

ABSTRACT

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Prevalence and Molecular Epidemiology of Enteric Viruses in HIV-infected Individuals in Uganda

Acute and chronic diarrhea are significant causes of morbidity and mortality among HIV-infected individuals. The importance of viruses as etiologic agents of diarrhea in HIV-infected individuals, especially in Africa, is not well documented. We examined the prevalence of enteric viruses among HIV-infected and uninfected individuals at baseline and during episodes of acute diarrhea in a prospective household study in rural Uganda. Of the 414 stool specimens analyzed, 27.5% tested positive for at least one virus, 16.4% tested positive for picobirnavirus, 6.5% for norovirus, 2.9% for sapovirus, 2.2% for astrovirus, and 5.1 % for adenovirus by RT-PCR. Picobirnaviruses could be grouped into two genetic clusters, Georgia and China, and significant genetic variability was observed within the China-cluster. This is the first household-based study describing the prevalence of enteric viruses among HIV-infected and uninfected individuals with acute diarrhea in Africa using state-of-the-art molecular methods. Surprisingly, picobirnaviruses are commonly found in stool from asymptomatic persons with and without HIV in Uganda. At this time, no significant association is noted between acute diarrhea and presence of viruses in stool, but testing of more specimens is pending to assess if these viruses are relevant etiological agents of diarrhea or simply commensals.

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LIST OF ABBREVIATIONS

Ad	adenovirus
AGE	acute gastroenteritis
EIA	enzyme immunoassay
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
HAsV	human astrovirus
HIV	human immunodeficiency virus
HuCV	human calicivirus
IEM	immuno electron microscopy
PAGE	polyacrylamide gel electrophoresis
PAHO	Pan-American Health Organization
PBV	picobirnavirus
PCR	polymerase chain reaction
RT-PCR	reverse transcription-polymerase chain reaction
SWS	Safe Water System

1. INTRODUCTION

Acute and chronic diarrhea are significant clinical manifestations of HIV-infection, especially in developing countries. It has been estimated that up to 90% of AIDS-infected patients in the developing world suffer from diarrhea (Smith, 1992; Mayer and Wanke, 1994). In Africa, the most frequently reported cause of death in HIV-infected children is diarrhea (Thea et al., 1993). Two to three million children less than five years of age die of diarrheal disease every year and a large proportion of the disease burden is caused by exposure to contaminated water (Centers for Disease Control and Prevention, CDC). The Safe Water System (SWS) is a household-based intervention that was developed in 1992 by the CDC and the Pan American Health Organization (PAHO) to provide a short-term solution to the drinking water problem in the developing countries (Mintz et al., 1995). The SWS is based on point-of-use disinfection of contaminated water using sodium hypochlorite solution purchased locally and safe storage of water in plastic containers with a narrow mouth, lid, and a valve to prevent recontamination.

Several viruses, bacteria and parasites cause diarrhea in HIV-infected individuals. Numerous viruses, including noroviruses, sapoviruses, rotaviruses, astroviruses, enteric (group F) adenoviruses, and picobirnaviruses (PBVs) have been suggested as being associated with acute gastroenteritis in HIV-infected individuals. Picobirnaviruses (PBVs) are a recently reported group of unclassified viruses with a bisegmented double-stranded RNA genome that were first discovered by polyacrylamide gel electrophoresis (PAGE) in fecal specimens from

children with gastroenteritis (Pereira et al., 1988a). Further studies showed that PBVs infect both humans and animals, including mammals and birds (review by Chandra, 1997). However, the pathogenic potential of PBVs has not been clearly established. The recent cloning of human PBV genomic segments and the development of primers (Rosen et al., 2000) will allow the application of sensitive RT-PCR assays to better determine if these viruses are associated with acute gastroenteritis or are simply commensals.

Unfortunately, only a few studies have described the prevalence of enteric viruses in Africa (Gassama et al., 2001; Cegielski et al., 1994; Thea et al., 1993) and children have been included in only few studies (Liste et al., 2000; Cegielski et al., 1994). In addition, the studies so far have been hospital-based and sensitive molecular detection methods have typically not been used. The current study is the first household-based study describing the prevalence of enteric viruses in HIV-infected individuals in Africa using state-of-the-art detection methods, such as RT-PCR and sequencing of the amplicons. In this study, we investigated the prevalence of enteric viruses among a cohort of HIV-infected and uninfected individuals at baseline and during episodes of acute diarrhea in a prospective household study in rural Uganda using sensitive molecular methods. The study population was divided into an intervention group that received the SWS and a comparison group that used normal water handling practices. Statistical analysis was performed to determine the possible association of the viruses with acute diarrhea, HIV-status, or the water intervention, among other factors.

2. LITERATURE REVIEW

2.1 ACUTE GASTROENTERITIS IN HIV-INFECTED INDIVIDUALS

2.1.1 Introduction

Acute and chronic diarrhea are common complications in AIDS-infected individuals. It has been estimated that nearly 60% of AIDS-infected patients in developed countries and as many as 90% in developing countries suffer from diarrhea (Smith, 1992; Mayer and Wanke, 1994). The majority of the morbidity and mortality associated with late AIDS is due to gastrointestinal disease (Sharpstone et al., 1996), especially in children (Liste, 2000). In Africa, the most frequently reported cause of death in HIV-infected children is diarrhea, accounting for more than half of the deaths in the first 18 months of life (Thea et al., 1993).

2.1.2 Causative agents of gastroenteritis

A wide range of bacterial, viral and protozoan pathogens excreted in feces are capable of causing waterborne infections in humans. Several factors, such as survival of the pathogen in water, latency, infectious dose, and the ability of the pathogen to multiply in the environment may affect the waterborne spread of infection (Leclerc et al., 2002). Importantly, immunocompromised individuals, such as HIV-infected patients, may be at increased risk of developing a symptomatic and subsequently, chronic illness. HIV-infected individuals may not be able to clear acute viral enteric infections as efficiently as immunocompetent hosts would do since gut mucosal immunity is closely linked to T-cell function and may be

remarkably disturbed in HIV-infected individuals (Cegielski et al., 1994; Smith et al., 1992). Opportunistic pathogens, organisms capable of causing disease only in individuals whose resistance to infection is decreased, often infect HIV-positive individuals. It has been estimated that opportunistic infections of the gastrointestinal tract account for 68-85% of both acute and chronic cases of diarrhea in HIV-infected patients (Laughon et al., 1988; Blanshard et al., 1991).

Studies that investigate various pathogens in HIV-infected individuals have produced inconsistent results about the relative importance of different pathogen groups and their association with diarrhea. It has been suggested that many parasites and bacteria, such as cryptosporidia, microsporidia, *Mycobacterium avium*, *Clostridium difficile*, *campylobacter* species, *Giardia lamblia*, and *Salmonella* species may be associated with diarrhea in HIV-infected individuals (Giordano et al., 1999; Celum et al., 1987; Horburg, 1991). In fact, enteric protozoal infection has been suggested to be the most common cause of diarrhea in HIV-seropositive individuals (Sharpstone et al., 1996). However, several studies have reported that emerging viral enteric pathogens may be more important etiologic agents of diarrhea in AIDS patients than either "classic viruses" or enteric bacteria or parasites (Giordano et al., 1999; Grohmann et al., 1993; Durepaire et al., 1995; Dionisio et al., 1997; Giordano et al., 1998). In contrast, numerous studies did not find an association between various enteric viruses and diarrhea in AIDS patients (Liste et al., 2000; Gassama et al., 2001; Gonzalez et al., 1998; Khoo et al., 1995; Cegielski et al., 1994; Thea et al., 1993). In

addition to enteric pathogens, functional and structural intestinal abnormalities and HIV itself may cause gastrointestinal symptoms (Pollock, 2001; Liste et al., 2000).

In a recent study in Senegal, the main etiologic agents of diarrhea were different according to the HIV-serostatus of patients (Gassama et al., 2001). In immunocompetent adults the main causes of diarrhea were *Shigella* sp, *Entamoeba histolytica*, *Salmonella enterica*, and *Giardia* but in the immunocompromised hosts the more frequent etiologic agents were enteroaggregative *E. coli*, *Microsporidium*, *Cryptosporidium* sp., *Rotavirus*, *Shigella* sp., *Candida albicans*, *E. histolytica*, *S. enterica*, and *Isospora belli*. The cause and effects of persistent diarrhea in HIV-infected children are largely unknown, especially in Africa (Thea et al., 1993). *Cryptosporidium* sp. has been the most common agent causing diarrhea in HIV-infected children, but enteroadherence-positive *E.coli* and non-enteric viruses, such as CMV and herpes simplex virus have also been found (Liste, 2000; Ramos-Soriano, 1996).

2.1.3 Clinical manifestations of gastroenteritis in HIV-infected individuals

Many gastrointestinal pathogens infecting HIV-infected individuals are resistant to treatment and can lead to severe weight loss and death (Sharpstone et al., 1996). In fact, weight loss is a major cause of death in most AIDS-patients and is most commonly caused by protozoal gastrointestinal infection (Sharpstone et al., 1996). While some pathogens, like CMV, can be treated with antiviral agents, there is no treatment for two protozoal pathogens, *Microsporidia* and *Cryptosporidia* (Sharpstone et al., 1996).

Testing stool samples for pathogens is the initial investigation method for HIV-seropositive individuals with diarrhea. After stool analysis, acutely ill individuals should be treated with ciprofloxacin that most enteric bacteria in HIV-seropositive individuals are sensitive to (Sharpstone et al., 1996). Symptoms of chronic diarrhea can be treated by giving antimotility agents and oral rehydration therapy may be necessary to maintain electrolyte balance (Sharpstone et al., 1996). Some viral infections can be treated with highly active antiretroviral therapy (HAART). Overall, the increased use of HAART has reduced the morbidity and mortality in HIV-infected individuals (Pollock, 2001). However, combination antiretroviral therapy is costly and thus it is not available for the majority of HIV-infected patients worldwide, especially in developing countries. Moreover, development of viral resistance and difficulties with compliance may cause the re-emergence of opportunistic gastrointestinal infections (Pollock, 2001).

2.1.4 Prevalence of Enteric Viruses in HIV-infected Individuals

Several viruses, including adenovirus, astrovirus, picobirnavirus, small round structured viruses, coronavirus, and rotavirus have been suggested to be related to acute gastroenteritis in HIV-infected individuals (Table 1). The association of CMV infection with diarrhea in HIV-infected individuals has been established but the role of enteric viruses is still controversial (Pollock, 2001). Nevertheless, it has been estimated that enteric viruses are likely to account for a significant proportion of pathogen-negative diarrhea in HIV-infected

individuals (Thomas et al., 1999). However, only a few studies have described the prevalence of enteric viruses in Africa (Gassama et al., 2001; Cegielski et al., 1994; Thea et al., 1993) and children have been included in only few studies (Liste et al., 2000; Cegielski et al., 1994). In addition, the studies so far have been hospital-based and sensitive molecular detection methods have typically not been used. The current study is the first household-based study describing the prevalence of enteric viruses in HIV-infected individuals in Africa using state-of-the-art detection methods, such as RT-PCR and sequencing of the amplicons.

Table 1. Prevalence of enteric viruses in HIV-infected and uninfected individuals in various studies.

<i>Study</i>	<i>Location</i>	<i>Cases/ controls</i>	<i>Methods</i> ¹	<i>Viruses detected</i>	<i>Association with diarrhea</i>	<i>Overall prevalence</i>	<i>Study Population</i> ³
Thea et al. 1993	Zaire	67/131	ELISA, EM	Adenovirus, coronavirus norovirus, rotavirus	No	17.0	Adults
Grohman et al. 1993	USA	109/113	EM, PAGE, EIA RT-PCR ²	Adenovirus ⁴ , astrovirus, Caliciviruses, PBV	Yes	27.9	Adults
Cegielski et al. 1994	Tanzania	59/0	EM	SRSV, rotavirus, CVLP	NA	19.0	Children
Durepaire et al. 1995	France	35/68	ELISA, CC	Adenovirus ⁴	Yes	8.7	Adults
Giordano et al. 1998	Argentina	116/81	PAGE	PBV	Yes	5.7	Adults
Gonzalez et al. 1998	Venezuela	41/84	EIA, latex aggl., EM, PAGE	Adenovirus, PBV	No	6.4	Adults
Giordano et al. 1999	Argentina	141/103	PAGE, latex aggl., EIA	PBV, astrovirus, adenovirus	Yes	20.0	Adults
Thomas et al. 1999	UK	377	EM	Coronavirus, adenovirus, Rotavirus, SRSV (CMV)	Yes	15.9	Adults
Liste et al. 2000	Venezuela	35/46	EIA, PAGE, RT-PCR	Enterovirus, astrovirus, Rotavirus (group A)	No (except rotavirus)	7.7	Children
Gassama et al. 2001	Senegal	279/215	latex agglutination	Rotavirus	No		Adults

¹ ELISA denotes enzyme-linked immunosorbent assay, EM electron microscopy, EIA enzyme immunoassay, CC cell culture, PAGE polyacrylamide gel electrophoresis, RT-PCR reverse-transcription polymerase chain reaction.

² RT-PCR was used to confirm positive results of astrovirus yielded with EIA.

³ All studies were hospital-based.

⁴ None of the adenoviruses were Ad40/Ad41.

2.1.5 Attempts to increase access to safe drinking water in developing countries

Despite of investments of billions of dollars in safe water by donor agencies and governments, lack of access to safe water remains a problem for more than a billion people in the developing world (CDC). Two to three million children less than five years of age die of diarrheal disease every year and a large proportion of the disease burden is caused by exposure to contaminated water (CDC). Several reasons have hindered the efforts to improve access to safe water. Population shifts from rural to urban areas, large population dislocations caused by armed conflicts and natural disasters, and dispersed populations and poor transportation infrastructure in rural areas have significantly complicated providing safe water and sanitation services (CDC).

Long-term efforts to increase the access to safe drinking water in developing countries include large-scale projects such as construction of deep wells or piped water systems (CDC). However, while these efforts are important in long term, this approach is expensive, time-consuming and will take decades to realize. In addition to these efforts to improve the infrastructure, other approaches are needed to address the immediate need for safe water. Several locally available methods to disinfect drinking water, such as boiling water or using commercially available bleach, are often impractical, expensive, environmentally unsustainable, or not appealing to local people (CDC).

The Safe Water System (SWS) is a household-based intervention that was developed in 1992 by CDC and the Pan American Health Organization (PAHO) in response to the cholera epidemic in Latin America (Mintz et al., 1995). The SWS is based on point-of-use disinfection of contaminated water using sodium hypochlorite solution purchased locally and safe storage of water in plastic containers with a narrow mouth, lid, and a valve to prevent recontamination. To increase awareness of the link between contaminated water and disease and to improve hygienic behavior, several behavior change techniques are employed, including social marketing, community mobilization, motivational interviewing, education, and communication (CDC). The SWS was designed to populations that must obtain their drinking water from sources including surface water, potentially contaminated shallow ground water, piped systems in which water is inadequately treated or discontinuous flow requires households to store water, and water tankers or vendors (CDC).

