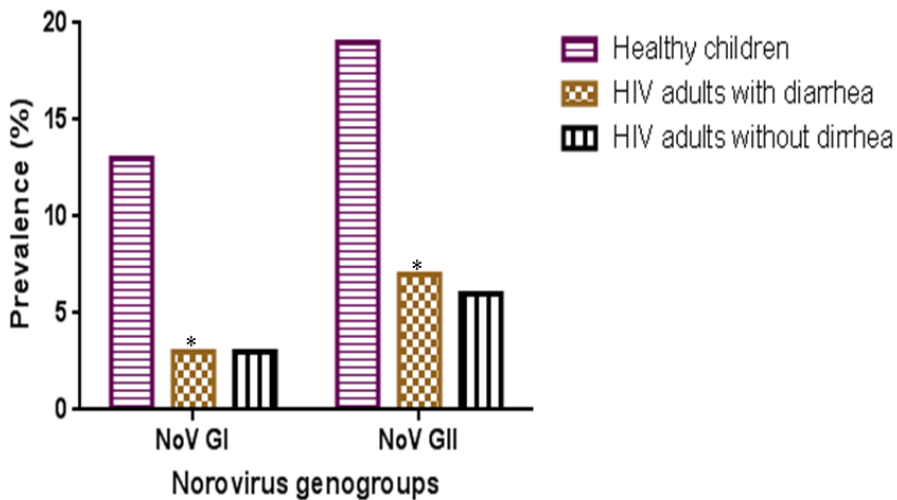


Figure 9. Prevalence of norovirus as detected by TaqMan PCR in fecal samples from HIV infected adults with diarrhea ($n=93$), without diarrhea ($n=93$) and healthy children ($n=54$). * denotes P -value >0.05 (Chi square test).

Surprisingly, the control group of healthy children without diarrhea had a higher prevalence (29%) of both GI and GII norovirus infection. In a one-year longitudinal prospective study, norovirus was detected in 3 (3%) of 100 diarrhea samples, and in 97 (4%) of 2384 samples collected during non-diarrheal episodes. This comparable detection rate of norovirus in both persons with and without gastroenteritis is compatible with findings from a study in Burkina Faso where similar high rate of norovirus detection (24.8% vs 21.2%) was observed among asymptomatic and gastroenteritis patients respectively [184]. In **paper III**, noroviruses and enteroviruses were frequently detected in healthy persons (monthly prevalence of 1-16% for

norovirus and 8-47% for enterovirus). Rotavirus detection among healthy persons was very low (monthly range between 0-3%). This finding that rotaviruses are rarely found as asymptomatic infections, suggests that the absence of circulating rotaviruses as natural infections provide a direct link of rotavirus infection with diarrhea [185-187].

4.1 Multiple enteric virus infection in healthy children in Cameroon

Detection of enteric viruses in asymptomatic children and adults influences our appreciation of the role of these pathogens as causative agents of diarrhea. In **paper I**, we found that 54% and 35% of healthy children and adults respectively in Cameroon were infected with at least one enteric virus. Interestingly, two third of infected children, showed multiple enteric virus infections (2-5 viruses), and the ratio of multiple/single infections was three times greater in children than adults. A similar observation was found in **paper IV** where children had a higher rate of enterovirus infection with multiple strains as compared to adults. In this study, a median of 2.0 (range 1-5) different enterovirus strains infected children per year. In **paper II**, one child was infected with 3 enterovirus types belonging to different species; EV-A, EV-B and EV-C. The same child was also infected with sapovirus, norovirus and adenovirus. This complex viral flora of enteric viruses here demonstrated in healthy children suggests that they may constitute a reservoir for these viruses and heighten concern about susceptibility factors in carriers of multiple infections. In order to make clinical judgments based on the detection of a specific enteric virus from a case of diarrhea, knowledge of the relative frequency with which such viruses (e.g. norovirus) is found in healthy persons without diarrhea is needed. Thus, our common finding of noroviruses in asymptomatic children and adults suggests that these agents may be less important as gastro enteric pathogens in Cameroon as compared to the industrialized world.

