Viral diarrhoea in children under two years of age in Dar es Salaam, Tanzania

Clinical and Molecular epidemiology

Sabrina Moyo

Dissertation for the degree philosophiae doctor (PhD)

at the University of Bergen

2014

Dissertation date: 08.12.2014

Scientific environment

This study was done in collaboration between University of Bergen, Norway and Muhimbili University of Health and Allied Sciences (MUHAS), Tanzania. The University of Bergen provided the funding for the work.

Acknowledgement

This work has been possible with the help, contributions, encouragements and support of a large number people. First and foremost I would like to thank my main supervisor Nina Langeland for believing in my abilities when I met her during the year of my specialisation in Microbiology (2006-2007), for that is when I started the journey to do research. She encouraged me not only to do research but also to pursue PhD. Words are not enough to express how thankful I am!. Thank you for believing in me and I highly appreciate your support and your guidance throughout the years of my training.

I also thank all my co-supervisors; Kurt Hanevik, thank you for being a good role model in research, for your contribution in my research studies and for always being there whenever needed help!;

Bjørn Blomberg for your guidance and support through different stages of this work I appreciate your support and contribution of this work;

Prof. Samuel.Y Maselle, thank you for being a mentor to me and advising me in my academic life. You have always encouraged me; I appreciate the guidance and support I received from you. Without the contribution from all my supervisors this work wouldn't be possible.

I am grateful to all members of the team who worked on this project of diarrhoea diseases in children; Øyvind Kommedal at the Department of Microbiology, Haukeland University Hospital and Vainio Kirsti at Division of Infectious Disease Control, Norwegian Institute of Public Health, Oslo, Norway. You have contributed ideas in making sure that the project goes well. Special thanks to Svein Arne Nordbø of Department of Microbiology, University Hospital of Trondheim for his collaboration in this work.

I wish to thank Mary Ngange and Elisabeth Manda from the Section of Microbiology of Central Pathology Laboratory, (MNH) and Steinar Sornes from the department of clinical science (UiB) for their technical support.

I thank all my office mates for being so good to me, Marit Gjerde Tellevik, Astrid Spjelnæs and Christel Gill Haanshuss) during the last months of my work at the Center for Tropical Infectious Diseases at the Haukeland University Hospital, Bergen,

I thank the nurses who helped data collection at the hospitals where the study was conducted.

I thank all my friends here in Bergen for their good company, especially Dr. Hawa Nalwoga, thank you for being a good friend and a sister to me.

I am grateful to all mothers/guardians of children who consented to participate in this study, without whom this work would have been impossible.

I wish to acknowledge the former and the current head of department Prof M.I Matee and Prof Said Aboud and other members of department of Microbiology and Immunology Prof F.S Mhalu, and Prof E.F. Lyamuya, for allowing me to come and further my education here in Norway.

I thank my parents Angelina Sepeku and Bernard Sepeku and my siblings Josephine and Edmund Sepeku for their support during all the period of my study.

I give special thanks to my dear husband Joshua, thank you for encouraging me to take this opportunity to advance my carrier, for your support and for your patience during my training.

Most of all, I give glory and praise to my lord and saviour Jesus Christ for the grace and comfort through all the hardships that I have gone through during all the time of my studies.

Summary

Introduction

Diarrhoeal disease is one of the leading causes of illness and death in children below five years, especially in developing countries. It is estimated to cause 0.7 million deaths among children younger than 5 years of age, most of which occur in children below two years age. Viruses such as rotavirus, norovirus and adenovirus are known to be major causes of infectious diarrhoea.

Objectives

This study was done investigate the molecular epidemiology of rotavirus, norovirus and adenovirus and their association with demographic and clinical parameters including HIV and in children under two years of age in Dar es Salaam, Tanzania.

Methods

This was a case control study conducted in Tanzania between August 2010 and July 2011. Cases were children admitted due to diarrhoea at three major hospitals of Dar es Salaam. Controls were children without history of diarrhoea for one month prior the study. These were either attending child health clinics for immunisation and growth monitoring or admitted due to diseases other than diarrhoea. Questionnaires were used to obtain demographic and clinical information. Stool specimens were collected from each study participant. We used ELISA methods for detection of rotavirus and adenovirus from stool specimens. Real time PCR was used for detection of norovirus. PCR followed by sequencing were used for typing all the three viruses.

Results

Viruses were detected in nearly half (48.8%) of the cases and in 17.5% of the controls. Dual viral infection was found in 7.4% and 8.1% in cases and controls respectively. Among cases, rotavirus was the most common detected virus (32.5%) followed by norovirus (18.3%), and adenovirus (3.5%). In controls the prevalence of rotavirus, norovirus and adenovirus was 7.7%, 9.2% and 2.4% respectively. The peak

prevalence of virus infection in cases was seen in the age group 7-12 months, 52.0%. Rotavirus G1P[8] predominated in cases and controls. Norovirus GII.4 predominated both in cases (87.9%) and controls (56.5%). Enteric adenoviruses contributed 50% in cases and 46% in controls. Rotavirus was more prevalent during cold than hot months during the study period. Rotavirus infection was significantly lower in HIV infected (15.4%) than HIV uninfected children (55.3%). There were no significant associations between HIV and either norovirus or adenovirus infection. There were no significant differences observed between viral infection and gender or parent level of education.