The CDC has tested the SWS in South America, Africa, and Asia and these field trials have shown the SWS to be practical, effective, relatively inexpensive, and potentially sustainable method to increase water quality and reduce waterborne diseases among users (CDC). In Bolivia, Ecuador, Nicaragua, Peru, Pakistan, and Zambia the use of SWS dramatically improved the quality of household drinking water (Quick et al, 1996, 1999; Macy and Quick, 1998; Luby et al., 1998). Families in Bolivia and Zambia who used the SWS had approximately 50% fewer episodes of diarrhea compared with families who did not use the SWS (Quick et al., 1997, 1999). Despite the fact that SWS has proved to be an effective means of improving the household water quality in many cases, household water treatment is not always the main concern. Safe feces disposal, improved hygienic behavior, and use of

sufficient amount of water can all reduce diarrheal diseases in a community more than improved water quality (Esrey et al., 1985). In addition, there are several alternative approaches to SWS that use simple and low cost technology. These methods include aeration, coagulation and flocculation, desalination, disinfection by boiling, chemical disinfection, solar disinfection, filtration, storage and settlement, and straining (CDC). For best outcome, it is important to carefully consider the circumstances and needs relevant to a particular community before deciding on the appropriate approach to improve the access to safe water and to reduce diarrheal disease in the community (CDC).

2.2 ENTERIC VIRUSES

2.2.1 Introduction

Viruses found in the human gut can be divided into three categories according to their epidemiological significance (Leclerc et al., 2002). The first category consists of recognized viral agents of gastroenteritis, including noroviruses, sapoviruses, rotaviruses, astroviruses, and enteric (group F) adenoviruses. The second category contains viruses whose causal relationship with gastroenteritis has not been confirmed. These assumed enteropathogens include coronaviruses, echovirus type 22, picobimaviruses, picotrimaviruses, toroviruses, parvoviruses and pestiviruses. The third category comprises of viruses that are either nonenteropathogenic or cause illness that is unrelated to the gut epithelium. These viruses include non-group F adenoviruses, poliovirus, coxsackieviruses A and B, echovirus, and hepatitis A and E virus. The causal relationship of enteric viruses to gastroenteritis is presented in Table 2. In the following subchapters the characteristics and molecular

epidemiology of picobirnaviruses, human caliciviruses (noroviruses and sapoviruses), group F adenoviruses, astroviruses, and rotaviruses is discussed.

Table 2. Causal relationship of various viruses with diarrhea in humans.

Group	Viruses
Causal relationship demonstrated ¹	Rotaviruses Human caliciviruses (noroviruses, sapoviruses) Astroviruses Enteric adenoviruses (group F)
Candidate agents (causal relationship not yet confirmed)	Enteric Coronaviruses Echovirus type 22 Picobirnaviruses Picotrimaviruses Parvoviruses Pestiviruses Toroviruses
Other agents (etiologic relationship not demonstrated)	Coxsackie A and B viruses Echoviruses Hepatitis A and B viruses Non-group F adenoviruses Poliovirus

Adapted from Atmar and Estes, 2001; Leclerc et al., 2002.

¹ Listed in order of relative clinical importance.

2.2.2 Picobirnaviruses

Picobirnaviruses are a recently reported group of unclassified viruses with a bisegmented double-stranded RNA genome that differ slightly from the existing members of the family *Birnaviridae*. They are nonenveloped and relatively small in size, 30-40 nm in diameter,

while birnaviruses are 60-65 nm. They have icosahedral symmetry with a triangulation number equal to 3 and their buoyant density in cesium chloride is 1.39-1.41 g/ml. The estimated size of the two genomic segments ranges from 2.3 to 2.6 and 1.5 to 1.9 kb while birnaviruses have larger genomic segments (3.3 and 3.8 kb). Picobirnaviruses infect mammals and birds but not fish as most birnaviruses do. The pathogenicity of birnaviruses for fish and birds has been established but the pathogenic potential of picobirnaviruses is still unconfirmed (Chandra, 1997).

Picobirnaviruses were initially discovered accidentally while investigating fecal specimens from children with gastroenteritis using polyacrylamide gel electrophoresis (PAGE) for detection of the characteristic 11 genome segments of rotavirus (Pereira et al. 1988a). The electrophoretic migration of the two picobirnavirus genomic segments falls within the range of migration of rotavirus genomic segments. Until recently, PAGE was the only available laboratory method for picobirnavirus detection; however, EM has occasionally been used (Grohman et al., 1993; Gallimore et al., 1995a). An example of an electrophoretic profile of picobirnavirus on a PAGE gel is shown in Figure 1.

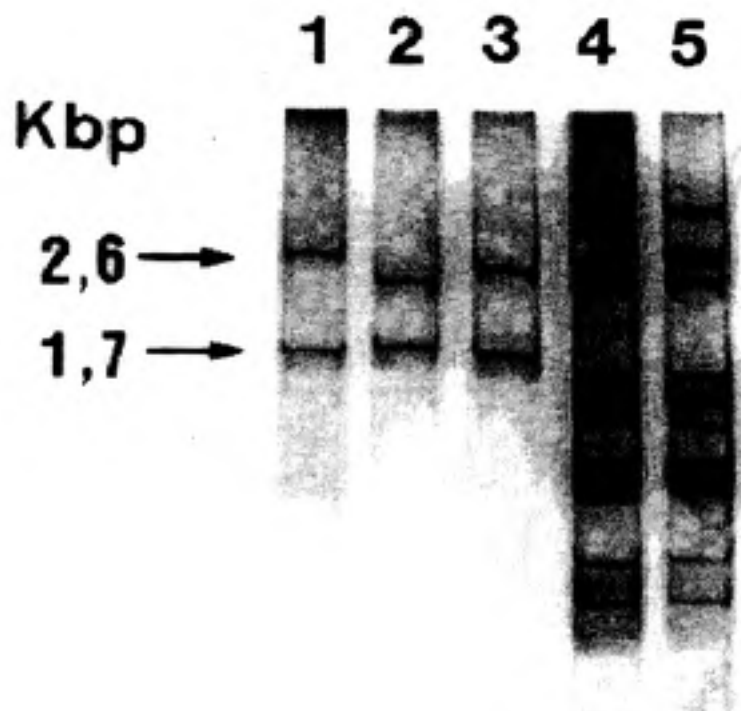


Figure 1. Electrophoretic profile of picobirnavirus on a PAGE gel. Examples of electrophoretic patterns of picobirnavirus strains (lanes 1, 2, and 3) and rotavirus double-stranded RNA (lanes 4 and 5) on a PAGE gel stained with silver nitrate. *Adapted from Gonzalez et al., 1998.*

Picobirnaviruses infect both humans and animals. They have been detected in fecal specimens from both domestic and wild animals, including rats (Pereira et al., 1998b), guinea pigs (Pereira et al., 1989), rabbits (Gallimore et al., 1993; Ludert et al., 1995), chickens (Leite et al., 1990; Alfieri et al., 1988), pigs (Gatti et al., 1989; Chasey et al., 1990; Ludert et al., 1991), calves (Vanopdenbosch and Wellemans, 1989) and foals (Browning et al., 1991).

The pathogenic potential of picobirnaviruses has not been clearly established. The virus has been detected in animals with and without diarrhea (Pereira et al., 1988b; Vanopdenbosch and Wellemans, 1989; Ludert et al., 1991; Browning et al., 1991), and its association with gastroenteritis has primarily been reported in studies with swine. Gatti et al. (1989) detected picobirnavirus in 15.3% of animals with diarrhea and 9.6 % in animals without diarrhea. However, Ludert et al. (1991) did not find a difference in picobirnavirus prevalence between animals with and without diarrhea.

In 1995, another bisegmented dsRNA virus was found in about one third of human fecal samples that tested positive for *Cryptosporidium spp.* but which was absent in samples that tested negative for this protozoan (Gallimore et al., 1995b). This 'atypical' picobirnavirus is similar to the 'typical' picobirnavirus but has smaller genomic fragments. The connection to *Cryptosporidium* raised a question about the host of picobirnaviruses since their ability to replicate in vertebrate hosts has not been confirmed (Rosen et al., 2000). However, Green et al. (1999) investigated the genomic characterization of the large segment of rabbit picobirnavirus genome and found no relationship in sequence or organization between rabbit picobirnavirus and the atypical picobirnavirus associated with *Cryptosporidium parvum*.

Several studies have suggested that picobirnaviruses are able to induce an immune response in the host animal (Vanopdenbosch and Wellemans, 1989; Gallimore et al., 1993; Ludert et al., 1991). In contrast, some studies have failed to detect an immune response (Grohmann et al., 1993; Ludert et al., 1995), but this may be due to the inability of the technique used to detect the low levels of antibodies (Chandra, 1997). Further characterization of

picobirnavirus has been hindered because the virus can not be cultivated in cell culture and no animal models are available.

In humans, picobirnaviruses are detected in individuals with and without diarrhea throughout an age range of 3 to > 65 years (Gallimore et al., 1995a). Association of these viruses with acute diarrhea in HIV-infected individuals has not been confirmed. Findings by Grohmann et al. (1993) and Giardano et al. (1999; 1998) suggest an association between picobirnavirus and diarrhea in HIV-infected individuals, whereas Gonzales et al. (1998) did not find an association. Liste et al. (2000) investigated the association between enteric virus infections and diarrhea in healthy and HIV-infected children in Venezuela and did not detect picobirnavirus in fecal samples using the PAGE method.

The frequency of detection of picobirnavirus in human fecal samples varies in different studies. In the first study of picobirnavirus in humans (Pereira et al., 1988a) the frequency was reported to be up to 20% in outbreaks of gastroenteritis. In contrast, Pereira et al. (1993) and Cascio et al. (1996) reported a lower frequency of 0.5% and 0.43%, respectively, and Liste et al. (2000) did not detect picobirnavirus at all.

Until recently, the methods for detecting picobirnavirus in fecal samples were limited to PAGE and rarely, electron microscopy (Grohman et al., 1993; Gallimore et al., 1995a). However, picobirnavirus is often present in low titers and the type of picobirnavirus-RNA extraction method has been reported to be crucial for successful detection by PAGE (Gallimore et al., 1995a; Giordano et al., 1998). Even though the sequence of the large

segment of rabbit picobirnavirus had been published (Green et al., 1999) there was no sequence information available on the human picobirnavirus strains until Rosen et al. (2000) cloned and sequenced the genomic segments of several human picobirnavirus strains and developed an RT-PCR assay. They reported the sequences of a partial length cDNA clone of the larger genomic segment of one human picobirnavirus strain (strain 3-GA-91) and several nearly full-length clones of the smaller genomic segment from two other human picobirnavirus strains (4-GA-91 and 1-CHN-97). The strains 3-GA-91 and 4-GA-91 were isolated from an HIV-infected individual in Georgia and the strain 1-CHN-97 was isolated from an HIV-negative individual in China.

The size of the larger RNA segment was estimated to be 2300 bp based on its electrophoretical mobility on a PAGE gel and the length of the partial clone of this fragment was 1527 bp. One possible open reading frame was proposed to be at a position of 1-725 nt at the 5' end. For the smaller RNA segment, nearly full-length consensus sequences were derived from several clones of 4-GA-91 and 1-CHN-97 strains and their sizes were 1674 and 1696 nt, respectively, which was very close to the estimated sizes on a PAGE gel. Both strains revealed a single ORF at position 90-1643 nt and 58-1650 nt, respectively, and the 5' non-coding regions were AT rich (Rosen et al., 2000). Comparison of the nucleic acid and deduced amino acid sequences of the small genomic segments with the sequences in the EMBL and GenBank revealed three motifs common among RNA-dependent RNA-polymerases (RDRP) of dsRNA and some ssRNA viruses. Of these motifs, motif 2 contained amino acid sequence SG-T that has been reported to be the most highly conserved region of the RDRP.

The primer pairs that were designed for RT-PCR were targeted towards the smaller genomic segment of the 4-GA-91 and 1-CHN-97 strains. When the authors tested the newly developed RT-PCR they found that the primers derived from the strain from China had the broadest reactivity. They produced an amplicon of the expected size when stool samples from individuals in Florida, Atlanta (two individuals other than 4-GA-91), Argentina, and Venezuela were tested. In two outbreaks of gastroenteritis in elder care facilities Florida a total of four samples tested positive for picobirnavirus. Amplicons of the expected size were produced only with the 1-CHN-97 primers. When partial length genomic segment 2 sequences of PBVs from these outbreaks were compared with those from HIV-infected individuals in the United States and Argentina and an HIV-negative individual in China, it was found that no picobirnavirus of one sequence type seemed to infect HIV-infected individuals rather than HIV-negative individuals. Strain 4-GA-91 was determined to be phylogenetically different from other picobirnavirus strains. Its nucleotide sequence differed by 60-63% from that of all other PBV strains and the predicted amino acid sequence differed by 72-83%.

2.2.3 Human Caliciviruses

Classification

Human caliciviruses are a member of the *Caliciviridae* family, which also contains two genera of animal caliciviruses, vesiviruses and lagoviruses. HuCVs are considered the most common etiologic agent of nonbacterial gastroenteritis outbreaks in individuals of all age groups worldwide (Green et al., 2001; Glass et al., 2000). HuCVs are assigned to two genera: noroviruses, previously called small round structured viruses (SRSV), and sapoviruses, a group of typical caliciviruses with distinctive morphology when view by electron microscopy (Green et al., 2001). Noroviruses can be classified into at least four different genogroups of which two (GI and GII) are most prevalent in humans. These genogroups can be further divided into at least 15 genetic clusters or genotypes (Vinjé et al., 2000). Sapoviruses consist of at least two genogroups (Schuffenecker et al., 2002). Reference strains and examples of representative strains of each genetic cluster of noroviruses and sapoviruses are presented in Table 3 and Table 4, respectively. Reference strains of norovirus genogroup I include Norwalk, Southampton, Desert Shield, Chiba, Musgove, Hesse, and Winchester viruses, whereas genogroup II includes Hawaii, Melksham, Toronto, Bristol, Hillingdon, Seacroft, and Leeds viruses (Table 3) (Green et al., 2001). Reference strains of sapoviruses include Manchester, Parkville, London, and Stockholm viruses (Table 4) (Green et al., 2001). While noroviruses cause gastroenteritis in all age groups, sapoviruses infect mainly children (Green et al., 2001; Schuffenecker et al., 2002).

Table 3. Classification of noroviruses by genetic relatedness in the complete capsid protein.

Genogroup	Reference virus	Genetic cluster	Examples of viruses having >80% amino acid identity with corresponding reference virus
I	Norwalk/1968/US	1	KY-89/1989/JP
	Southampton/1991/UK	2	Whiterose/1996/UK
	Desert Shield 395/1990/SA	3	Birmingham/1993/UK
	Chiba 407/1987/JP	4	Thistlehall/1990/UK
	Musgrove/1989/UK	5	
	Hesse 3/1997/GE	6	Sindlesham/1995/UK
	Winchester/1994/UK	7	
II	Hawaii/1971/US	1	Wortley/1990/UK
	Melksham/1994/UK	2	Snow Mountain/1976/US
	Toronto 24/1991/CA	3	Mexico/1989/MX
	Bristol/1993/UK	4	Lordsdale/1993/UK, Grimsby/1995/UK
	Hillingdon/1990/UK	5	
	Seacroft/1990/UK	6	
	Leeds/1990/UK	7	
	Amsterdam/1998/NL	NA ^a	

Adapted from Green et al., 2001.