The main viral association found in mixed infections was of noroviruses and enteroviruses. Indeed, noroviruses were detected in almost all cases of mixed infections (**paper I**), thus supporting evidence of a role as major circulating enteric viruses. A plausible explanation for the high prevalence of enteric viruses among children may be attributed to the fact that they generally live in close contact with other children, and that their hygienic habits are less than optimal [188]. Adults are less infected probably due to immunity from previous exposure. In the current study, we conclude that the high prevalence of noroviruses found prospectively among asymptomatic persons indicates a circulation of these viruses as natural infections in the community.

4.2 Viral cycle threshold values in natural infection

Cycle threshold values (Ct values) may be used for a determination of the viral load (amount of RNA/DNA) in the sample analyzed. A high Ct value corresponds to a relatively small amount of virus and vice versa. The question has been posed whether the viral load could discriminate gastro enteric disease from health. In a study on intestinal infectious diseases, Phillips G et al, suggested an optimal Ct value cut-off of 31 for attributing disease due to norovirus infection [189]. However, such direct comparison of Ct values should be interpreted with caution since the amplification efficiencies may not be identical for different real-time PCR assays. In spite of this reservation as shown in Figure 10, the monthly mean Ct values of norovirus infection among participants were above 30, except for children in the month of June when greater viral quantities were detected. Although it is known that noroviruses are highly infectious with only a small amount of viral particles required to infect and cause disease [32, 95], our finding of low viral load of natural norovirus infections may be related to presence of specific intestinal secretory immunoglobulin A (IgA) antibodies as previously described [190]. We hypothesize that such IgA antibodies may control replication and spread in the mucosa without necessarily inducing degradation of the virus. Therefore, clinical disease is precluded, while still allowing asymptomatic carriage and shedding of the virus in feces.

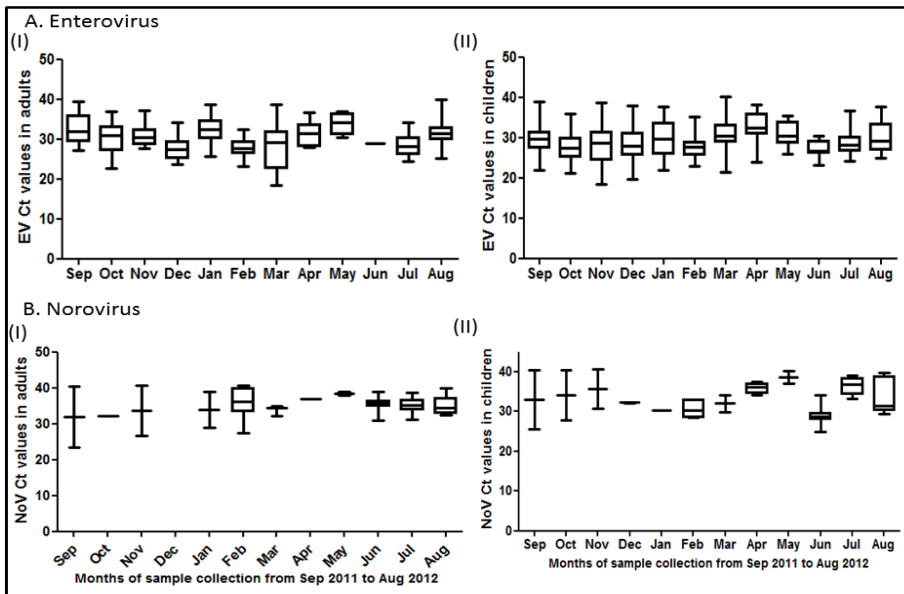


Figure 10. Monthly cycle threshold (Ct) values among healthy children and adults obtained after TaqMan PCR analysis of RNA from enteroviruses (A) and noroviruses (B).

A similar finding of relatively low viral load was observed also during natural infection of enteroviruses.

4.3 Norovirus and enterovirus diversity in Cameroon

A characteristic feature of noroviruses and enteroviruses is their great genetic diversity. There are at least 35 norovirus genotypes and 113 human enterovirus types [105, 151]. In **paper I**, a marked norovirus diversity within the main genogroups GI and GII was observed, involving a total of 4 different genotypes (GI.3, GII.4, GII.8, and GII.17). These genotypes were homologous to strains previously associated with outbreak of gastroenteritis from other parts of the world, suggesting a rapid global spread of noroviruses probably by human mobility. In **paper II**, we observed a significant diversity of the detected enteroviruses. In all 28 different types were found, including a new enterovirus type, designated EV-A119, which was isolated from a healthy 6-year old girl. In **paper IV**, this diversity was even more pronounced, with more than 121 different enteroviral strains identified. At least 25% (28 types) of the 113 currently identified human enterovirus types were detected in the **paper II** within a limited population in a small community in Limbe, Cameroon. The study revealed a complex enterovirus ecosystem, which may be a driving force for enterovirus evolution through recombination [134, 173, 191].