Conclusion

Viruses contribute to a high burden of diarrhoea in hospitalised children of Dar es Salaam, Tanzania. We found a high frequency of asymptomatic carriage of diarrhoea-associated viruses in healthy children; these children may be a source of infection in the community. We observed a large genetic diversity of the three viruses investigated in this study. Pre-rotavirus vaccination data showed predominance of rotavirus genotype G1. Our study suggests that rotavirus may not be an opportunistic pathogen in children infected with HIV.

List of Publications

Paper I

Moyo SJ, Blomberg B, Hanevik K, Kommedal O, Vainio K, Maselle SY, Langeland N. Genetic diversity of circulating rotavirus strains in Tanzania prior to the introduction of vaccination. N. *PLoS One.* 2014; **20**;9(5)

Paper II

Moyo S, Hanevik K, Blomberg B, Kommedal O, Vainio K, Maselle S, Langeland N. Genetic diversity of norovirus in hospitalised diarrhoeic children and asymptomatic controls in Dar es Salaam, Tanzania. *Infect Genet Evol*. 2014; 26:340-7.

Paper III

Moyo SJ, Kurt Hanevik, Bjørn Blomberg, Oyvind Kommedal, Svein Arne Nordbø, Samuel Maselle, Nina Langeland. Detection and molecular characterisation of human adenovirus in diarrhoeic children of Dar es Salaam, Tanzania; a case control study (Submitted)

Abbreviations

cAMP	Cyclic-AMP
CBC	Community based controls
DNA	Deoxyribo Nucleic Acid
ELISA	Enzyme Linked Immunosorbant Assay
EM	Electron Microscope
HAdV	Human adenovirus
HBC	Hospital based controls
HIV	Human Immunodeficiency Virus
HBGAs	Histo-blood group antigens
IgA	Immunoglobulin A
LA	Latex agglutination
MUHAS	Muhimbili University of Health and Allied Sciences
MNH	Muhimbili National Hospital
ORF	Open reading frame
ORS	Oral Rehydration Solution
PCR	Polymerase chain reaction
RT-PCR	Reverse Transcriptase - Polymerase chain reaction
RNA	Ribo Nucleic Acid
RdRp	RNA–dependent RNA polymerase
RRV-1	Rhesus rotavirus vaccine candidate
VP	Viral proteins
VLPs	Virus-like particles
WHO	World health organization

Table of contents

Scientific environment………… 2

Acknowledgement………… 3

Summary………… 5

List of publications………… 7

Abbreviations………… 8

1. Introduction………… 11

1.1 Burden of diarrhoeal disease………… 11

1.2 Risk factors for viral diarrhoea………… 12

1.3 Seasonality of viral diarrhoea………… 12

1.4 Viral causes of diarrhoea………… 13

1.4.1 Historical background………… 13

1.4.2 Rotavirus………… 14

1.4.3 Norovirus………… 18

1.4.4 Adenovrius………… 22

1.5 Mechanisms of virus induced diarrhoea………… 24

1.6 Management of viral diarrhoea………… 25

1.6.1 Treatment of viral diarrhoea………… 25

1.6.2 Preventive measures………… 25

2. Aims of the thesis………… 26

3. Rationale of the study………… 27

4. Materials, methods and methodological considerations………… 27

4.1 Study design and study sites………… 27

4.2 Study population………… 27

4.3 Specimen collection and storage………… 28

4.4 Study subjects………… 28

4.5 Laboratory methods………… 30

4.5.1 Extraction of total nucleic acid………… 30

4.5.2 Detection of rotavirus (Paper I)………… 31

4.5.3 Detection of human adenovirus (Paper III)………… 32

4.5.4 Detection of norovirus (Paper II)………… 32

4.5.5 PCRs for typing rotavirus and adenovirus………… 33

4.5.6 PCRs for genotyping of norovirus………… 34

4.5.7 Sequencing and phylogenetic analysis of rotavirus, noro- 35

virus and adenovirus……………………………………………
4.6 Statistical analysis……………………………………………………….. 35
4.7 Ethical consideration………………………………………………………. 35
5. Summary of results………………………………………………………… 37
5.1 Characteristics of the study population………………………………........... 37
5.2 Overall prevalence of viruses……………………………………………… 37
5.3 Prevalence of enteric viruses in different age groups……………… 37
5.4 Paper I………………………………………………………………………… 38
5.5 Paper II……………………………………………………………………….. 39
5.6 Paper III……………………………………………………………………… 40
6. Discussion……………….. 41
6.1 Discussion of the main findings……………………………………….. 41
6.1.1 Prevalence of enteric viruses……………………….............. 41
6.1.2 Mixed viral infections……………………………………….. 43
6.1.3 Distribution of enteric viruses by age groups…………….. 43
6.1.4 Molecular epidemiology of viral infections ….…………….. 44
6.1.5 Seasonality of viral infections…………………………….. 46
6.1.6 Association between enteric viruses and HIV………......... 47
6.2 Methodological discussion……………………………………….. 49
6.2.1 Study design, study population and study participants … 49
6.2.2 Laboratory methods………………………………………. 49
7. Study limitations………………………….…………………………… 52
8. Conclusions……………..……………………………………………… 53
9. Recommendations…………………………………………………….. 53
10. Future perspectives……………...………………………………………… 54

References…………………………………………………………...……. 56
Papers 1-III…………………………………………………………...…....
Appendices………...………
i) Questionnaire for cases……….…………………………………
ii) Questionnaire for controls……………………………………….....