^a NA, not assigned

Table 4. Classification of human sapoviruses by genetic relatedness in the complete major capsid protein.

Reference virus	Examples of viruses having >80% amino acid identity with corresponding reference virus
Manchester/1993/UK Parkville/1994/US London/1992/UK Stockholm 318/1997/SE	Sapporo/1982/JP; Plymouth/1993/UK Houston 27/1990/US

Adapted from Green et al., 2001.

Morphology

HuCVs are small (28-35 nm), non-enveloped viruses with a positive sense single-stranded RNA genome of 7.3-7.6 kb. The virion is composed of a single major capsid protein (Atmar and Estes, 2001). The key characteristic of the capsid architecture is 32 cup-like depressions

visible by EM that are more common in some strains, especially sapoviruses (Atmar and Estes, 2001). In both genera the non-structural proteins are encoded at the 5' end of the genome and the major capsid protein is coded at the 3' end. However, the genome of sapoviruses is organized slightly differently. In noroviruses, ORF 1 codes for non-structural proteins, ORF 2 encodes for the single major capsid protein and ORF 3 encodes for a minor structural protein whose function is uncertain (Atmar and Estes, 2001). In sapoviruses, ORF 1 encodes for the non-structural proteins as well as the capsid protein forming a long polyprotein. Sapovirus genomes also contain a small ORF at the 3' end encoding for a small protein of unknown function.

Clinical symptoms and pathogenesis

The incubation period for gastroenteritis associated with HuCVs is usually between 24 and 48 hours, but in volunteer studies symptoms have occurred as soon as within 10 hours of exposure (Green et al., 2001). The typical symptoms of norovirus infection include rapidly beginning vomiting, watery non-bloody diarrhea with abdominal cramps, and nausea. Characteristically, norovirus infection has a high attack rate (45% in average) and secondary spread is significant (Kaplan et al., 1982; Hedberg et al., 1993). HuCV infection usually does not cause serious complications in immunocompetent individuals, but dehydration is common and may require medical attention especially among the young and the elderly. Symptoms typically last 24 to 60 hours and there is no evidence of any serious long-term sequelae. However, a recent study in the Netherlands revealed that up to 20% of individuals in a community-based cohort reported symptoms lasting longer than two weeks (Rockx et al.,

2002). Viral shedding usually begins with the onset of symptoms and may continue for up to two weeks after recovery.

The mechanism by which HuCVs cause diarrhea is not well understood. The changes observed in the intestinal epithelium of volunteers infected with noroviruses included blunting of the villi, abnormal epithelial cells, and loss of and inflammatory response in lamina propria (Koopmans et al., 2002). HuCVs do not cause any known systemic effects.

Diagnostic methods

Noroviruses cannot be propagated in cell culture and in the past the diagnosis has been made by visualization of virus particles in stool by EM (Koopmans et al., 2002). However, EM is relatively insensitive because this technique requires virus concentrations of at least 10^6 per ml of stool (Doane, 1994) and noroviruses are present in feces in small numbers compared to other enteric viruses (Atmar and Estes, 2001; Koopmans et al., 2002). Immune EM (IEM) has been used to detect Norwalk-virus in stool filtrates (Kapikian et al., 1972) and since then several other SRSVs has been detected with IEM in stool samples (Kaplan et al., 1982; Thornhill et al., 1977). Even though IEM is an important diagnostic tool for the non-cultivable caliciviruses, like direct EM, the use of IEM is limited and it is not readily available for large epidemiological studies (Atmar and Estes, 2001).

Although noroviruses cannot be propagated in cell culture, several antigen-based methods have been developed, including RIA and EIA with hyperimmune animal sera (Koopmans et al., 2002; Atmar and Estes, 2001). However, noroviruses are a diverse group of viruses and

the problem is that hyperimmune responses are mainly type-specific and thus these assays have a narrow scope (Koopmans et al., 2002). ELISAs based on the use of hyperimmune animal sera raised against recombinant capsid proteins are also mainly type-specific (Jiang et al., 1995). The production of monoclonal antibodies to recombinant NLV capsids has allowed the production of antibody panels that are reactive to a broad range of NLV genotypes within the two NLV genogroups (Jiang et al., 2000; Nakata et al., 2000). The evaluation of a commercial ELISA that uses pools of these monoclonal antibodies showed that this assay could distinguish reliably between strains in genogroups 1 and 2 and it displayed a broad reactivity to a wide range of NLV genotypes (Richards et al., 2003). However, sensitivity of the ELISA was only 55.5% when compared with RT-PCR, and it failed to detect some of the genetic clusters currently or recently co-circulating in UK (Richards et al., 2003).

Over the last 10 years, diagnosis of HuCV illness in outbreaks has improved with the increasing use of reverse transcriptase polymerase chain reaction (RT-PCR), and now considered the gold standard for norovirus detection (Green et al., 1995a,b). After RT-PCR, the PCR-products can be confirmed with hybridization assays or sequencing. Sequence analysis allows further characterization of circulating strain types and is also useful in linking and differentiating outbreaks (Fankhauser et al., 1998). The genetic diversity of noroviruses has complicated the development of single primer pair that detects all strains of these viruses (Atmar and Estes, 2001). Most primers amplify the most conserved region of the genome, the RNA-dependent RNA polymerase region (Atmar and Estes, 2001, Ando et al., 1995; Green et al., 1995; Jiang et al., 1999; Vinjé et al., 1996). Other regions of the genome have also

been targeted, like the helicase, the capsid region, and ORF3, but in general, these assays that amplify nonpolymerase regions are less broadly reactive (Atmar and Estes, 2001).

Epidemiology

The development of molecular methods to detect noroviruses has revealed that these viruses are one of the most common etiologic agents causing gastroenteritis in adults, both in outbreaks of gastroenteritis and sporadic cases (Koopmans et al., 2002; De Wit et al., 2001; Pang et al., 1999). Moreover, noroviruses are the second most common cause of gastroenteritis in children after rotavirus (Koopmans et al., 2002; De Wit et al., 2001; Pang et al., 1999). The estimated proportion of foodborne norovirus outbreaks varies greatly between countries. In the US, 96% of non-bacterial gastroenteritis reported to the CDC between January 1997 and June 1998 were attributable to noroviruses (Koopmans et al., 2002). In the Netherlands, approximately 80% of gastroenteritis outbreaks were caused by norovirus (Vinjé et al., 1997; Vennema et al., 2001) and in England and Wales, norovirus caused 27% of all outbreaks of intestinal infectious disease between 1992 and 1994 (Djuretic et al., 1996).

Immunity

The mechanisms of immunity to HuCVs are not well understood, but it has been reported that immunity may be strain-specific and lasts only a few months (Noel et al., 1997; Green et al., 2001). Infection by noroviruses induces a specific, IgG, IgA, and IgM serum antibody response, even if there has been previous exposure, and most individuals are resistant to reinfection for 4-6 months (Green et al., 2001). Long-term protection has not been observed (Wilhelmi et al., 2003). Due to the significant genetic variability among noroviruses and

sapoviruses, individuals become repeatedly infected with these viruses. There is also evidence suggesting that susceptibility to norovirus infection may be genetically determined. Susceptibility to norovirus infections seems to be associated with the ABO blood type, suggesting that noroviruses might recognize histo-blood group antigens on intestinal epithelial cells as receptors for infection (Huang et al., 2003; Hutson et al., 2002).

Modes of transmission

HuCVs are transmitted primarily through the fecal-oral route, either by consumption of fecally contaminated food or water or by direct person-to-person contact, but they may also be spread through environmental and fomite contamination as well as airborne droplets (Sawyer et al., 1988). Person-to-person contact is the most common mode of transmission (Green et al., 2001). HuCVs are highly contagious, and the infectious dose for noroviruses may be as low as 10-100 viral particles (Glass et al., 2000). Several foodborne outbreaks have been described, many of which resulted from an infected food handler. However, contamination may occur at almost any step of the food distribution chain (Koopmans et al., 2002). Waterborne outbreaks have occurred both directly as a result of ingestion of contaminated water and indirectly via washed fruits and contact with recreational waters (Gray et al., 1997; Kukkula et al., 1999). Volunteer studies have shown that asymptomatic infection may occur in as many as 30% of infections, although the role of asymptomatic infection in norovirus transmission is unclear (Green et al., 2001). Several studies have indicated that norovirus infections show a peak in the winter months (reviewed by Mounts et al., 2000). This seasonality was independent of the study setting (winter peak was demonstrated in both outbreaks and sporadic cases), the age of patients, the detection

methods used, and was present even when outbreaks occurred in institutions or were traced to contaminated food (Mounts et al., 2002). The predominant winter seasonality and the rapidity by which norovirus infection can spread in a community suggest that airborne transmission is an important secondary route of transmission (Mounts et al., 2000).

Until recently, humans were considered the only host of noroviruses but new data suggests the possibility of zoonotic transmission of noroviruses (Liu et al., 1999; Sugieda et al., 1998). Noroviruses were found in pigs and calves, and even though the two bovine enteric calicivirus strains and the pig enteric calicivirus were genetically distinct from human strains, they are sufficiently enough related to human norovirus strains so that under favorable conditions zoonotic transmission could occur (Koopmans et al., 2002).

2.2.4 Adenoviruses

Classification

Adenoviruses are classified in the family *Adenoviridae* that consists of two genera. Genus *Aviadenoviruses* is limited to birds and genus *Mastadenoviruses* includes viruses that infect mammals including humans. Human adenoviruses currently comprise of 49 different serotypes divided into six subgenera (A through F), but candidate serotypes 50 and 51 have recently been reported (De Jong et al., 1999). Depending on the serotype, adenoviruses can cause various respiratory illnesses (species B, C, and E), gastroenteritis (A and F), conjunctivitis (D and E), cystitis, and rash. In some cases adenoviruses can also infect the

urinary tract, liver and other organs such as the pancreas and central nervous system (Horwitz, 2001). Some adenovirus serotypes are oncogenic, including Ad12, Ad18 and Ad31 that are highly oncogenic in hamsters (Horwitz, 2001). Enteric adenoviruses have been associated with diarrhea in several population groups, but infants and young children are at highest risk of getting infected (Horwitz, 2001). The principal serotypes causing gastroenteritis include serotypes Ad40 and Ad41 in subgenus F that are believed to be an important cause of childhood gastroenteritis worldwide. Studies in Europe, Asia, and North and South America have found adenoviruses to be associated with 3.1-13.5% of cases of pediatric diarrhea, and serotypes 40 and 41 reportedly comprise a substantial proportion of these adenoviruses (Brown et al., 1990; Harsi et al., 1995; Scott-Taylor et al., 1995; Moore et al., 2000). Subgenus A adenoviruses (especially Ad31) are also commonly isolated from infants with gastroenteritis in small outbreaks (Pring-Åkerblum et al., 1999).

Morphology

Adenoviruses are nonenveloped, medium-sized (80-100 nm) icosahedral viruses with a double-stranded DNA genome. The linear 36 kb genome is organized into eight supercoiled domains. The capsid consists of 240 hexon capsomers and 12 penton capsomers. The penton capsomers are made of the penton base from which the fiber protein projects. The unusual stability of adenoviruses to chemical and physical factors and pH conditions allows them to survive long periods outside the body (CDC online). Their infectivity is stable between pH 6.0-9.5 and is not adversely affected after 70 days at +4°C (www.nlv.ch/Adenovirus/Adenofactsheet.htm).

Clinical symptoms and pathogenicity

Adenovirus infections are usually mild and require no therapy or only treatment of the symptoms. However, gastroenteritis caused by Ad 40 and Ad41 can be severe and in general, adenovirus infections tend to be more severe in immunocompromised hosts (Horwitz, 2001). There is no virus-specific remedy for adenovirus infection and thus the illnesses can only be managed by treating the symptoms (CDC). Some adenovirus types can establish persistent asymptomatic infections in tonsils, adenoids, and intestines of the hosts and viral shedding can continue for months or years (CDC and Horwitz, 2001).

Diagnostic methods

Adenoviruses were first cultured and identified as distinct viral agents in 1953 (Horwitz, 2001). Since then, adenoviruses have been extensively studied by serological and molecular methods. Development of PCR assays has made it possible to rapidly detect adenoviruses in clinical specimens and to distinguish between different serotypes. Viral isolation is the gold standard for direct detection of adenovirus but it is often slow and can thus be of limited value in clinical decisions (Avellón et al., 2001). Direct antigen detection methods based on EIA, immunofluorescence (IF), and specific latex agglutination are also widely used but they can be less sensitive than tissue culture (Avellón et al., 2001).

Since different Ad species and serotypes are often associated with distinct clinical outcomes and epidemiological features (Xu et al., 2000), it is usually important in clinical settings to differentiate Ads on the subgenus level or even identify the serotype. Methods used to identify different subgroups or serotypes include serotype-specific neutralization tests, RE

analysis of DNA extracted from infected cells, and the hemagglutination inhibition test (Elnifro et al., 2000). Faster identification methods include the use of serotype-specific monoclonal antibodies (Wood et al., 1997), detection of subgenus-specific antibodies (Akalu et al., 1998), and PCR (Allard et al., 1994; Kidd et al., 1996; Akalu et al., 1998; Pring-Åkerblom et al., 1999; Xu et al., 2000). PCR assays have been shown to be at least as good as or better than classic cell culture or immunodiagnostic methods for detection of adenoviruses in clinical samples (Xu et al., 2000). PCR is also useful for classification of adenoviruses to species and serotype levels using primers targeted to the hexon (Avellón et al., 2001; Pring-Åkerblom et al., 1999) or the fiber gene (Xu et al., 2000).

Epidemiology

Enteric adenoviruses cause acute and severe diarrhea especially in young children. Subgenus F adenoviruses (types 40 and 41) are considered to be an important cause of infantile gastroenteritis, and subgenus A adenoviruses are also commonly found in infants with gastroenteritis in small outbreaks (Pring-Åkerblom, 1999; Hammond et al., 1985; Adrian et al., 1987). The clinical symptoms are difficult to distinguish from rotavirus infection; however, adenovirus is less prevalent than rotavirus in children (Horwitz, 2001). All adenovirus strains are transmitted by direct contact, fecal-oral transmission and in some cases water-borne transmission (CDC). The likelihood of viral transmission by both direct contact and fecal-oral route is moderate (CDC).

The relationship of adenoviruses and diarrhea has been controversial since many adenoviruses replicate efficiently in the intestine and are excreted in stool. Adenoviruses are

commonly found in stools of asymptomatic patients, and shedding of the virus can continue for months or years (Horwitz, 2001). Only Ad40 and Ad41 have been certainly linked with gastroenteritis in several population groups (Horwitz, 2001). However, it has been reported that even during epidemics of enteric adenovirus infection, many infected individuals do not develop gastroenteritis but shed virus in their stool (Van et al., 1992).