4.4 Alternating predominance of enterovirus species and norovirus genogroups

In samples collected in 2009, enterovirus types belonging to species EV-A dominated. As shown in Figure 11, in the first part of 2011 (September and October), most of the detected enteroviruses instead belonged to species EV-B (**paper II**). Subsequently, from November 2011 to August 2012 species EV-C constituted the predominant species (**paper IV**).

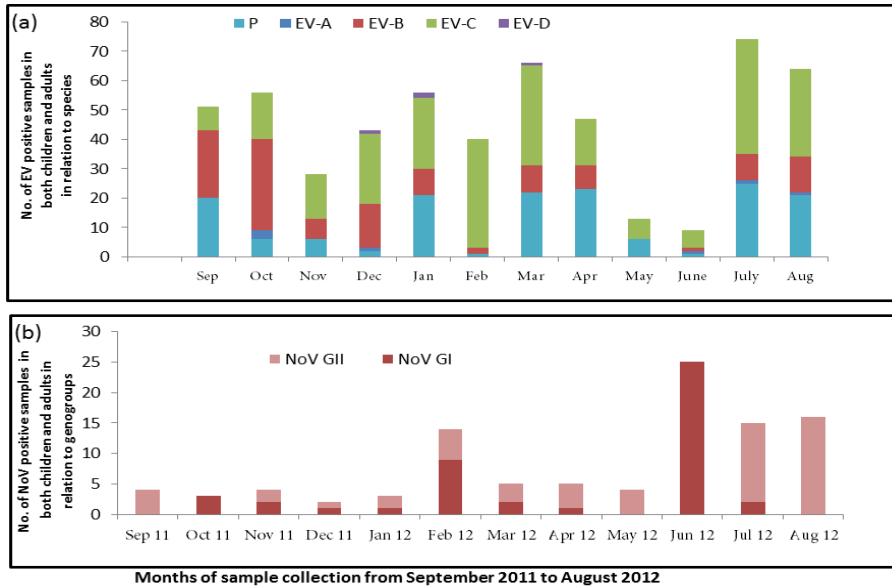


Figure 11. Monthly enterovirus (EV) species (a) and norovirus (NoV) genogroups (b) detected in fecal samples from children and adults in Limbe, Cameroon from September 2011 to August 2012. P denotes positive by TaqMan PCR but not sequenced.

We suggest that the high rate of infection of many different types in the population may induce herd immunity against the circulating types. Also, in the prospective longitudinal study reported in **paper III**, we found a seasonal predominance of a particular norovirus genogroup. In June, infections were predominantly associated with genogroup GI. Thereafter, a shift to GII predominance was observed during the subsequent months from July through September and then replaced again by genogroup GI in October (Figure 11). This cyclical pattern of prevalence of genetic clusters of noro- and enteroviruses is a novel finding which may suggest a viral turnover within the population related to herd immunity of limited duration.

4.5 Seasonality of norovirus and enterovirus in Cameroon

Norovirus and enterovirus seasonality have been extensively studied in temperate regions of the northern hemisphere [126, 192]. In contrast, very limited data are available from tropical regions of the world due to lack of adequate laboratory capacity to carry out such surveillance. Although norovirus infections occur round the year in temperate regions, peak of disease outbreak (most often caused by genogroup GII viruses) have been associated with the winter period of the year [124], while the peak of enterovirus infections occur in late summer or autumn [41]. In **paper III** we

sought to investigate the seasonality of these two viruses in Cameroon over a period of 12 months among 200 participants. We observed that norovirus and enterovirus circulated throughout the year (Figure 12). The incidence peak of norovirus infection was observed in the rainy season from June through August. Although enterovirus infection occurred around the year, May and June were identified as a low enterovirus season in Limbe, Cameroon (Figure 12).