1. Introduction

1.1 Burden due to diarrhoea diseases

Diarrhoeal disease is one of the leading causes of illness and death in children under-five years in the world especially in developing countries. Studies conducted at different times have shown a substantial decrease in mortality due to diarrhoea in developing countries over time (Figure 1) [1-5]. In the year 1976 diarrhoea was estimated to cause five million deaths in children in developing countries [6], but recent studies have shown a decrease in mortality due to diarrhoea, with current estimates of 700,000 deaths annually among children younger than 5 years of age [5]. Of these deaths, 72% occur in children below two years of age. Despite the observed 13 each child experiencing an average of three episodes of diarrhoea per year [5, 7]. Apart from high morbidity and mortality associated with diarrhoeal diseases,

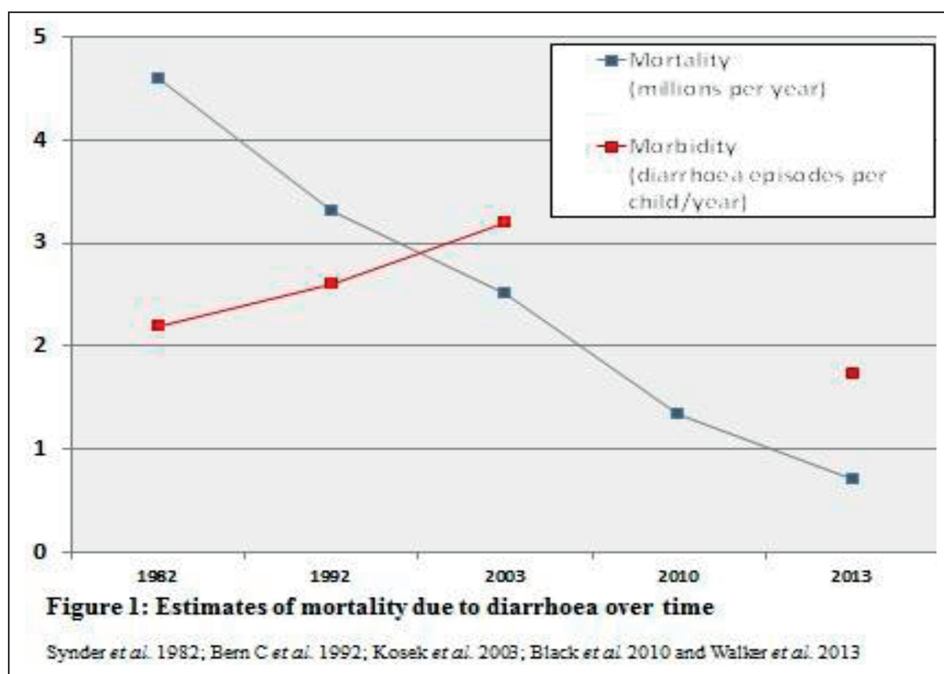

Figure 1: Estimates of mortality due to diarrhoea over time

Synder *et al.* 1982; Bern C *et al.* 1992; Kosek *et al.* 2003; Black *et al.* 2010 and Walker *et al.* 2013

diarrhoea also present an economic burden for the developing countries, with more than a third of the hospital beds for children being occupied by patients with diarrhoea, often involving the use of expensive intravenous fluids and drugs [8].

1.2 Risk factors for diarrhoea

Unlike in developed countries, children living in developing countries experience more diarrhoea episodes, severe episodes with dehydration, and a higher death rate. Several factors have contributed to this. Lack of accessibility to clean and safe drinking water, lack of appropriate sewage disposal, crowding and exposure to farm animals (free roaming chickens and pigs), poor hand hygiene and poor sanitary conditions, inaccessibility to medical care and low educational level [9, 10]. Poor handling of weaning food is also a factor which predisposes children to diarrhoea. If weaning food is prepared under unhygienic conditions, it is frequently heavily contaminated with pathogens and hence becomes a major factor in the cause of diarrhoeal diseases [11].

Lack of exclusive breast feeding was found to be associated with diarrhoeal diseases in young infants [12, 13]. Exclusively breastfed children get less diarrhoea episodes compared to no- breastfed infants for the first three months of life. This is because exclusively breast fed children are unlikely to take any other foods or fluids which are likely to be contaminated. Prolonged breast feeding also reduces the incidence or severity of certain types of diarrhoea diseases such as shigellosis and cholera [14-18].