The incidence of gastroenteritis caused by adenoviruses varies considerably in different clinical settings (Horwitz, 2001). In a study in Iran, 6.7% of stool specimens from 872 children contained enteric adenoviruses and 2.0% non-enteric adenoviruses when analyzed with monoclonal antibody-based EIA (Saderi et al., 2001). In Bangladesh, adenoviruses were detected in 2.8% of 4,409 specimens but the percentage was as high as 12.3% in some months when diagnosed with Ad40- and Ad41-specific monoclonal antibodies (Jarecki-Khan et al., 1993; Horwitz, 2001). In a study conducted at day care centers in Houston, stool samples were collected weekly from children 6 to 24 months of age who were present during 10 separate epidemics of adenovirus-associated diarrhea. Enteric adenoviruses were detected in 38% of 249 children (Van et al., 1992). However, nearly half of these samples were obtained from asymptomatic children. In another study at day care centers in Atlanta, stool samples were collected from 565 children with diarrhea and 129 controls. Enteric adenoviruses 40 and 41 were detected in 2% of samples in each group (Lew et al., 1991).

Adenoviruses commonly cause persistent and generalized infections in immunocompromised hosts, and these infections can sometimes lead to death (Horwitz, 2001). Many patients with AIDS shed adenoviruses that are rarely or never isolated from immunocompetent individuals

(De Jong et al., 1999; Hierholzer et al., 1988, Khoo et al., 1995), and the seven most recently described adenovirus serotypes in subgenus D were first isolated from HIV-infected individuals (Hierholzer et al., 1988; Schnurr et al., 1993). However, causal relationship between group D adenoviruses and diarrhea in immunocompromised hosts has not been demonstrated (Horwitz, 2001). The first reported case of intestinal infection caused by Ad40 in a patient with AIDS was described recently with shedding of the virus continuing for 13 months (Dionisio et al., 1997). No other enteropathogens were found in this study, which suggested a causal relationship between Ad40 and gastroenteritis.

Immunity

Human adenoviruses, as well as most adenoviruses infecting other mammals and birds, are species specific; however, some cross-infectivity may occur between closely related species (Horwitz, 2001). Most patients develop both strain- and type-specific antibodies to the infecting adenovirus strain (Horwitz, 2001). Patients may continue to shed adenoviruses in their stool sporadically for months following a successful humoral immune response (Horwitz, 2001). Neutralizing antibodies may protect against reinfection with the same serotype but they do not eliminate the carrier stage (Horwitz, 2001).

2.2.5 Astroviruses

Classification

Astroviruses were initially discovered by electron microscopy in fecal samples from children with diarrhea (Madeley et al., 1975) and are now classified in the family *Astroviridae*

(Monroe et al., 1993). The name astrovirus was derived from the distinctive star-like motif (Astron, "star" in Greek) observed on the surface of some of the virus particles (Matsui and Greenberg, 2001). Astroviruses comprise the third group of nonenveloped human and animal viruses with a plus-sense, single stranded RNA, in addition to *Picornaviridae* and *Caliciviridae*. Human astroviruses are currently classified into seven serotypes (HAstV 1-7) using immune EM, immunofluorescence techniques, EIA, and ELISA (Lee and Kurtz, 1982; Kurtz and Lee, 1984, 1994; Noel et al., 1995, Belliot et al., 1997). Subsequently, 7 human astrovirus genotypes have been identified based on sequence analysis of ORF2 (Noel et al., 1995; Cunliffe et al., 2002). These genotypes correlate with the serotype assigned by EIA (Noel et al., 1995; Cunliffe et al., 2002). The existence of 8th human astrovirus serotype has been suggested (Belliot et al., 1997; Mendez-Toss et al., 2000; Taylor et al., 2001). According to Belliot et al. (1997) astroviruses can also be classified into two genogroups based on the sequence of the ORF 1a, genogroup A consisting of serotypes 1 through 5 and genogroup B consisting of serotypes 6 and 7. Epidemiological studies have shown that serotype 1 is the main cause of astrovirus-related gastroenteritis worldwide (Konno et al., 1982; Koopmans et al., 1998; Lee and Kurtz, 1994), although serotypes 6 and 3 have also caused large outbreaks (Matsui and Greenberg, 2001).

Morphology

Astroviruses are 28-30 nm particles with smooth margin and a characteristic five or six-point star on the surface. The plus-sense, single stranded RNA genome of astroviruses is approximately 6,800 nt in length, excluding the poly (A) tail at the 3' end. The viral genome consists of three ORFs. ORF1a and ORF1b at the 5' end of the genome contain highly

conserved sequences and encode nonstructural proteins (protease and polymerase, respectively) (Cunliffe et al., 2002). In contrast, ORF2 at the 3' end of the genome is highly variable and encodes the capsid precursor protein (Cunliffe et al., 2002; Matsui and Greenberg, 2001). Studies characterizing the structural proteins of human and animal astroviruses have reported conflicting results, but it is believed that human astroviruses are composed of two or three capsid proteins (P1, P2, and P3), depending on the serotype (Belliot et al., 1997). During infection, both the full genomic and a subgenomic (2.4 kb) RNA of the ORF-2 are produced (Matsui and Greenberg, 2001). The use of subgenomic messengers is an efficient way to quickly produce abundant capsid proteins and stretches of conserved sequences in noroviruses suggest a similar mechanism for these viruses (Green et al., 2001).

Clinical illness and pathology

Human astroviruses cause gastroenteritis primarily in young children, although other important groups include the elderly, institutionalized patients and immunocompromised individuals (Matsui and Greenberg, 2001). They cause mild, watery diarrhea that usually lasts for 2-3 days, accompanied with vomiting, fever, anorexia, abdominal pain, and various constitutional symptoms that typically last less than 4 days (Matsui and Greenberg, 2001). In children, diarrhea caused by astrovirus may be difficult to distinguish from a diarrhea caused by rotavirus on clinical basis alone but in general, astrovirus-related diarrhea is milder and does not usually lead to extensive dehydration or hospitalization (Matsui and Greenberg, 2001). According to volunteer studies, unlike noroviruses, the pathogenicity of astroviruses is relatively low in adults. However, it has been suggested that astroviruses are important

etiologic agents of diarrhea in immunocompromised patients (Grohmann et al., 1993; Giordano et al., 1999; Matsui and Greenberg, 2001) and HIV-infection may increase the risk of more severe infection (Cunliffe et al., 2002).

Viral pathogenesis of astrovirus infection in humans is not well understood. It has been suggested that astrovirus replicates in intestinal tissue of humans (Phillips et al., 1989), but the human volunteer studies have not investigated the histological effects of astrovirus-related diarrhea (Kurtz et al., 1979; Midthun et al., 1993). The role of astrovirus in persistent diarrhea is not clear (Matsui and Greenberg, 2001).

Diagnosis

The astrovirus genome has been cloned and sequenced (Willcocks et al., 1992; Jiang et al., 1993; Lewis et al., 1994) and several diagnostic methods are available for astrovirus detection, including EM, EIA, IF, ELISA, RT-PCR. There is no animal model for studying human astrovirus infection (Matsui and Greenberg, 2001) but astroviruses can be propagated in cell culture (Lee and Kurtz, 1981), which clearly distinguishes them from Norwalk virus and other human caliciviruses. RT-PCR assays directed to different regions of the genome have been developed with primers targeting the nonstructural protein coding region in ORF 1a and 1b (Belliot et al., 1997), the capsid protein region in ORF 2 (Noel et al., 1995; Nadan et al., 2003; Sakon et al., 2000) or the 3' untranslated region (Jonassen et al., 1995; Mitchell et al., 1995; Saito et al., 1995). Single primer pairs targeted to either ORF1a or ORF2 have been used to detect reference strains of seven astrovirus serotypes and a wide selection of wild astrovirus strains from samples collected around the world (Belliot et al., 1997; Noel et

al., 1995). In addition, sequence analysis of the ORF1a region showed that astroviruses form two distinct genogroups (Belliot et al., 1997).

Epidemiology

Epidemiological studies using the electron microscope (Leite et al., 1991; Monroe et al., 1991) or EIA (Herrmann et al., 1991; Cruz et al., 1992; Mitchell et al., 1993) show that astroviruses have a worldwide distribution (Noel et al., 1995). They have been isolated from humans and several animal species, and in most species astroviruses are found in association with gastroenteritis (Matsui and Greenberg, 2001), including lambs (Snodgrass et al., 1977), calves (Woode and Bridgen, 1978), deer (Tzipori et al., 1981), piglets (Bridger et al., 1980), kittens (Hoshino et al., 1981), mice (Kjeldsberg et al., 1985), dogs (Marshall et al., 1984; Williams, 1980) and turkey poults (McNulty et al., 1980). In humans, astroviruses are associated with gastroenteritis in children (Herrmann et al., 1988; Lew et al., 1990; Mitchell et al., 1993), adults (Belliot et al., 1997; Pager et al., 2002), the elderly (Lewis et al., 1989; Midthun et al., 1993), and immunocompromised individuals (Grohmann et al., 1993; Giordano et al., 1999). Typically, astroviruses cause less severe gastroenteritis than other enteric viruses and is thus usually detected more often in community-based surveys (Cunliffe et al., 2002; Glass et al., 1996).

Globally, the prevalence of astrovirus in young children with sporadic episodes of diarrhea has been reported to be between 2.1% and 10% (Glass et al., 1996). Studies using EIA for detecting viruses in stools have demonstrated that astroviruses are significant etiologic agents of diarrhea in developing countries. In Thailand and Guatemala astroviruses were found to be

the second most common cause (after rotavirus) of viral diarrhea in young children and were associated with diarrhea in 8.6% and 7.3% of diarrhea cases, respectively (Herrmann et al., 1991; Cruz et al., 1992).

Only few studies of astrovirus infections have been conducted in Africa (Pavone et al., 1990; Steele et al., 1998; Naficy et al., 2000) or in HIV-infected individuals (Grohman et al., 1993; Giordano et al., 1999; Liste et al., 2001). In Malawi, astroviruses were detected by EIA in 1.9% of 786 inpatients and in 2.3% of 400 outpatients in a hospital-based study of pediatric gastroenteritis (Cunliffe et al., 2002). Gastroenteritis was more severe in children with HIV. In South Africa, astroviruses were found at a similar level in adults (3.1%) and children (4.8%) (Pager et al., 2002). In a study in Nigeria, astrovirus was detected by ELISA in 6.7% of diarrheal stools from young children compared to 5.7% of the control specimens (Pennap et al., 2002). Serotype 1 is the most common serotype worldwide, but the distribution of serotypes may vary by year and geographic location. Studies from developed countries usually report a limited spectrum of astrovirus serotypes (Noel et al., 1995; Glass et al., 1996), whereas studies from developing countries have identified multiple cocirculating serotypes (Cunliffe et al., 2002; Naficy et al., 2002; Walter et al., 2001).

Immunity

The mechanism of immunity to astrovirus is not yet clear. Symptomatic astrovirus infection is found primarily in young children and the elderly (Matsui and Greenberg, 2001). This suggests that antibodies acquired early in life protect from illness through adulthood but the immunity declines later in life (Matsui and Greenberg, 2001). Volunteer studies have

indicated that detectable levels of serum antibody to astrovirus protect individuals from diarrhea (Kurtz et al., 1979).

Modes of transmission

Astroviruses are transmitted through the fecal-oral route, as shown by human volunteer studies (Kurtz et al., 1979; Midthun et al., 1993). Contaminated food (Oishi et al., 1994; Walter et al., 2000) and water (Cubitt, 1991) have occasionally been linked to outbreaks associated with astrovirus, although it is not clear which proportion of cases can be attributed to this mode of transmission (Nadan et al., 2003). In a study in South Africa, genetically identical astrovirus strains were detected in wastewater and in hospitalized children, suggesting that astroviruses in the environment may pose a potential health risk in communities in which fecally contaminated water is used for domestic purposes and recreation (Nadan et al., 2003).

The capsid protein of astroviruses infecting different hosts is reportedly highly different and astrovirus infection appears to be species-specific (Matsui and Greenberg, 1996; Nadan et al., 2003). However, there are similarities among human astrovirus and feline and porcine astrovirus capsid proteins, suggesting that zoonotic transmission between pigs, cats and humans could occur even though no interspecies transmission has been documented so far (Nadan et al., 2003; Jonassen et al., 2001). Similarly to rotaviruses and noroviruses, astrovirus infections are detected more often in the winter months in temperate regions or the rainy season in more tropical climates (Matsui and Greenberg, 2001).

2.2.6 Rotaviruses

Rotaviruses are considered the single most important etiologic agent of severe acute diarrhea in infants and young children in both developed and developing countries (Kapikian et al., 2001). They were discovered in 1973 by thin-section EM of duodenal mucosa and were detected by EM in feces shortly thereafter (Flewett et al., 1973). Subsequent studies showed that rotaviruses are a major cause of severe infantile diarrhea throughout the world. In the developed countries, rotavirus infection is common but the mortality is low. Rotaviruses account for about 5-10% of all episodes of diarrhea in young children in the US, but they cause up to 50% of the cases of severe diarrhea in this population group (Kapikian et al., 2001; CDC, 1999). In the developing world, rotaviruses are the leading cause of diarrheal illness in infants and young children. Rotavirus infection is many times life-threatening because effective means of replacing fluids and electrolytes lost during severe diarrhea are not available as routinely as in the developed countries (Bern et al., 1994). In cases of diarrhea requiring treatment, rotavirus is usually the virus detected most frequently. In a hospital-based study in Bangladesh, rotaviruses were detected in 46% of 6,325 patients under 2 years of age with diarrhea for which treatment was sought (Black et al., 1980). In Egypt, rotaviruses accounted for 34% of cases of fatal or potentially fatal diarrhea in children under 18 months of age (Shukry et al., 1986). In contrast, rotavirus has not been detected in several studies investigating etiology of diarrhea in HIV-infected adults (Grohman et al., 1993; Gonzalez et al., 1998; Giordano et al., 1999) or children (Liste et al., 2000). However, in these studies, patients were hospitalized for reasons other than severe diarrhea.

Rotaviruses are members of the family *Reoviridae* (Kapikian et al., 2001). The virions are about 70 nm in diameter and have a characteristic double-layered protein capsid (Kapikian et al., 2001). The capsid consists of inner and outer layer, and within the inner layer is the third layer, the core, that encloses the genome (Kapikian et al., 2001). The rotavirus genome comprises of 11 segments of double-stranded RNA, which range from 0.6 to 3.3 kbp in size (Kapikian et al., 2001). Six of these genes are structural and four are nonstructural proteins (Kapikian et al., 2001). Rotaviruses are currently classified in seven groups, A-G (Bridger, 1994), and these can be further classified into subgroups and serotypes. Serogroups A, B, and C are associated with acute gastroenteritis in humans (Saif, 1990) and of these serogroup A rotaviruses are the most important etiologic agents of diarrhea causing an estimated 580,000 deaths annually worldwide (Murray and Lopez, 1997).