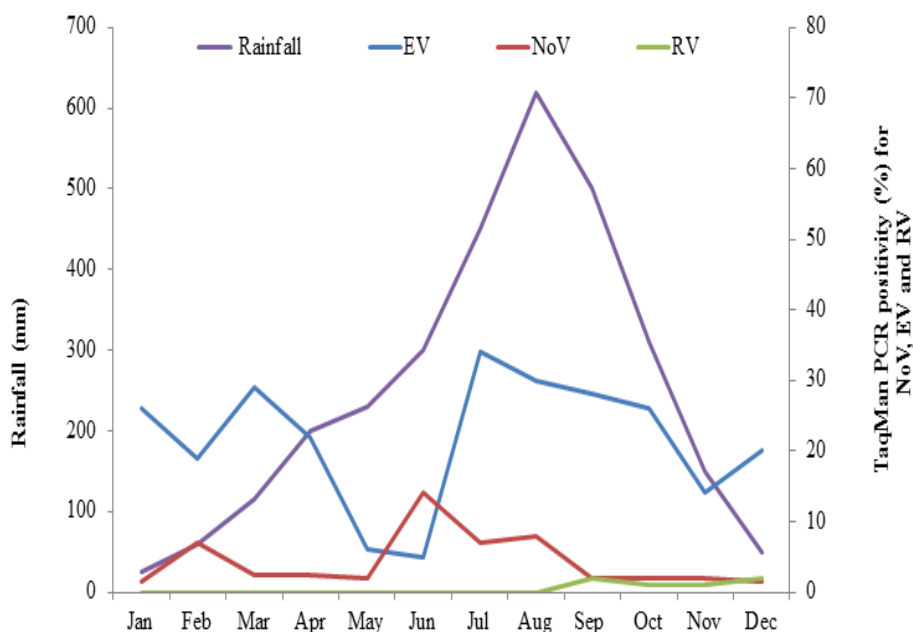


Figure 12. Variation of monthly rainfall and Taqman PCR positivity (%) of norovirus (NoV), rotavirus (RV) and enterovirus (EV) in Limbe, Cameroon from September 2011 to August 2012. Rainy season; June – November, Dry season; December – May.

A positive correlation of temperature and relative humidity with respect to norovirus and enterovirus prevalence was also observed ($r = 0.4$). This is the first comprehensive prospective study on norovirus and enterovirus seasonality in tropical Africa. Taken together, the findings support the observation that future climate change associated with increase in precipitation and temperature may likely be associated with increase in transmission of these enteric viruses [192].

4.6 Duration of enterovirus and norovirus shedding

Excretion of enteroviruses in feces may persist for several weeks [163], the exact duration of shedding, however, is unknown. **In paper IV**, the duration of enterovirus excretion in natural infection of human enteroviruses was investigated. We observed a prolonged enterovirus RNA shedding in feces for 10 months in two children and an adult. An extended excretion of type 1 vaccine-derived poliovirus (VDPV) has been reported in a healthy child in an Irish study [193]. However, our finding of 10 months shedding in healthy participants is longer than previously reported for non-polio enterovirus shedding among children hospitalized for herpangina and hand foot and mouth disease [163]. Deficiency in humoral immunity has been associated with extended viral shedding in previous studies [194]. For norovirus, the comprehensive genotyping data is lacking, however detection of the same norovirus genogroup in multiple samples from the same person was observed for a maximum of 2 consecutive months. This suggests that norovirus shedding during natural infection may not be of more than one month of duration. Taken together, this finding suggests that asymptomatic chronic shedders may serve as reservoir for dissemination of enteroviruses, and maybe also noroviruses, in the studied population.

4.7 Enterovirus transmission pattern

A hallmark of this thesis was the ambition to unravel the complex mechanisms of circulation of enteric viruses in the studied population. To address this question, we recruited two participants within each household. A child and an adult from the same household were selected as they are likely to share the same transmission routes such as fomites, water and or food [37, 119], and proximity may facilitate transmission as well [195]. Contrary to our expectation, in **paper IV** only 11 (8.5%) of 128 families demonstrated the same enterovirus strain in both the child and adult in the same month, which suggested that spatial proximity did not increase enterovirus transmission. In most cases, the two individuals in the same household were infected with two different strains. Maybe as important, certain enterovirus strains such as those belonging to species EV-B were more commonly infecting children than adults. Analyses of the individual time line of enterovirus positive samples revealed a high frequency of infection with different enterovirus strains. We also found that re-infection with the same enterovirus strain, after a period of carriership of other enterovirus strains, was rare, suggesting the occurrence of a protective homotypic, but not heterotypic, immune response. The duration of such homotypic immune protection after sub clinical infection is still not entirely clear.