Underlying conditions or host factors such as malnutrition, which is more common in developing countries, increase the risk of diarrhoea and severity [19, 20]. Diarrhoea is also a major source of morbidity and mortality in HIV infected children, particularly in developing countries [21-23].

1.3 Seasonality of viral diarrhoea

Seasonality is an important feature of rotavirus diarrhoea. Reports have shown that changes in the environmental conditions, such as humidity, temperature cycles, rain patterns and winds, are associated with seasonality of infectious diseases [24]. Globally, rotavirus infection is present all the year round, which suggests that low-level transmission could maintain the chain of transmission. Before rotavirus was discovered, the disease was called 'winter diarrhoea and winter gastroenteritis [25-27]. In temperate climates seasonality of rotavirus infection has been well demonstrated [28, 29], where rotavirus is most frequently detected in the winter and rarely identified

in the summer months [25]. In contrast, in the tropics, it is detected all year round, with seasonal trends that are not well defined [25]. However, recently two studies demonstrated the inverse relationship between temperature and rotavirus incidence in the tropics, and the findings concur with those of temperate countries [26, 30].

Norovirus infections occur in epidemics as well as in sporadic cases. Seasonality of norovirus is poorly understood [24]. In temperate countries, norovirus epidemic characteristics, and timing, are consistent from year to year, with a peak incidence during the winter time. However, outbreaks of norovirus also occur during the summer [31], although at a reduced rate and with an absence of epidemic spread to geographically remote areas [24]. In contrast, in tropical countries, norovirus infection is observed all year round and does not show a clear seasonal pattern [32]. The observed temporal distribution of norovirus infections is most likely based on biological, environmental and behavioural factors that regulate the transmission, virulence and persistence of the virions in host populations [24].

Adenovirus of all types are circulating year round, but sometimes marked seasonal pattern has been reported, especially for non-enteric adenovirus types [33]. A recent study conducted in Bangladesh reported high prevalence of adenovirus infection during the rainy season [34]. Other studies have shown no consistent seasonal pattern of enteric adenovirus infection [35-37].

1.4 Viral causes of diarrhoea

Infectious diarrhoea can be caused by a wide range of viruses, bacteria, or parasites [38]. Studies have shown that diarrhoea can be caused by a single pathogen or a mixture of two or more pathogens [39, 40]. This thesis is dealing with diarrhoea caused by three common viruses, rotavirus, norovirus and adenoviruses.

1.4.1 Historical background

Viruses have been suspected of being important causes of gastroenteritis since the 1940s, but the aetiology remained unknown in most cases [41, 42]. In 1972 **Norwalk virus** became the first viral agent identified to cause diarrhoea, using immune electron microscopy (EM) after an outbreak of diarrhoea [43]. One year later **rotaviruses** were

identified as a cause of diarrhoea in children [44, 45]. In 1975 **astroviruses** were detected in the stools of children with acute diarrhoea [46, 47]. This was followed by the discovery of **enteric adenoviruses** [48], and **human caliciviruses** of two different genogroups now called **Norwalk- like viruses** (NLVs) and **Sapporo-like viruses** (SLVs) [49]. Other viruses which have been linked with gastroenteritis in humans are coronaviruses, Aichi viruses, picobirnaviruses, pestiviruses and toroviruses which produce diarrhoea in animals, and they are emerging as causes of viral gastroenteritis in humans, according to several studies [50-59]. The detailed structure and classification of the three viruses rotavirus, norovirus and adenovirus, which form this thesis, are described below.

1.4.2 **Rotavirus**

Rotavirus is the leading cause of severe diarrhoea in children worldwide [60]. It is estimated to cause 36% of diarrhoea hospitalizations among children aged less than 5 years, according to WHO rotavirus surveillance networks [61]. Rotavirus diarrhoea is estimated to cause 453,000 deaths in children younger than five years, more than half occur in developing countries of Africa and Asia [62].

Structure and classification

Rotaviruses are non-enveloped with icosahedral symmetry belonging to the genus Reoviridae [63]. Figure 2 shows the structure of rotavirus. Rotavirus possesses a genome consisting of 11 segments of double-stranded RNA, which are 18,555 nucleotides in total, enclosed in three protein layers. **The genome segments** encode six structural viral proteins which make viral particles (VPs) and five non-structural proteins (NSPs). Most segments encode a single polypeptide. Rotavirus NSPs have various functions in the virus replication cycle, in pathogenesis and evasion of the host immune response [64, 65].