Rotaviruses are transmitted via the fecal-oral route, but airborne transmission has also been suggested (Kapikian et al., 2001). Rotavirus infections display a seasonal pattern, with peaks occurring during the cooler months of the year (Kapikian et al., 2001; Bishop et al., 1994). The incubation period is estimated to be less than 48 hours. Clinical symptoms can vary from mild to severe dehydrating diarrhea that may be accompanied by vomiting and fever (Kapikian et al., 2001). Rotaviruses can induce a chronic symptomatic infection in immunodeficient children (Saulsbury et al., 1980; Oishi et al., 1991) but they do not seem to be an important cause of diarrhea in HIV-infected adults (Grohmann et al., 1993; Gonzalez et al., 1998; Giordano et al., 1999). Several diagnostic methods are available for rotavirus detection, including EM, PAGE, latex agglutination, ELISA with polyclonal and/or monoclonal antibodies, and RT-PCR. Many animal rotaviruses can be easily grown in cell

culture whereas human rotaviruses are fastidious and have not been grown in cell culture until fairly recently (Sato et al., 1981). There is some cross reactivity between human and animal rotaviruses, as some human rotavirus strains have been shown to induce diarrhea in experimental animals (Mebus et al., 1976; Mitchell et al., 1977).

3. OBJECTIVES

The importance of viruses as etiologic agents of gastroenteritis in HIV-infected individuals, especially in Africa, is not well documented. Several viral agents have been suggested as causative agents of gastroenteritis in HIV-infected individuals but their role has not been confirmed. The genetic variability of novel enteric viruses, such as picobirnaviruses, has not been documented in an epidemiological investigation before. In the developing world, access to safe drinking water is a significant problem and as a short term solution, the so-called Safe Water System of household chlorination and storage in a protected container has been developed by PAHO and CDC to provide safer water in the communities. However, the effectiveness of the SWS in preventing viral diarrhea has not been assessed before, and furthermore, it has not been tested specifically with HIV-infected individuals.

To address the aspects mentioned above, the objectives of this study are to:

- Determine the prevalence of enteric viruses (PBVs, noroviruses, sapoviruses, adenoviruses, and astroviruses) in HIV-infected and uninfected individuals in Uganda.
- Investigate the possible association of these viruses with various factors, the main ones being acute gastroenteritis, HIV-status, and the water intervention, and other factors such as age, sex, presence of bacteria and/or parasites in stool, and seasonality.
- Identify the virus strains circulating in the study population.
- Characterize the genetic variability of PBVs in the study population.

4. MATERIALS AND METHODS

4.1 Study Population

This study was part of an investigation conducted by the Centers for Disease Control and Prevention (CDC) to evaluate the effectiveness of a Safe Water System (SWS) and cotrimaxole prophylaxis in the prevention of diarrhea among persons with HIV in Uganda. The study design and population are described in the report by the CDC (*manuscript in progress*). Briefly, this study was a prospective, randomized household level intervention in rural communities in Uganda. The study population consisted of 400 individuals with HIV registered with The AIDS Support Organization (TASO) in Tororo, Uganda, and their household members regardless of their HIV-status. The population was randomized into an intervention group, which received a Safe Water System, and a comparison group using normal water handling practices. Data on gastrointestinal symptoms and diarrhea incidence were recorded during weekly visits to participants' homes. Fecal samples were collected between January 2001 and February 2002 from anybody who had diarrhea and antimotility treatment was provided as needed.

4.2 Clinical specimens

In total, 414 fecal specimens collected from HIV-infected and uninfected individuals with gastroenteritis as well as samples from asymptomatic individuals to assess the baseline shedding of enteric viruses were included in this study for virological examination. The stool samples were stored at -20°C and kept frozen during shipment to UNC. The samples had

been analyzed previously for bacteria parasites and rotavirus group A by a latex agglutination test which results will be published elsewhere.

4.3 Viral RNA extraction and RT-PCR

The fecal samples were prepared as 10% extracts in PBS, extracted with chloroform to inactivate HIV possibly present in the samples, and stored at -80°C until tested. Viral nucleic acid was purified after lysis with guanidinium isothiocyanate and binding to silica-based membranes using a Qiagen™ stool extraction kit (QIAamp Viral RNA Mini Kit) following manufacturer's instructions (Qiagen, Valencia, CA). Initial experiments showed an excellent recovery of adenoviral DNA using these kits. Therefore, this procedure was used for extraction of both viral RNA and DNA. Samples were tested for PBV, norovirus, sapovirus, and astrovirus using a Qiagen™ One-Step RT-PCR kit. For PBV, a combination of four primers in a multiplex assay was used to detect the two known genetic clusters (China and Georgia) using primers Pico B23 and Pico B24 (China) and Pico B25 and Pico B43 (Georgia) as described by Rosen et al. (2000) (Table 5). Prior to reverse transcription (RT), the ds-RNA template ($2.5\ \mu\text{l}/\text{reaction}$) was denatured with primers ($37.5\ \text{pmol}/\mu\text{l}$ each) at 99°C for 8 minutes. The RT-PCR was performed in a final volume of $50\ \mu\text{l}$ consisting of 1 X Qiagen RT-PCR buffer, $0.4\ \text{mM}$ dNTP mix, 1 X Q-solution, $2\ \mu\text{l}/\text{reaction}$ enzyme mix containing reverse transcriptase and Taq polymerase, 20 U RNase inhibitor (Promega, Madison, WI) and RNase free water. RT was carried out for 1 hour at 42°C followed by 15 minutes at 95°C to activate the TaqGOLD (AmpliTaq Gold® DNA Polymerase). The PCR consisted of 40 cycles of 1 minute at 94°C , 2 minutes at 49°C , and 3 minutes at 72°C , followed by a single incubation of 7 minutes at 72°C , as described by Rosen et al., 2000.

Table 5. Oligonucleotide primers used in multiplex RT-PCR of PBV.

<i>Primer¹</i>	<i>Sequence</i>	<i>Target region</i>	<i>Amplicon size (bp)</i>
PicoB23(+)	CGG TAT GGA TGT TTC	Genomic segment 2	368 bp
PicoB24(-)	AAG CGA GCC CAT GTA	of 4-GA-91	
PicoB25(+)	TGG TGT GGA TGT TTC	Genomic segment 2	201 bp
PicoB43(-)	ART GYT GGT CGA ACT T	of 1-CHN-97	

¹ Rosen et al. 2000

RT-PCR of noroviruses and sapoviruses was performed using primer pairs MJV12/RegA and SR80/JV33, respectively, targeted to the RNA-polymerase region in ORF1 (Table 6). The RT-PCR mix for noroviruses consisted of 1X Qiagen RT-PCR buffer, 0.4mM dNTP mix, 50 pmol/ μ l of each primer, 2 μ l/reaction enzyme mix containing reverse transcriptase and Taq polymerase, 20 U RNase inhibitor (Promega), and RNase free water in a final volume of 25 μ l. The RT-PCR mix for sapoviruses was similar, but included 1X Q-solution and the primer concentration was 30pmol/ μ l. For noroviruses, the RT was performed for 60 minutes at 42°C followed by 15 minutes at 95 °C to activate the ampliTaq GOLD. The PCR consisted of 40 cycles of 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72 °C, followed by a final extension of 10 minutes at 72°C. The RT-PCR for sapoviruses was identical, except that the annealing temperature was 37 °C.

For astroviruses, primers Mon340 and Mon 348 (Belliot et al., 1997) targeted to the protease gene in ORF1a were used (Table 6). The RT-PCR was performed in final volume of 25 μ l consisting of 1 X Qiagen RT-PCR buffer, 0.4 mM dNTP mix, 1 X Q-solution, enzyme mix containing reverse transcriptase and Taq polymerase, 20 U RNase inhibitor (Promega) and

RNase free water. The reverse transcription consisted of 30 minutes at 42°C followed by 15 minutes at 95 °C. The PCR consisted of 40 cycles of 1 minute at 94°C, 1 minute at 50°C, and 1 minute at 72 °C, followed by a final extension step of 10 minutes at 72°C.

Primers Ad1 and Ad2 (Xu et al., 2000), targeted to the hexon gene, were used in adenovirus PCR (Table 6). The PCR mix consisted of 1.5 mM MgCl₂, 0.2mM PCR nucleotide mix (Roche, place, state), 30 pmol each primer, 2.5U/reaction Taq polymerase (New England Biolabs), 1X PCR buffer in pH 9.0, and RNase free water in a final volume of 50 µl. The PCR consisted of 5 minutes at 94°C followed by 40 cycles of 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72 °C, followed by a single incubation of 5 minutes at 72°C.

Table 6. Oligonucleotide primers used in (RT)-PCR of adenoviruses, astroviruses, noroviruses and sapoviruses.

<i>Virus</i>	<i>Primer</i>	<i>Sequence 5'→ 3'</i>	<i>Target region</i>	<i>Amplicon size (bp)</i>
Adeno	Ad1 (+) ¹	TTC CCC ATG GCI CAY AAC AC	Hexon	482
	Ad2 (-)	CCC TGG TAK CCR ATR TTG TA		
Astro	Mon340 (+) ²	CGT CAT TAT TTG TTG TCA TAT	Protease	289
	Mon348 (-)	ACA TGT GCT GCT GTT ACTA TG		
Noro	MJV12 (+) ³	TAY CAY TAT GAT GCH GAY TA	Polymerase	327
	RegA (-)	CTC RTC ATC ICC ATA RAA IGA		
Sapo	SR80(+) ⁴	TGG GAT TCT ACA CAA AAC CC	Polymerase	320
	JV33(-)	GTG TAN ATG CAR TCA TCA CC		

¹ Pring-Åkerblom et al., 1999.

² Belliot et al., 1997.

³ Vinjé et al., unpublished.

⁴ Vinjé et al., 2000.

Agarose gel electrophoresis

Following amplification of viral nucleic acid by PCR or RT-PCR, amplified DNA was visualized on 2% agarose gels stained with ethidium bromide. A 100 bp DNA ladder and known positive control samples were run with the samples to confirm the correct size of the bands.

Rotavirus group A

In addition, 50 RNA extracts from children under 5 years of age were tested for rotavirus group A at the DGS Division of Consolidated Laboratory Services in Richmond, VA (Dr. Vince R. Hill) by real-time RT-PCR using primers RV1/RV6 (Grinde et al., 1995) targeting gene 6 and Beg9/R4 (Le Guyader et al., 2000) targeting gene 9.

4.4 DNA Sequencing of RT-PCR products

All amplicons of appropriate size in agarose gels were confirmed by DNA sequencing. For this, the products were gelpurified using a Qiagen™ gel extraction kit when multiple bands were visible on the agarose gel or purified directly using Qiagen™ PCR purification kit when a clear single band was visible and sent for sequencing at the UNC DNA Sequencing Core Laboratory. Sequences were aligned using Clustal-W (v1.4). Particular strains were determined with BLAST search (www.ncbi.nlm.nih.gov/BLAST). For PBVs, sequences were also translated and deduced amino acid (AA) sequences of China and Georgia clusters were aligned separately with picobirnavirus prototype strains (FIG 7 and 8, respectively). Due to considerable genetic variation among the China strains, PBVs of the China cluster were considered positive only when 4 conserved AA regions were present. Phylogenetic

trees (UPGMA) were generated using Treecon software (Van de Peer and De Wachter, 1994) and confidence intervals of the internal nodes were calculated by 100 bootstrap analyses (FIG 10 and 11).

4.5 Statistical analysis

Results were analyzed using SPSS v11.0. (SPSS Inc., Chicago, IL). Chi-Square test was used to determine the association between different viruses and various factors, such as acute gastroenteritis (AGE), HIV-status, SWS (water intervention), age (adults versus children 5 years of age or younger), gender, and other pathogens (bacteria and parasites) in stool. Seasonality of the viruses was determined by comparing the prevalence of viruses in samples collected during the rainy season (Mar-May, Oct-Nov) and dry season (Dec-Feb, Jun-Sep).

5. RESULTS

5.1 Prevalence of enteric viruses

Enteric viruses were detected in 114 (27.5%) of the 414 samples analyzed. Of these samples, 68 (16%) tested positive for PBVs, 27 (6.5%) for noroviruses, 12 (2.9%) for sapoviruses, 21 (5.1%) for adenoviruses, and 9 (2.2%) for astroviruses (Table 7). Seventeen (4.1%) of the samples tested positive for more than one virus. Only two of the specimens tested positive for enteric adenoviruses, Ad 40/41. No rotaviruses were detected in the 50 specimens that were tested by real-time PCR.

Enteric viruses were detected in a similar frequency in the 317 samples from individuals with gastroenteritis and in the 52 samples from asymptomatic individuals (30.8% versus 26.8%, $p=0.553$). Samples from individuals with diarrhea were not more likely to have PBV (17.7% versus 13.5%, $p=0.692$), norovirus (6.3% versus 11.5%, $p=0.374$), sapovirus (2.8% versus 3.8%, $p=0.910$), adenovirus (4.7% versus 9.6%, $p=0.336$), or astrovirus (2.2% versus 1.9%, $p=0.981$) (Table 8).

At least one virus was detected in 40% of the 226 individuals with HIV and in 22.1% in the 136 individuals uninfected with HIV ($p=0.066$). Therefore, the difference in virus occurrence in HIV-positive and negative individuals is not quite significant ($0.05 < p < 0.1$). Samples from HIV-infected individuals were statistically not more likely than those from HIV-negative individuals to have PBV (19.5% versus 12.5%, $p=0.086$), norovirus (7.1% versus

6.6%, $p=0.876$), sapovirus (4% versus 1.5%, $p=0.178$), adenovirus (4.9% versus 4.4%, $p=0.620$), or astrovirus (1.8% versus 2.2%, $p=0.770$) (Table 9).

Overall, enteric viruses were detected in 31.4% of the 70 samples from individuals in the intervention group and in 26.4% of the 299 samples from individuals in the comparison group ($p=0.398$). Specimens from individuals using the SWS were not more likely to have PBV (20.0% versus 15.7%, $p=0.385$), norovirus (10.0% versus 6.0%, $p=0.233$), sapovirus (2.9% versus 3.0%, $p=0.946$), adenovirus (8.6% versus 4.0%, $p=0.111$), or astrovirus (1.4% versus 2.0%, $p=0.750$) (Table 10).

When comparing the prevalences of the viruses in HIV-infected and uninfected individuals with and without diarrhea, no association was found (Table 11). Enteric viruses were detected in 20% of the 198 samples collected from HIV-infected individuals with gastroenteritis and in 19% of the 37 samples from HIV-infected individuals without gastroenteritis ($p=0.903$). In HIV-negative individuals, enteric viruses were detected in 13.3% of the 113 samples from individuals with gastroenteritis and in 9.0% of the 23 samples from individuals without gastroenteritis. No association with diarrhea in either HIV-infected and uninfected individuals was observed with any of the individual viruses ($p>0.05$), except for adenoviruses (Table 11). They were detected in 1.8% (2/133) of samples from HIV-negative individuals with diarrhea and in 17.4% (4/23) of samples from HIV-negative individuals without diarrhea ($p=0.001$). This is likely to be an artifact due to small sample size and the fact that many non-enteric adenoviruses replicate in the gut even though they are not associated with diarrhea.