4.8 Demographic and risk factors to norovirus and enterovirus infections

Understanding the relationship between demographic factors and risks of infection with enteric viruses is an important tool towards effective control and prevention of infection. In **paper III**, we observed that the risks of norovirus infection was greater among children <5 years than in older children and adults, suggesting a possible increase in immunity with age. This observation is compatible with a previous report by Phillips et al [196]. For enteroviruses, the risk of multiple episodes of infection was also significantly higher among children aged <5 years (**Paper IV**). A plausible explanation is that older children and adults may have developed a better immune protection due to exposure of these viruses earlier in life [197]. Males had significantly ($p = 0.003$) high number of multiple episodes of enterovirus infection than females. Similar to this finding, an increased risk of enterovirus 71 infection was reported among male children in Taiwan [176]. The reason for this gender difference in the risk of single or multiple episode of enterovirus infection is presently not clear. The mean household size in the current study was 5.1 persons. A large household size could probably increase the circulation of strains resulting in enhanced herd immunity and thereby reducing the risk of developing symptomatic infection. Conversely, during norovirus associated hospital outbreaks of gastroenteritis in Gothenburg, Sweden, exposed personnel that were living alone showed an increased risk of developing symptomatic norovirus infection compared to those living in larger households (unpublished observation).

4.9 Role of water in the spread of enteric viruses

Water is undoubtedly one of the major sources of human exposure to enteric viruses [33, 198]. Water may be involved either directly through consumption of contaminated water, or indirectly through contamination of food with polluted water (Figure 13) [199-201]. Surprisingly, we found that consumption of water from the tap in Limbe was associated with higher prevalence of norovirus infection, compared to consumption of water from household borehole wells. Such wells in the study region were mostly protected and built upstream of toilet facilities. A high diversity of enterovirus strains detected in **paper IV** is also suggestive of a probable contamination of drinking water source in the study region. This finding is compatible to a massive Swedish norovirus GI water-related outbreak of great genetic diversity previously reported by our group [42].

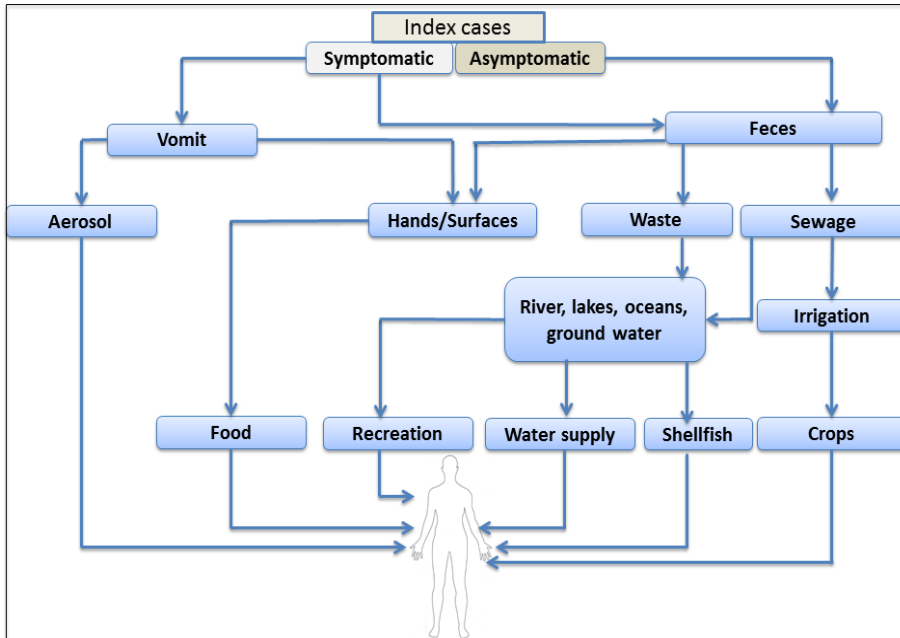


Figure 13. Routes of transmission of human enteric viruses.