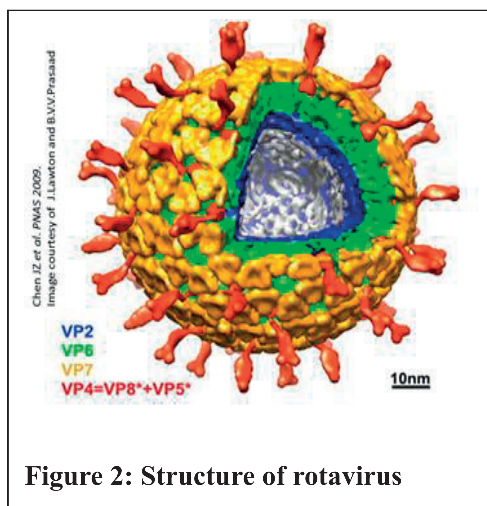

Figure 2: Structure of rotavirus

The inner layer is composed of the core shell (VP2), the viral RNA-dependent RNA polymerase (VP1), and RNA capping enzyme (VP3), as well as the 11 double stranded RNA genome segments. **The middle protein layer,** also known as inner capsid, is composed of VP6 which surrounds the core shell. The VP6 is used for rotavirus classification and determines reactivity for the seven major groups (A through G) of rotavirus [64]. Rotavirus groups A, B and C are known to infect humans and various animals. Rotaviruses D, E, F and G so far have only been identified in animals, mostly birds. Epidemiologically, rotavirus group A is the most important for human infection and disease, responsible for more than 90% of all rotavirus infections. **The outer protein layer,** also called outer capsid, is composed of two structural proteins, VP4 and VP7. The VP7 makes up the outer capsid protein shell while VP4 forms spikes that protrude through the outer capsid shell [65].The VP4 (a protease cleaved protein, P type antigen) and VP7 (a glycoprotein, G type antigen) induce neutralizing antibodies, correlate with protective immunity and serve as the basis of rotavirus typing system [66-68]. VP7 types are classified as **serotypes** by neutralization assays or as **genotypes** by sequencing. The 2 assays produce the same results, therefore rotaviruses are referred to by their G serotype alone e.g., G1, G2, G3, etc. [65, 69]. VP4 serotypes are also classified by neutralization and sequencing assays, but the results does not always agree, so there is a dual system for P typing. P **serotypes** are referred to by their serotype numbers (e.g., P1, P2) and P **genotypes** are denoted in brackets (e.g., P[8], P[4]). P genotyping is the most widely used method for classification because of difficulties in standardizing VP4 serotype assays [65, 69]. Currently there are 27 G genotypes and 35 P genotypes identified in human rotaviruses [70].

Molecular epidemiology of rotavirus

Globally, 80–90% of the childhood rotavirus disease burden are caused by genotypes G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8] [71]. Recently rotavirus genotype G12 has been identified as an emerging genotype [72]. Three African countries; South Africa, Malawi and Nigeria, has reported the emergence of G12 occurring in combination with P[6] or P[8] [73-75]. Rotavirus genotype G8 has been reported worldwide, with high prevalence in some African countries [71, 76-83].

Vaccine status

Efforts to produce rotavirus vaccine started in early 1980s. The first rotavirus vaccine candidate was RIT4237, a live bovine rotavirus vaccine [84]. A single dose of this vaccine candidate was given orally before the winter epidemic season. The vaccine was given at birth and was able to induce 82-88% protection against severe disease in the first 2-3 years of life. Breastfeeding did not suppress the uptake of vaccine [84]. The vaccine was not pursued because of its failure in clinical trials conducted in the African region [85, 86]. A second vaccine RRV-1 Rhesus rotavirus vaccine candidate was an alternative to bovine rotavirus vaccine. This vaccine was later reasserted with human rotavirus to express human rotavirus antigen G1, 2, 3 and 4 and Rhesus G3. It was then known as tetravalent Rotavirus vaccine RRV-TV or Rotashield ™ [87]. In trials this oral vaccine was given in 3 doses and was able to induce 80-100% protection against severe disease. The vaccine was licenced in USA in 1998 and was discontinued one year later because it increased the risk of intussusception (at least 25-fold) within the first 10 days after administration [88].

From 2006 there are two licensed oral rotavirus vaccines which as in use, namely RotaTeq™ and Rotarix™. RotaTeq™ is a Bovine-Human re-assorted rotavirus vaccine produced by Merck. This pentavalent vaccine express human G1, 2, 3, 4 and human P8 on the Bovine W3 backbone [89]. The vaccine is given in three doses, trials in USA and developed countries have shown that the vaccine can induce 74% protection against any rotavirus diarrhoea, 87% protection against diarrhoea requiring a visit to physician and approximately 100% protection against severe rotavirus diarrhoea. The vaccine is not affected by breastfeeding and vaccine shedding has been

reported as infrequent and at a low level. Rotarix™ is a human rotavirus vaccine produced by GlaxoSmithKline. This monovalent vaccine is given in two doses; it gives 85% protection against severe diarrhoea and 100% protection against most severe diarrhoea [90, 91]. This vaccine is shed in greater amounts than RotaTeq, the bovine-derived vaccine. A decrease in morbidity and mortality attributable to rotavirus diarrhoea has been reported in high and middle income countries that have introduced rotavirus vaccines [92-99].