Additional information, such as the results of parasitological and bacteriological analysis and the demographics of the study population, was available on 371 samples. Of these specimens, 50 samples (13%) tested positive for at least one bacteria and 188 (51%) tested positive for at least one parasite. Of the 101 (27%) samples that tested positive for viruses within these 371 samples, 68 (67%) were mixed infections with bacteria and/or parasites. Specifically, 14 (14%) samples tested positive for viruses and bacteria, 51 (50%) tested positive for viruses and parasites, and 4 (4%) tested positive for viruses and both bacteria and parasites. The male-to-female ration was 1:2 and approximately 20% of the samples were obtained from children aged 5 years or younger. Samples were collected throughout the year, 167 (45%) of the samples being collected during rainy season (Mar-May, Oct-Nov) and 202 (55%) during the dry season (Dec-Feb, Jun-Sep). The prevalence of the viruses was not associated with gender or age, and there was no significant difference between the prevalence of viruses during rainy or dry season ($p>0.05$).

Table 7. Prevalence of adenoviruses, astroviruses, PBVs and caliciviruses in 414 stool specimens analyzed.

<i>Virus</i>	<i>Number of positive samples</i>	<i>% (N=414)</i>
Any virus	114	27.5
PBV	68	16
Caliciviruses	39	9.4
Norovirus	27	6.5
Sapovirus	12	2.9
Adenovirus [†]	21	5.1
Astrovirus	9	2.2

[†] Only two specimens were positive for Ad40/41

Table 8. Number (%) of specimens positive for different viruses in symptomatic and asymptomatic individuals (baseline).

<i>Virus</i>	<i>Asymptomatic N=52</i>	<i>Symptomatic N=317</i>	<i>P-value</i>
Any virus	16 (30.8%)	85 (26.8%)	0.553
PBV	7 (13.5%)	56 (17.7%)	0.692
Norovirus	6 (11.5%)	20 (6.3%)	0.374
Sapovirus	2 (3.8%)	9 (2.8%)	0.910
Adenovirus [†]	5 (9.6%)	15 (4.7%)	0.336
Astrovirus	1 (1.9%)	7 (2.2%)	0.981

[†] Only two specimens were positive for Ad40/41

Table 9. Number (%) of specimens positive for different viruses in HIV-infected and uninfected individuals.

<i>Virus</i>	<i>HIV-positive</i> <i>N=226</i>	<i>HIV-negative</i> <i>N=136</i>	<i>P-value</i>
Any virus	70 (40.0%)	30 (22.1%)	0.066 ¹
PBV	44 (19.5%)	17 (12.5%)	0.086 ¹
Norovirus	16 (7.1%)	9 (6.6%)	0.867
Sapovirus	9 (4%)	2 (1.5%)	0.178
Adenovirus ²	11 (4.9%)	6 (4.4%)	0.620
Astrovirus	4 (1.8%)	3 (2.2%)	0.770

¹Values between 0.05 and 0.1 were considered almost statistically significant

²Only two specimens were positive for Ad40/41

Table 10. Number (%) of specimens positive for different viruses in the intervention group and the comparison group.

<i>Virus</i>	<i>Intervention</i> <i>N=70</i>	<i>Comparison Group</i> <i>N=299</i>	<i>P-value</i>
Any virus	22 (31.4%)	79 (26.4%)	0.398
PBV	14 (20%)	47 (15.7%)	0.385
Norovirus	7 (10%)	18 (6.0%)	0.233
Sapovirus	2 (2.9%)	9 (3.0%)	0.946
Adenovirus ¹	6 (8.6%)	12 (4.0%)	0.111
Astrovirus	1 (1.4%)	6 (2.0%)	0.750

¹Only two specimens were positive for Ad40/41

Table 11. Prevalences of viruses in HIV-infected and uninfected individuals with and without acute gastroenteritis (AGE).

Virus	HIV positive			HIV negative		
	AGE n=198	No AGE n=37	p-value ^a	AGE n=113	No AGE n=23	p-value ^a
PBV	39 (20%)	7 (19%)	0.903	15 (13.3%)	2 (9.0%)	0.241
Noro	13 (6.6%)	3 (10.7%)	0.423	6 (5.3%)	3 (13%)	0.174
Sapo	8 (4.0%)	1 (3.6%)	0.905	1 (0.9%)	1 (4.3%)	0.209
Adeno ¹	10 (5.1%)	1 (2.7%)	0.734	2 (1.8%)	4 (17.4%)	0.001
Astro	4 (2%)	0 (0%)	0.448	2 (1.8%)	1 (4.4%)	0.443

^a Chi square test

¹ Only two specimens were positive for Ad40/41

5.2 PBVs

PBVs were detected in 68 (16.4%) of 414 samples analyzed (Table 7). Seven samples (1.7%) were co-infected with other enteric viruses (norovirus, sapovirus, or astrovirus). In this study, the prevalence of PBVs in samples obtained from symptomatic individuals was not statistically higher than the prevalence in samples from asymptomatic individuals at baseline. Fifty-six (17.7%) of the 317 samples collected from symptomatic individuals and seven (13.5%) of the 52 samples collected from asymptomatic individuals tested positive for PBV ($p=0.692$) (Table 8). Also, the prevalence of PBVs in HIV-positive individuals was not statistically higher than in HIV-negative individuals. Of the 226 samples from HIV-positive individuals, 44 (19.5%) tested positive for PBV, compared with 17 (12.5%) of the samples from HIV-negative individuals ($p=0.086$) (Table 9). This was considered a not quite significant difference. The prevalence of PBVs was not significantly associated with the

water intervention either. Of the 70 samples obtained from individuals in households having the SWS, 14 (20%) tested positive for PBV in comparison with 47 (15.7%) of the 299 samples obtained from individuals in the control group ($p=0.385$) (Table 10).

In both HIV-infected and uninfected individuals, the prevalence of PBVs was similar in asymptomatic and symptomatic persons. Of 37 stool specimens collected from asymptomatic persons with HIV at baseline, 7 (19%) tested positive for picobirnavirus compared with 39 (20%) of 198 acute diarrhea specimens during follow-up. Of the specimens from HIV-negative persons with AGE, 14 (12%) of 113 tested positive for picobirnavirus (Table 11). There was no significant difference between PBVs in adults (17.8%) and children (11.7%), $p=0.198$, and no seasonality was observed. The prevalence was 16.3% in the dry season and 16.7% in the rainy season.

5.2 Genetic variability of PBVs

Phylogenetic analysis showed that PBVs belonged to two different genogroups, 1-CHN-97 (China-like strains) and 4-GA-97 (Georgia-like strains), in relation to the primers used in RT-PCR. Most of the PBVs were China-like strains (51 China-like versus 15 of Georgia-like strains). Two samples were positive for both Georgia- and China-like strains. Deduced amino acid sequences of China- and Georgia-like strains were aligned separately with PBV prototype strains (FIG 2 and FIG 3, respectively). There was considerable variation among the deduced amino acid sequences of the China-like strains. As an arbitrary criterion for confirmation, PBVs of China cluster were considered positive only when four conserved amino acid regions were present (indicated with boxes I-IV in FIG 2). In contrast, the

deduced amino acid sequences of Georgia-like strains were almost identical. There was co-circulation of multiple genetically diverse China-like picobimaviruses with sequence diversity up to 60% (FIG 4). In contrast, Georgia-like PBVs were genetically very similar (maximum of 5% diversity), as previously noted (FIG 5).



Figure 2. Deduced amino acid sequences of China-like PBV strains. Because of the high nucleotide diversity, arbitrary conserved amino acid regions (boxed I, II, III, and IV) were used to confirm PBV origin of field strains.

4-GA-91 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico023 WIRDYLS WRGFEHVKETLIATWPHAKIVGGDTTKMDAHMRF AQIRIVFEI
 Pico065 WIRDYLS WRGFEHVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico085 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico106 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico019 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico455 WIRDYLS WRGFEHVKETLSATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico226 WIRDYLS WRGFEHVKETLSATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico234 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico235 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico240 WIRDYLS WRGFEHVKETLSATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico256 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico264 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico273 WIRDYLS WRGFEHVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico316 WIRDYLS WRGFEHVKETLSATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico353 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico360 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI
 Pico207 WIRDYLS WRGFEYVKETLIATWPHAEIVGGDTTKMDAHMRF AQIRIVFEI

Figure 3. Sequence variation of Georgia-like PBV strains in genomic segment 2.

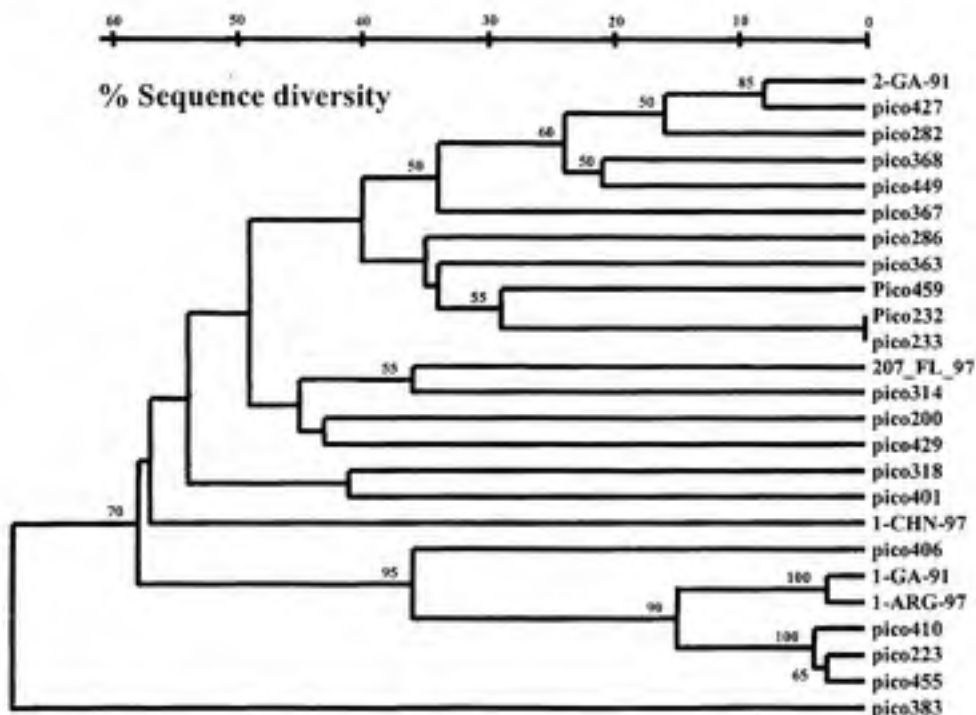


Figure 4. Predicted phylogenetic relationship among PBVs based on a 140 nt stretch of genomic segment 2 of China-like strains. Bootstrap values $\geq 50\%$ are shown.

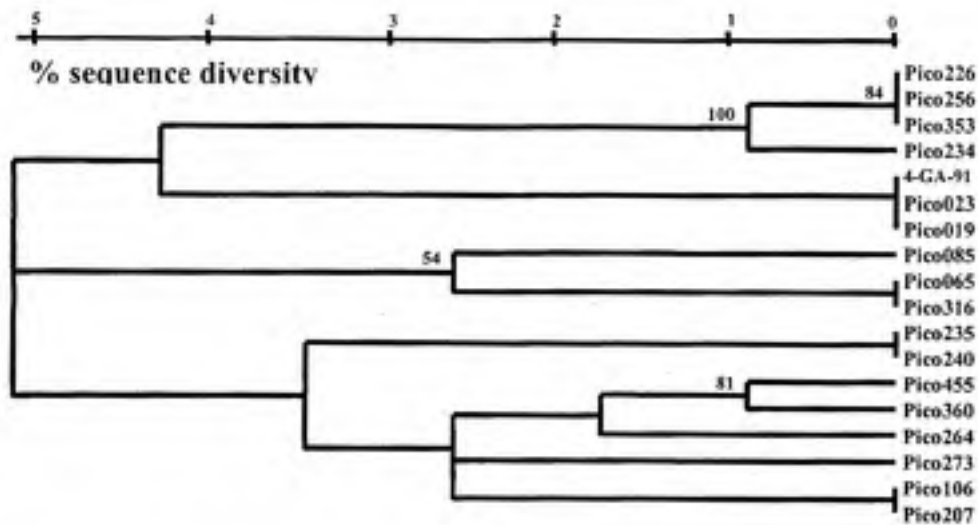


Figure 5. Predicted phylogenetic relationship among PBVs based on a 298 nt stretch of genomic segment 2 of Georgia-like strains. Bootstrap values $\geq 50\%$ are shown.

5.3 Noroviruses

Of the 414 samples analyzed, 27 (6.5%) tested positive for noroviruses (Table 7). The prevalence of noroviruses was not associated with AGE, HIV-status, or SWS. Of the 317 samples collected from symptomatic individuals, 20 (6.3%) tested positive for norovirus while six (11.5%) of the 52 samples from asymptomatic individuals tested positive for norovirus ($p=0.374$) (Table 8). These results that indicated that noroviruses were more prevalent in asymptomatic than symptomatic individuals, but this difference was not statistically significant ($p>0.05$). Sixteen samples (7.1%) of the 226 samples from HIV-infected individuals tested positive for norovirus compared with nine (6.6%) of the 136 samples from HIV-infected individuals ($p=0.374$) (Table 9). Noroviruses were not associated with SWS either. Of the 70 samples obtained from individuals in households having the SWS, seven (10%) tested positive for norovirus in comparison with 18 (6.0%) of the 299 samples obtained from individuals in the control group ($p=0.233$) (Table 10).

The prevalence of noroviruses in HIV-infected individuals with AGE was 6.6%, compared with 10.7% in HIV-infected individuals without AGE ($p=0.423$). In HIV-negative individuals, the prevalence of noroviruses in individuals with AGE was 5.3% and 13% in individuals without AGE ($p=0.174$) (Table 11). No seasonality was observed. The prevalence of noroviruses was 7.2% in the rainy season and 6.4% in the dry season ($p=0.775$). The prevalence of noroviruses in adults (6.5%) did not differ from that in children (7.8%) ($p=0.690$).

Phylogenetic analysis showed that most of the norovirus strains belonged to genogroup II and only four strains were genogroup I (FIG 6). Norovirus genotypes identified included Bristol (GII.4), Melksham (GII.2), Leeds (GII.7), Musgrove (GI.5), and Wortley (GII.1b).