It is important to note that microbial quality assessment of tap water is often based on bacterial indicators such as coliforms, enterococci and *Escherichia coli* [202, 203]. These bacterial indicators may not accurately predict the presence of enteric viruses [204-207]. Rain fall was also statistically associated ($P < 0.02$) with an increase in norovirus prevalence. The peak of norovirus infection, at the beginning of the rainy season, was dominated by genogroup GI. This finding is in agreement with the previously mentioned Swedish waterborne GI outbreak reported from our lab [42]. These data suggest that water is a major vehicle of norovirus transmission, especially of GI viruses, and highlight the fact that at least GI noroviruses may be very stable in water [208].

5 CONCLUSIONS AND HYPOTHESIS

Gastroenteritis caused by norovirus infection has been suggested to be an important cause of global mortality in young children. The present study includes the first report of norovirus in Cameroon. Findings of similar norovirus prevalence in HIV-positive adults with and without gastroenteritis, together with a high prevalence in healthy children and adults, raised the question of whether asymptomatic infection of noroviruses is an important part of their natural infection in this sub-Saharan country. Indeed, in the follow-up study, we discovered that both noro- and enteroviruses circulated as asymptomatic infections in children as well as in adults.

We consider the ubiquitous presence of diverse enteric viruses in healthy children and adults and the shift in circulating enterovirus species and norovirus genogroups from one period to another as important findings of the present study. This observed viral 'turn-over' may suggest that a short-lived but constantly boosted immunity can prevent disease manifestations although allowing for infection. In support of this, we found relatively low viral quantities of noro- and enteroviruses in fecal samples from the asymptomatic study subjects.

Evidence against transmission of enterovirus strains within a household due to proximity suggests other routes of transmission. The rainy season brought increased prevalence of enteroviruses and of noroviruses of genogroup I, and the risk of infection was increased in families using tap water. Thus, drinking water was strongly linked to the transmission of these viruses.

Based on these findings and on relevant literature, the following hypothesis on the dynamics of circulation of enteric viruses and on the protection against symptomatic disease was formulated.

Dissemination of enteric viruses in the study population mainly occurs through asymptomatic individuals shedding viruses in feces. Viral particles in excrements are disseminated through wash-out during rainy seasons and then taken up by wastewater treatment plants. Current treatment practices such as filtration, chlorination, activated sludge, and lime coagulation seems unable to guarantee virus-free wastewater effluents [188]. Especially non-enveloped enteric viruses may resist treatment and a significant viral load may be released into effluent discharge. Treated sewage containing viable viral particles is commonly discharged into rivers. The subsequent intake of river water by municipality water treatment plants followed by distribution and consumption of tap water for drinking and irrigation of vegetables may constitute efficient means for reinfection of the population.

The lack of symptoms in infected individuals is likely to be due to innate or adaptive immunity response induced by the naturally circulating noroviruses. We suggest that IgA antibodies secreted by the mucus layer of the intestinal lumen may control replication and spread without necessarily killing the virus. Therefore, clinical disease is precluded, while still allowing asymptomatic carriage and shedding of the virus in feces. In this context, the gut architecture and natural homeostasis may constitute obstacles to disseminated norovirus infection. The identification of a plethora of noroviruses and enteroviruses, including the new EV-A119, that were found to be dynamically circulating in our prospective study of healthy persons suggests that these viruses are colonizing the intestinal lumen as resident 'virobiota' that would require a loss of immunity or otherwise compromised host immune system to cause a disease. However, only a comprehensive metagenomic analysis of fecal materials from inhabitants in both low and high-income countries will help to characterize the composition of such human 'virobiota' and will improve understanding of their role in health and disease.

In contrast, such naturally occurring circulation was not observed for rotaviruses whose detection instead was statistically associated with symptomatic gastroenteritis. Future studies in Cameroon including levels of viral immunity and of genetic markers such as histo-blood group antigens may help to better understand the division between asymptomatic and symptomatic infection of enteric viruses [112].

Also, prospective epidemiological surveillance of enteric viruses in sewage and environmental samples may help to better appreciate the natural circulation of enteric viruses in the population.

The obtained insight into the diversity, seasonality and dynamics of circulation of these viruses can hopefully be used to develop adequate preventive and control strategies in order to eliminate infections caused by these viruses. Paradoxically, such improved control measures may lead to a transient increase of symptomatic disease caused by noroviruses and maybe also by enteroviruses, as the herd immunity decreases. This possibility is not an argument against improvement of water and food hygiene, but a factor that should be considered in the struggle for improved public health in sub-Saharan Africa.

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