Transmission and clinical symptoms of rotavirus infection

Rotaviruses are mainly transmitted by the faecal-oral route [100]. Some studies speculate that transmission also occur through respiratory route [25, 100-102]. This theory is supported by the fact that there is rapid acquisition of rotavirus antibodies in the first few years of life in all settings regardless of hygiene standards [42].There has also been reports of large outbreaks in which faecal-oral route could not be documented [100, 103]. In addition, there are reports of respiratory symptoms among patients with rotavirus diarrhoea [102].

The incubation period of rotavirus diarrhoea is estimated to be less than 48 hours [100]. Rotavirus produces spectrum of responses from subclinical infection to mild and severe rotavirus diarrhoea with dehydration [100].Vomiting may occur and may precede diarrhoea symptoms. Diarrhoea symptoms can last between one and nine days [100].

Rotavirus can produce chronic symptoms in children who are immunocompromised causing prolonged shedding of the virus in stool [100, 104].

Rotavirus infection has also been associated with intussusception, which may occur when a child is infected with wild type rotavirus [105, 106]. The theory is supported by the report of intussusception following oral administration of quadrivalent rhesus rotavirus based vaccine [88]. A study conducted in Japan has also found that infants and young children with intussusception had evidence of rotavirus infection, which was also detected in stool [105, 106]. The shedding of rotavirus in stool can last between four to 29 days when measured by ELISA or four to 57 days if PCR is used to measure the virus in stool [107].

Immune response to rotavirus infection

The immunological mechanisms responsible for protection against infection by rotavirus are still not well known. Intestinal IgA is probably the most important mechanism for long-term protection against rotavirus [108]. Studies showed that the first infections are generally the most severe, with severity decreasing as the number of infections increases [109-113]. Furthermore, protection conferred by infection was greatest against moderate to severe disease, less against mild illness, and least against asymptomatic infection. Asymptomatic infection provides protection to a degree comparable to that achieved by symptomatic infection [109, 110, 114, 115]. A trend was observed where repeated infections with the same G serotype is less likely to occur, suggesting the first infection with rotavirus elicits a homotypic neutralizing antibody response [116], with heterotypic responses in subsequent infections [109, 110, 116]. The presence of neutralizing antibodies directed towards the proteins VP4 or VP7 does not correlate with protection against this disease [117]. Another study has also shown that protein NSP4 produces a cellular immunity-mediated response [118].

1.4.3 Norovirus

Noroviruses are among the leading cause of sporadic cases and outbreaks of acute gastroenteritis across all age groups [119-121]**.** It is estimated to cause 18% of all cases of sporadic acute gastroenteritis in children younger than 5 years and older children and adults were associated with norovirus [122]. In developing countries, noroviruses are estimated to cause more than 1.1 million hospitalisations and 200,000 deaths in children <5 years of age each year [123]. When norovirus infection occurs in outbreaks, they are usually difficult to control because of the prolonged shedding of the virus, environmental stability and resistance to disinfection, low infectious dose, and contribution of virus-induced vomiting to the spread of the infectious agent [124].

Structure and classification

Noroviruses belong to the genus Norovirus in the family *Caliciviridae*. The virions are composed of a single structural capsid protein, with icosahedric symmetry [125]. This

capsid protein has a continuous shell with protrusions in the shape of an arch (Figure 3). A key characteristic is the existence of cup-shaped depressions, situated on the axes of the icosahedron, from whose Latin designation, *calyx*, the virus derives its name [126]. The genome of the norovirus consists of positive-sense, single-stranded RNA of approximately 7.5 kbp. The genome is organized into three open reading frames (ORFs). ORF1 at the 5' end is the largest and encodes non-structural polyproteins such as RNA–dependent RNA polymerase (RdRp), proteinase and NTPase (Figure 3B). It is followed by ORF2 encoding a single viral capsid protein [127] and ORF3 at the 3' terminus encoding a basic protein which regulate the expression and stability of the viral capsid protein [128]. Based on RNA polymerase