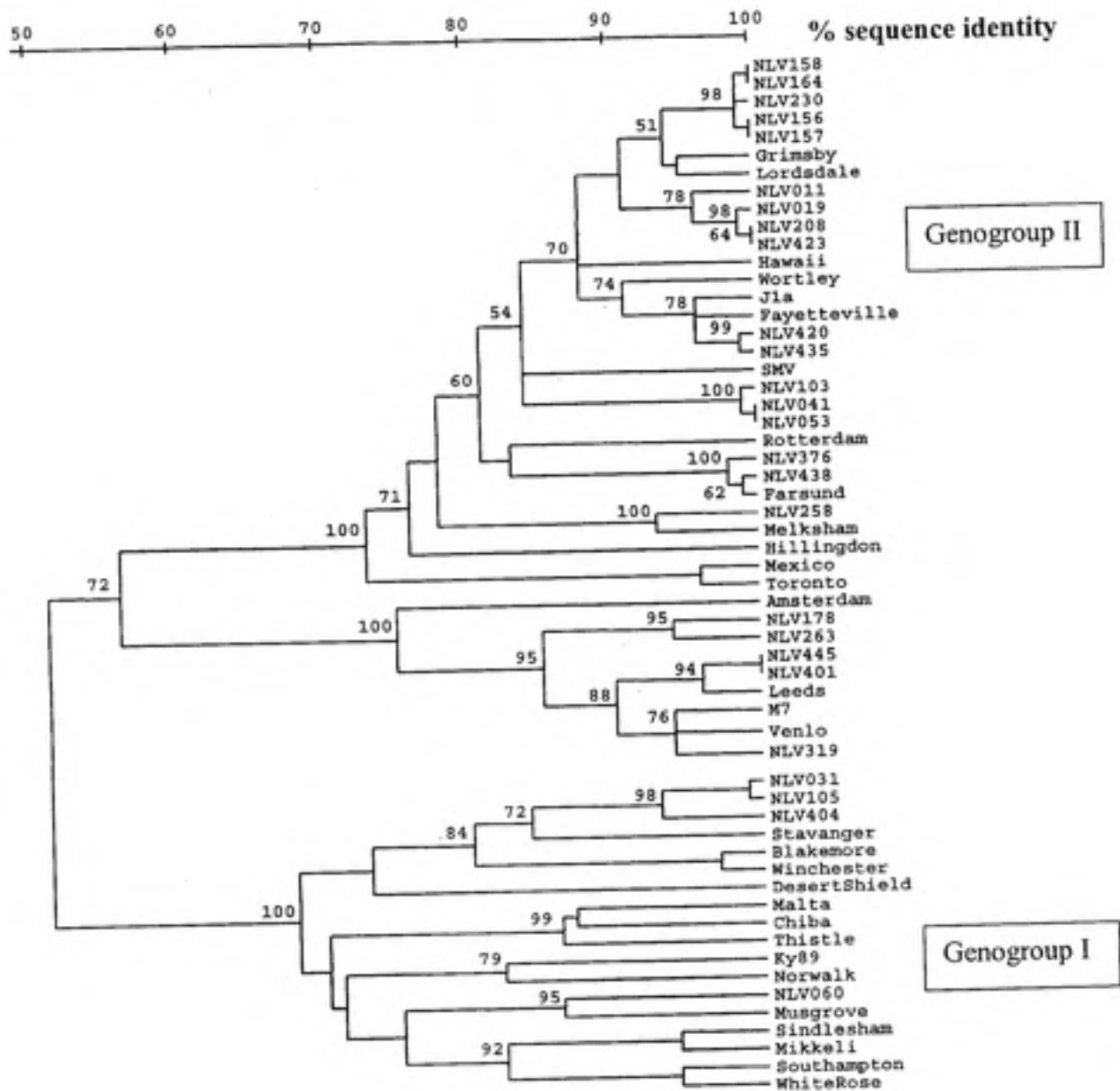


Figure 6. Dendrogram of norovirus strains detected in this study including the major reference strains. Phylogenetic relationships are based on a 145 bp region of the polymerase gene. Twenty-three (85.2%) of the 26 strains detected in this study were genotype GI.

5.4 Sapoviruses

Overall, 12 samples (2.9%) tested positive for sapoviruses (Table 7). Sapoviruses were not statistically more prevalent in individuals with AGE (2.8%) than in individuals without AGE (3.8%), $p=0.910$ (Table 8), nor in HIV-infected individuals (4%) than in HIV-negative individuals (1.5%), $p=0.178$ (Table 9). No difference was observed between the prevalence of sapoviruses in individuals using the SWS (2.9%) and the control group (3.0%), $p=0.946$ (Table 10). There was no statistically significant difference between the prevalence of sapoviruses in individuals with acute gastroenteritis, neither in HIV-infected individuals ($p=0.905$) nor in HIV-negative individuals ($p=0.209$) (Table 11). No seasonality (2.4% during rainy season and 3.5% during dry season, $p=0.547$) or association with age (2.6% in children under 5 years and 3.1% in adults, $p=0.824$) was observed either.

The sapovirus strains detected in this study included Parkville (7), Potsdam (2), Lyon (1), London/Van Deer Bijl (1), and London/Mexico.

5.5 Adenoviruses

Of the 414 specimens analyzed, 21 (5.1%) tested positive for adenovirus (Table 7).

No statistically significant association was found between adenoviruses in symptomatic individuals (4.4%) and asymptomatic individuals (9.6%) ($p=0.336$) (Table 8). Samples from HIV-infected individuals were not more likely to have adenovirus than those from HIV-negative individuals (4.9% and 4.4%, respectively, $p=0.620$) (Table 9). No statistically significant difference was observed between the prevalence of adenoviruses in individuals using the SWS (8.6%) and the control group (4.0%), $p=0.111$ (Table 10). The prevalence of

adenoviruses in HIV-infected individuals with AGE was 5.1%, compared with 2.7% in HIV-infected individuals without AGE ($p=0.734$) (Table 11). In HIV-negative individuals, the prevalence of adenoviruses in individuals with AGE was 1.8% (2/113) and 17.4% (4/23) in individuals without AGE ($p=0.001$). This is likely to be an artifact due to small sample size and the fact that many non-enteric adenoviruses replicate in the gut even though they are not associated with diarrhea.

Only two of the 21 adenoviruses detected were enteric adenoviruses Ad40/41. Both were detected from individuals with diarrhea. One was detected in a sample from an HIV-negative individual in the comparison group and the other in a HIV-positive individual in the intervention group.

5.6 Astroviruses

Nine (2.2%) of the 414 samples analyzed tested positive for astrovirus (Table 7). The prevalence of astroviruses was similar in symptomatic individuals (2.2%) and in asymptomatic individuals (1.9%), $p=0.981$ (Table 8), as well as in HIV-infected individuals (1.8%) and in HIV-negative individuals (2.2%), $p=0.770$ (Table 9). No difference in astrovirus prevalence was observed between the intervention group (1.4%) and the comparison group (2.0%), $p=0.750$ (Table 10).

The prevalence of astroviruses in HIV-positive individuals with AGE was 2.0% while no astroviruses were detected in HIV-positive individuals without AGE, $p=0.448$. In HIV-

negative individuals, the prevalence was 1.8% in symptomatic individuals and 4.4% in asymptomatic individuals, $p=0.443$ (Table 11).

Of the nine astroviruses detected, five were type 8, two were type 7, and one could be classified to genogroup A (contains types 1-5) and one to genogroup B (contains types 6-7).

5.7 Rotaviruses

No rotaviruses were detected by real-time RT-PCR.

6. DISCUSSION

6.1 Overview

The etiology of acute diarrhea in HIV-infected individuals in Africa is not well understood. This study describes the prevalence and molecular epidemiology of various enteric viruses (PBVs, noroviruses, sapoviruses, adenoviruses, and astroviruses) in HIV-infected and uninfected individuals in rural households in Uganda, and investigates their potential association with diarrhea, HIV-status and the SWS, as well as age, gender, and seasonality. In summary, at least one of these viruses was observed in 27.5% of the stool samples analyzed by RT-PCR. With 16.4% of samples positive, PBVs were detected most frequently, followed by 6.5% for noroviruses, 5.1% for adenoviruses, 2.9% for sapoviruses, and 2.2% for astroviruses. None of the 50 samples analyzed for rotavirus group A tested positive. No statistically significant association of the presence of enteric viruses with HIV-status was observed. The overall prevalence of enteric viruses, however, was higher in HIV-infected individuals (40%) compared to HIV-negative individuals (22%), but this association was not quite statistically significant ($p=0.066$). In addition, PBVs were the only viruses whose association with HIV-status was near being statistically significant (19.5% versus 12.5%, $p=0.086$). Viruses were not detected more often in individuals with diarrhea than in individuals without diarrhea, and no association was noted between the use of the SWS versus normal water handling practices and virus presence in stool. These results are different from those obtained in some earlier studies which reported that enteric viruses were detected more frequently in HIV-infected individuals with diarrhea than without diarrhea (Grohmann et al., 1993; Durepaire et al., 1995; Giordano et al., 1998, 1999). On the other hand, these findings are consistent with other earlier studies reporting no association between enteric

viruses and diarrhea in HIV-infected individuals (Thea et al., 1993; Gonzalez et al., 1998; Liste et al., 2000).

It is important to notice that the findings in the current study are limited by the relatively small sample size and the fact that a high number of samples tested positive for multiple pathogens including bacteria and/or parasites. In fact, 67% of the samples that tested positive for any virus were mixed infections with bacteria and/or parasites. In addition, the association with acute gastroenteritis was difficult to determine because individuals may have been shedding virus for a prolonged period of time. These and other reasons may have prevented us from seeing the possible associations between viruses in stool and various factors including acute diarrhea, HIV-status, and the SWS, and they are discussed in chapter 6.7. To be able to reliably assess these possible links, it is crucial to increase the sample size. The number of additional samples will be determined with statistical power calculations, based on the diarrhea rates and virus associations already observed.

6.2 PBVs

In previous studies, the prevalence of PBVs has varied between 20% in outbreaks of gastroenteritis (Pereira et al., 1988a) and 0% in sporadic cases of gastroenteritis (Liste et al., 2000) using PAGE for PBV detection. The prevalence of 16.4% found in the present study is at the higher end of this spectrum, even though we tested sporadic cases of gastroenteritis.

This relatively high rate of PBV detection also may be due to the use of RT-PCR, a relatively sensitive detection method, compared to PAGE of viral genome segments, a relatively insensitive virus detection method. RT-PCR is a more promising detection method

for PBVs because its sensitivity is relatively high, whereas the sensitivity of PAGE is limited and may vary depending on the method used to extract viral RNA. Furthermore, PBVs are usually present in low titers, which limits their detectability by relatively insensitive methods (Gallimore et al., 1995a; Giordano et al., 1998).

Picobirnavirus RNA was detected in 17.7% of individuals with diarrhea and in 13.5% of individuals without diarrhea, but these numbers are not statistically significant ($p=0.692$). These prevalences were slightly higher than those observed by Grohmann et al. (1993) when using PAGE (9% and 2%, respectively). Importantly, in that study an association was found between PBV and diarrhea ($p=0.017$). In two Argentinean studies, PBVs were detected in up to 14.6% of HIV-infected individuals with diarrhea but not in HIV-infected individuals without diarrhea, or in HIV-negative individuals (Giordano et al., 1998, 1999). The results of the present study did not support this finding, since the prevalence of picobirnaviruses was almost the same in HIV-infected and uninfected individuals with diarrhea, and PBVs were also detected in HIV-negative individuals (9-13.3%). Therefore, the association of PBVs with acute diarrhea is difficult to conclusively establish. Infected individuals may shed the virus for a prolonged period of time, up to 107 days (Gallimore et al., 1995a), and this may also have affected the results of Grohmann et al. (1993).

Performance of RT-PCR for PBVs

The picobirnavirus primers seemed to perform effectively in a One-Step RT-PCR format. When developing the primers, samples from two outbreaks of gastroenteritis in elder care

facilities were included as controls (Rosen et al., 2000). However, this is the first time that RT-PCR was used for PBV detection in an epidemiological study in which samples were not pre-selected for outbreaks. Even though the frequency of detection among different viruses was highest for PBVs, positive samples could have been missed. Importantly, sequence information was available only on a limited number of PBV isolates when these primers were designed (Rosen et al., 2000), and thus they may not be optimal. Now that additional sequence information can be obtained from the isolates detected in this study, the performance of the RT-PCR could be improved with new primers. Primers with higher annealing temperature would lead to more specific binding and decrease the unspecific background binding observed in this study. In addition, the relative sensitivity of PBV detection can be determined for the current and newly developed primer sets using both conventional single step and nested (or hemi-nested) RT-PCR.

Genetic variability of PBVs

In the samples from the elder care patients of a previous study, only China-like PBVs were detected (Rosen et al., 2000). Similarly, majority of the PBVs detected in this study were China-like strains (85%, 51/58). One explanation could be that the primer set B23/B24 is less widely reactive, and thus it captures only a limited number of viruses in which the target area is highly conserved. The PBV RT-PCR was done in multiplex and it is possible that the selected amplification conditions may have affected the performance of the primers.

However, to ensure accurate results, before screening the samples the RT-PCR assay was tested with control samples known to be positive for China-like PBV and Georgia-like PBV, respectively. The performance of two-step RT-PCR was compared with that of one-step RT-

PCR, in both multiplex and for each primer pair separately. Since the performance of the two-step assay was comparable to that of one-step RT-PCR in multiplex format, we decided to screen the samples using multiplex one-step RT-PCR.

When deduced amino acid sequences of China- and Georgia-like strains were aligned separately with PBV prototype strains, considerable variation was noted among the deduced amino acid sequences of the China-like strains. Subsequently, PBVs of the China-cluster were considered positive only when four conserved amino acid regions were present. By setting this criterion we wanted to make sure that there are no false positives. On the other hand, this restrictive definition of China-like strains may have led to underestimation of the prevalence of these viruses. In contrast, the deduced amino acid sequences of Georgia-like strains were almost identical, indicating that the genetic variability among the Georgia-like strains was minimal. There was co-circulation of multiple genetically diverse China-like picobirnaviruses with sequence diversity up to 60%. On the contrary, Georgia-like PBVs were genetically very similar (maximum of 5% diversity).

It is hard to say why the genetic variability among Georgia-like strains is so limited compared to that of China-like strains. We don't know how long and in which geographic regions these viruses have been circulating. The populations in which China-like strains have circulated may have been different from those in which Georgia-like strains have been circulating, allowing more recombination to occur during the evolution of the viruses and thus resulting in greater genetic variability. China-like strains have been detected in Florida, Georgia, Argentina, and Venezuela, whereas after the initial finding of the Georgia-like

isolate in the HIV-patient in Atlanta, they have been detected only in Uganda. This doesn't mean that Georgia-like strains circulate only in the US, but the RT-PCR system may be favoring detection of China-like strains and a system using a new primer set with perhaps broader as well as more sensitive detection could give different recovery rates.

In addition, positive-sense primers B23 and B25 differ from each other by only two nucleotides, and it was demonstrated that if primers B23 and B43 were mixed in one reaction and B25 and B24 in another, they produced amplicons that were the expected size of the China-cluster and Georgia-cluster, respectively. This led us to suspect that the double infections with China- and Georgia clusters might not be infections involving two different strains but instead be artifacts due to the primers. However, as the description of the primers in Table 5 shows, the target regions of the B23 and B25 are almost the same, and it is primers B24 and B43 that define how long or short the amplicon is going to be. So the two primer sets are amplifying about the same stretch of the genome, but the amplicon with primer pair B23/B24 is longer. The genetic variability between the amplicons of China- and Georgia-cluster, when looking at the deduced amino acid sequences, was significant. Thus it is unlikely that the similarity of the positive sense primers was interfering with the results of double infections. Due to these reasons the suspected possibility that the two different primer pairs could amplify the same virus and produce two different amplicons was not experimentally tested.

Since no molecular methods have been used in epidemiological investigations of PBVs before, there is limited information available on the genetic variability of PBVs.

After more studies investigating the prevalence of PBVs in various parts of the world using molecular methods become available it will be easier to evaluate the genetic diversity of PBVs around the world.

6.3 Human caliciviruses

Noroviruses were detected in 6.5% and sapoviruses in 2.9% of the samples by RT-PCR. This prevalence is relatively low, since RT-PCR as a detection method is sensitive and the primers used in this study were widely reactive. In comparison, noroviruses were detected in 20.2% of 873 and sapoviruses in 9.3% of 776 stool samples from young children with diarrhea by RT-PCR in Finland (Pang et al., 2000). These prevalences are considerably higher than the prevalence of noroviruses and sapoviruses in children (< 5 year of age) in our study (7.8% and 2.6%, respectively). In contrast, the prevalence of noroviruses detected in HIV-infected individuals in Atlanta by EM was low, 2% in individuals with diarrhea and none in individuals with no diarrhea (Grohmann et al. 1993). In two studies in Venezuela, no Norwalk virus was detected in HIV-infected and uninfected patients by EIA (Liste et al. 2000; Gonzalez et al., 1998), but the EIA used in these studies was specific for only Norwalk virus and therefore these results can not be extrapolated to being representative of all noroviruses. The prevalence of noroviruses in the current study was similar to that obtained in a study in Zaire, where noroviruses were detected in 5.1% of 198 stool specimens from HIV-infected and uninfected hospitalized adults by EM (Thea et al., 1993). Furthermore, like in our study, noroviruses were not associated with acute or chronic diarrhea or HIV-status. In a study in Kenya that investigated the prevalence of enteric viruses in children with acute gastroenteritis in outpatient clinics, noroviruses were detected in 2.2% of 1,431 stool

samples by EIA during 1991-1994 (Nakata et al., 1998). Sapoviruses were detected in 1% to 4% of the samples each year, while Norwalk virus and Mexico virus were rarely detected or not detected at all. Again, the explanation for this might be that the EIAs used were too specific or lacked sensitivity compared to RT-PCR.

During the last 8 years it has become clear from many outbreak studies that GII noroviruses, especially those related to Bristol and Lordsdale strains, are the predominant viruses associated with outbreaks of gastroenteritis in several countries, including Canada, Finland, Japan, South Africa, the Netherlands, and the US (Noel et al., 1997; Nakata et al., 1998; Wright et al., 1998; Levett et al., 1996; Pang et al., 2000, Vinjé et al., 1997, Wolfaardt et al., 1997). Our results are in concordance with these since 85% of the 27 noroviruses were GII and the majority were genetically similar to Lordsdale virus. The relatively low prevalence of HuCVs in this study compared with some previous studies may be due to differences in the settings of the studies, differences between the geographic regions, and different living conditions. It is important to notice that our data is based on sporadic cases of gastroenteritis, and thus the results are not comparable with studies in which data from outbreaks are used. In rural Uganda, living conditions may have been less crowded and thus the typical secondary spread of noroviruses may have been lower. In addition, there is evidence that some population groups are less susceptible to norovirus infection due to genetically mediated differences in the receptor binding sites on the epithelial cells (Huang et al., 2003; Hutson et al., 2002). It has been shown that even adults in developing countries, like Kenya, show a low prevalence of antibody to NV (Nakata et al., 1998), which could be explained by a difference in susceptibility to infection in different races. The climate in Kenya is similar to

that of Uganda in that they both have two rainy seasons each year. During the 4-year study period in Kenya, the rate of sapovirus infections in children was equal in dry and rainy seasons, which is in concordance with the observations in our study.

6.4 Adenoviruses

Adenoviruses were detected in 5.1% of the samples by RT-PCR, and sequence analysis revealed that only two (0.5%) were enteric adenoviruses Ad 40/41. These results are comparable with those from a study in Zaire, in which enteric adenoviruses were detected in only two (1.0%) of 198 stool specimens from HIV-infected and uninfected individuals by ELISA (Thea et al., 1993). Relatively low prevalence of adenoviruses has been reported in other studies too. In a study in Kenya investigating the prevalence of enteric viruses in children with acute gastroenteritis in outpatient clinics, enteric adenoviruses were detected in only 1.4% of 1,431 stool samples by EIA (Nakata et al., 1998). In Venezuela, the prevalence of adenoviruses in separate studies has been 0% (Liste et al., 2000), 4.0% (Gonzalez et al., 1998) and 5.6% (Giordano et al., 1999). Enteric adenoviruses have been found rarely or not at all (Giordano et al., 1999; Grohmann et al., 1993; Thea et al., 1993). However, the prevalence of enteric adenoviruses detected in the present study is very low when compared with a study in which PCR was used: enteric adenoviruses were detected in 6.3% of 811 samples from children with diarrhea under 2 years of age by PCR in Finland (Pang et al., 2000). However, enteric adenoviruses are detected with higher frequencies in young children and the study population in our study was predominantly adults (80%).

In the current study, adenoviruses were not associated with diarrhea, HIV-status, or the water

intervention. Similar findings have been obtained previously in studies with HIV-infected and uninfected patients (Thea et al., 1993; Gonzalez et al., 1998; Giordano et al., 1999). However, even though Grohmann et al. (1993) did not detect enteric adenoviruses, non-group F adenoviruses were detected more often in diarrhea specimens (9%) than non-diarrhea specimens (3%), $p=0.047$.

6.5 Astroviruses

The prevalence of astroviruses was the lowest of all viruses, except for rotaviruses that were not detected at all. Astroviruses were detected in 2.2% of samples, and like the other viruses, they were not associated with acute gastroenteritis, HIV-status, the SWS, or the other factors. These results are similar to the prevalence of 1.9% in inpatients and 2.2% in outpatients in 2-year hospital based study of astrovirus infection in children with AGE in Malawi (Cunliffe et al., 2002). On the other hand, the prevalence was lower than that observed in other studies. Grohmann et al. (1993) detected astroviruses in the US more often in individuals with diarrhea than without diarrhea (12% versus 2%, $p=0.003$), while Giordano et al. (1999) detected astroviruses at similar frequency in Argentina in HIV-positive individuals with diarrhea (4%) and in HIV-positive individuals without diarrhea (5.3%) ($p>0.05$). In a study in Venezuela, 6% of samples from HIV-infected and uninfected individuals were positive for astrovirus by EIA (Liste et al., 2000). All were found in HIV-infected children but were equally common in patients with and without diarrhea. In contrast, Gonzalez et al. (1998) did not detect any astroviruses by RT-PCR in 125 stool samples from HIV-infected individuals with and without diarrhea in Venezuela. In a study in Finland in which RT-PCR was used,

astroviruses were detected in 8.8% of 811 samples from young children with diarrhea (Pang et al., 2000). RT-PCR was used also in a study in Brazil, and astroviruses were detected in 2.8% of 351 samples (Cardoso et al., 2002). These results are similar to ours.

Serotype 1 astroviruses have been reported to be the most common worldwide (Matsui and Greenberg, 2001) and studies in developing countries have identified multiple astrovirus serotypes cocirculating in the community (Cunliffe et al., 2002, Naficy et al., 2002; Walter et al., 2001). In our study, astroviruses in both genogroups A and B were detected, with HAstV8 and HAstV7 most commonly found. This is noteworthy because serotype 8 is not detected very commonly (Nadan et al., 2003; Glass et al., 1996; Monroe, 1996). However, HAstV-8 seems to be more frequent in Africa (Nadan et al., 2003; Taylor et al., 2001) and in Barcelona, Spain (Guix et al., 2002). In a 2-year hospital-based study in Malawi, six astrovirus serotypes were detected, the most common ones being HAstV1, HAstV2, and HAstV3 (Cunliffe et al., 2002). Of the 24 astroviruses isolated from fecal specimens from children in South Africa, 63% were HAstV1, 13% were HAstV3, and HAstV5, HAstV6 and HAstV8 made up 8% each (Nadan et al., 2003). Since primers Mon267/270 (Sakon et al., 2000) targeting ORF2 are better suited for serotyping, confirmation of the rather unusual serotypes, HAstV7 and HAstV8, detected in this study using these capsid primers is suggested.

6.6 Rotaviruses group A

Rotaviruses group A are one of the most common etiologic agents of acute diarrhea in developing countries with peaks during the cooler winter season and lower incidence during

the warmer season. Thus, rotavirus incidence can be used as an indication of whether or not samples were collected during an unusual sampling period. Their absence in fecal specimens from individuals with diarrhea can be considered an indication of an atypical sampling period. To assess if the samples in this study were collected during an unusual period, a subset of samples were tested for rotavirus group A by RT-PCR. Fifty specimens from children under 5 years or younger were selected and sent for rotavirus analysis by real-time PCR. All samples tested negative for rotavirus. This is surprising because rotavirus infection is very common in children under 5 years of age, and especially in developing countries, rotavirus is a leading cause of non-bacterial gastroenteritis. They have been detected in up to 10.1% of samples from HIV-infected adults in Zaire (Thea et al., 1993). In contrast, several studies have failed to detect rotavirus in HIV-infected individuals (Giordano et al., 1999; Gonzalez et al., 1998; Liste et al, 2000; Grohmann 1993). In Kenya, rotaviruses have been detected in 22.2% of 1,431 stool samples by EIA in (Nakata et al., 1998). The absence of rotaviruses suggests that the period in which the specimens were collected is not representative of the "real" burden of these viruses circulating in Uganda.

6.7 Association with AGE, SWS, and HIV

In this study, no statistically significant association was observed between any of the enteric viruses and gastroenteritis. There are several factors that may have influenced this. First, a parasite was detected in 45% and a bacterial agent in 12% of the samples tested. Sixty-seven percent of the samples positive for enteric viruses also were positive for bacteria and/or parasites, indicating co-infections, and 15% tested positive for multiple enteric viruses. Parasites in particular, such as *Cryptosporidium*, are known to cause chronic diarrhea in

HIV-infected individuals (Sharpstone et al., 1996). Second, some patients may have shed viruses for prolonged periods of times, and thereby masked the true cause of their diarrhea. Their diarrhea may have been caused by a bacterium, a parasite or another virus. For instance, several studies have shown that patients may shed PBVs for a long time, such as 45 days (Giordano et al) and up to 107 days (Gallimore et al., 1995). Shedding of norovirus may continue up to 3 weeks after onset of illness, and sapovirus up to 2 weeks (Rockx et al., 2002). Third, it is unknown how long patients in the baseline study had been without diarrhea before the specimen was collected. Thus, they could still be shedding virus from previous episodes of diarrhea. And importantly, the relatively small sample size may have prevented the observation of the potential associations between etiological agents and diarrhea. To be able to better address the question whether these enteric viruses are associated with diarrhea, HIV-status or SWS in individuals in Uganda, analysis of more specimens is pending. The number of samples needed to reliably show possible associations will be determined by statistical power calculations based on virus positivity and diarrhea prevalence. Finally, HIV itself may cause gastrointestinal symptoms thereby confounding possible associations between diarrhea and other viruses as well as other microbes (Pollock et al, Liste et al., 2000).

6.8 Conclusions

Overall, limited information is available on the prevalence of enteric viruses in HIV-infected individuals in Africa, and sensitive molecular methods have not been commonly used for the wide range of enteric viruses capable of causing diarrhea and other AGE symptoms. This study is the first in which prevalence of enteric viruses and genetic variability of PBVs is

described among HIV-infected and uninfected individuals in Africa using state-of-the-art molecular detection methods. Additionally, this is the first study evaluating the effectiveness of the SWS in preventing viral diarrhea in HIV-infected individuals.

In this study, the prevalence of enteric viruses except for PBVs was relatively low, considering that RT-PCR was used for screening the samples. Importantly, no rotaviruses were detected even though they are the leading cause of non-bacterial gastroenteritis in developing countries. In previous studies, the prevalence of enteric viruses has varied depending on the method used to detect viruses, geographic location, and the clinical and epidemiological features of the study population. Several factors, such as race, immunological status, nutritional status, genetic factors of the study population and environmental conditions may impact the susceptibility of individuals to infection and the ability of the virus to spread in the population. Rotaviruses are usually common pathogens in individuals with diarrhea in developing countries, and their absence in this study suggests that the sampling period (Jan 2001-Feb 2002) may have been unusual. No other data on the prevalence of rotavirus in other East African countries at the same time period were available to assess if the absence of rotavirus could be confirmed.

Enteric viruses, particularly PBVs, were commonly found in both symptomatic and asymptomatic individuals, regardless of their HIV-status. The effectiveness of the SWS in preventing diarrhea has been shown in previous studies (Quick et al., 1997, 1999), but its usefulness in preventing viral diarrhea has not been investigated before. In this study, viruses were not detected more commonly in the comparison group than in the intervention group.

No seasonality was observed, and the viruses were not more common in adults than children. The relatively small sample size and the high frequency of mixed infections, among other factors, may have masked any potential associations.

7. CONCLUSIONS

- Enteric viruses were detected in 27.5% of the samples. The prevalence of viruses other than picobirnaviruses was relatively low.
- None of the viruses could be linked with
 - Gastroenteritis
 - HIV-status
 - The water intervention (Safe Water System)
 - Other factors, such as age, sex, presence of bacteria and/or parasites in stool and seasonality.
- Possible reasons for not observing associations include:
 - Small sample size
 - Low frequency of detection
 - High number of mixed infections
 - Possible prolonged shedding of viruses.
- The absence of rotavirus suggests that the sampling period may have been unusual.
- Testing of more specimens is pending to assess if these viruses are relevant etiologic agents of diarrhea or simply commensals.
- This is the first report on the molecular epidemiology of picobirnaviruses in humans.
- Picobirnaviruses are found in individuals with and without diarrhea regardless of HIV-status in Uganda.
- The picobirnavirus primers performed effectively in One-Step RT-PCR format.
- Picobirnaviruses could be grouped two genogroups, Georgia and China.
 - Majority of PBVs belonged to China-cluster
 - The genetic variability was high within China-cluster and low within Georgia-cluster.

8. SUGGESTED FUTURE STUDIES

- Increase sample size based on statistical power calculations to better assess the relationship between PBVs and diarrhea
 - Samples from Uganda and Malawi
- Confirmation by PAGE of a panel of samples that tested positive for PBV with RT-PCR
- Picobirnaviruses
 - Further optimization of RT-PCR
 - Molecular epidemiology and genetic variability
 - Cell culture system
 - Investigation of the immunological response to PBV infection
- Evaluation of the Safe Water System in preventing viral diarrhea
 - Include HIV-infected individuals

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