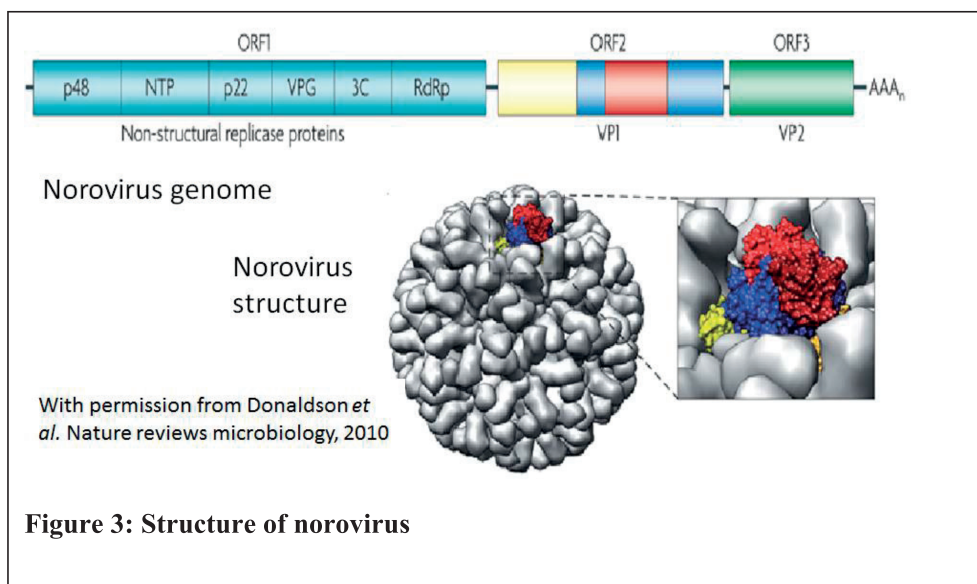

Figure 3: Structure of norovirus

or capsid gene sequence analysis noroviruses in humans have been divided into three genogroups (G), GI, GII and GIV, whereas GIII and GV Noroviruses have been found in animals only. Within GI and GII, several genotypes are recognized [129, 130]. To date 14 GI and 29 GII polymerase genotypes and 8 GI and 23 GII capsid genotypes have been described [131].

Molecular epidemiology of norovirus

Most human norovirus infections are caused by genogroup GII, which account for an average of 96.0% [131, 132]. Norovirus genotype (GII.4) predominates and is

responsible for approximately 55–85% of the norovirus gastroenteritis cases worldwide [131, 132]. Norovirus GII.4 has also been reported to cause severe outcomes of hospitalizations and deaths [133]. Within norovirus GII.4 seven different variants have been reported since 1990s to early 2013, these variants were associated with global epidemics of gastroenteritis [132]. A review by Hoa Tran and co-workers has shown that norovirus GI occurs less common, at an average of 3.6% [131]. Norovirus GI is also reported to be associated with water-borne outbreaks [132]. Mixed norovirus infections of GI and GII were also reported with an average of 0.4% [131]. Some countries have reported high prevalence of norovirus GI in approximately one third of norovirus strains; in Madagascar, Egypt, Yemen and Thailand [134-136].

Vaccine status

Currently there is no licensed norovirus vaccine. Promising vaccine candidates are under development. Studies have shown that both orally and nasally administered virus-like particles (VLPs) can be well tolerated and are immunogenic in phase 1 trials [137, 138]. A nasally administered investigational GI.1 norovirus VLP vaccine was also shown to confer 47% protection against norovirus gastroenteritis in healthy adults in a human experimental challenge model [139]. Recently, a bivalent intramuscular norovirus virus-like particle (VLP) vaccine was reported to be well tolerated and immunogenic in adults [124].

Transmission and clinical symptoms of norovirus infection

Noroviruses can infect humans via multiple routes such as the oral route, through contact with fecal matter or aerosolized vomitus from infected people, as well as contaminated surfaces, food, or water [140-142]. Transmission of norovirus is very efficient because of certain characteristics of the virus that enhance their ability to spread. The virus is highly infectious, and a very small inoculum, as few as 10–100 virions, is sufficient to cause infection [141, 143]. In addition, the virus is relatively stable in the environment. It survives freezing, heating to 60° C, and exposure to chlorine [141]. Norovirus can cause food-borne related outbreaks in healthcare settings such as nursing homes and hospitals where the virus is predominantly spread

from person to person [142, 144]. Norovirus is also among major causes of sporadic gastroenteritis in children and adults [142].

After an incubation period of 12-48h, the illness may start with one or all of the following symptoms: projectile vomiting, non-bloody diarrhea, nausea, abdominal cramps, and low-grade fever [142, 145] . In healthy individuals the duration of symptoms is usually 12-72 hours [141] and in most patients the disease is self-limiting [142]. However, young children are at increased risk for more severe and prolonged illness leading to hospitalization [142, 146], while for immuno-compromised patients the disease is increasingly recognized as an important cause of chronic gastroenteritis [146]. The shedding of the virus in stool can continue for up to three weeks [144].

Immune response to norovirus infection

Protective immunity to norovirus is complex and also not well understood. It is hypothesised that innate host factors and acquired immunity contributes to the susceptibility of norovirus infection [147]. Some studies have shown that infected volunteers were susceptible to reinfection with the same or different strains [147, 148]. Furthermore, it was demonstrated that people with pre-existing antibodies are not protected from infection unless repeated exposure to the same strain occurs within a short period of time. In these studies it was also noted that homologous antibody protection is short lived lasting from 8 weeks to 6 months [147, 148]. Proposed receptors for norovirus are Histo-blood group antigens (HBGAs), including H type, ABO blood group, and Lewis antigens. Strain-specific susceptibility to norovirus infection has been associated with the expression of HBGAs [144, 149-154]. In addition, resistance to norovirus infections has been associated with mutations in the 1, 2-fucosyltransferase (FUT2) gene which lead to lack of expression of HBGAs on the surface of intestinal cells [149-151, 155]. People with normal FUT2 gene and express HBGAs antigens are termed as "secretors". People with mutations in the FUT2 gene who will not express HBGA are termed as "non-secretor", these are people who are less susceptible to norovirus infection. However, secretor status alone does not entirely explain the observed differences among infected and uninfected people, for all strains of norovirus. Therefore it is most likely that other mechanisms

of immunity are involved and this needs further research. There are also evidence suggesting that new GII.4 variants evolve to escape the build-up of both acquired and innate immunity resistance in the human population [156, 157].

1.4.4 Adenovirus

Human adenovirus causes acute diarrhoea sporadically, as well as in outbreaks. Besides acute diarrhoea, adenoviruses cause other diseases such as respiratory diseases, conjunctivitis and haemorrhagic cystitis [158]. Adenoviruses have also been associated with persistent infections in both immuno-competent and immuno-compromised individuals [158]. In young children enteric adenoviruses are reported with variable prevalence of infection. In developed countries, the prevalence varies from 1% to 8% [36, 159-162], whereas in developing countries, prevalences from 2–31% has been reported [163-167].

Structure and classification

Adenoviruses represent the largest non-enveloped viruses measuring 90–100 nm, icosahedral in shape. The nucleocapsid contains a double stranded DNA genome. They belong to the family Adenoviridae and genus *Mastadenovirus*. Their name derives from their initial isolation from human adenoids in 1953 [158].

Figure 4 shows the structure of adenovirus. The protein capsid is composed of 252 capsomers with hexones/ pentones and structures called fibers that protrude to the outside. The hexones contain proteins II, VI, VIII, and IX, which participate in the stability and assembly of the viral particle. The pentone proteins (III and IIIa) have the function of cellular penetration. The protruding fibers are hemagglutinins and are responsible for binding the virus to receptors [168-170]. The core is made of at least eight proteins which maintain the integrity of the genome, and participate in enzymatic activity [158]. The adenovirus genome is linear, non-segmented double-stranded (ds) DNA that is between 26 and 48 Kbp [158, 171].

Adenoviruses have a broad range of vertebrate hosts; in humans, there are 52 types of adenoviruses identified, grouped into seven species A to G on the basis of their resistance to neutralisation by antisera to other known human adenoviruses [171, 172] or genome analysis [172].

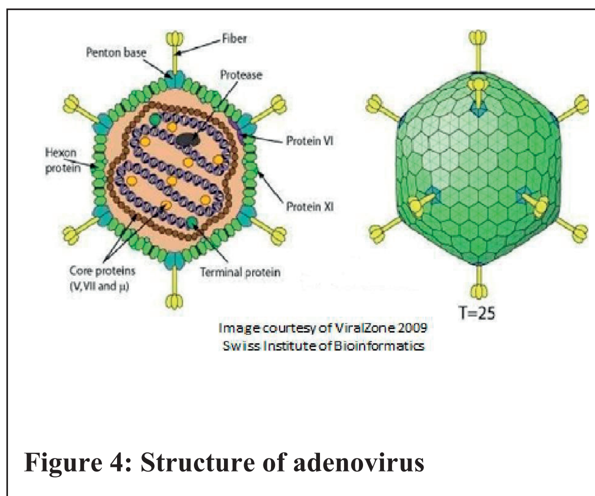

Figure 4: Structure of adenovirus

Vaccine status

Currently there is no vaccine for enteric adenovirus in children. [173]. However adenoviruses are commonly used as vectors for various other vaccines [173, 174].

Transmission and clinical symptoms of adenovirus infection

Enteric adenoviruses are transmitted directly through person-to-person contact via the fecal- oral route, as well as indirect transfer through contact with contaminated surfaces or shared utensils [166]. Human adenovirus causes acute diarrhoea sporadically, especially in children, as well as in outbreaks [175]. The incubation period is approximately 7-8 days [175, 176]. Symptoms include non- bloody watery diarrhoea with or without vomiting. Low grade fever may occur, dehydration and respiratory tract infection may be present [175]. The infection last for 5-12 days, which is longer than other types of viral gastroenteritis, but usually the disease is milder compared to other viruses [175, 177].

Molecular epidemiology of adenovirus

The disease pattern of adenoviruses varies according to species. Adenovirus species F, types 40 and 41, has been found to be regularly associated with gastroenteritis and they are referred to as enteric adenoviruses and are responsible for most cases of acute gastroenteritis. Other species such as A (types 12, 18 and 31) [169] , C (types 1, 2 and

5) and D (types 28, 29, 30, 32, 37, 43 to 46) [158, 178, 179] have also been associated with diarrhoea.

Immune response to adenovirus diarrhoea

After adenovirus infection, type specific neutralizing antibodies develop in most cases. These can provide protection both in the current illness and against reinfections by the same serotype, although patients may continue to eliminate the virus in their faeces for months after an effective humoral response [158].

1.5 Mechanisms of virus induced diarrhoea

Enteric viruses, namely rotavirus, norovirus and enteric adenoviruses, are stable at low pH and resistant to digestive enzymes. The viruses infect the intestinal villus and spare the crypt cells [180]. Once the virus is inside the cell, virus replication occurs, which later causes cell lysis and release of viral progeny which will infect neighbouring cells [180]. This cytolytic infection leads to villus atrophy, which will cause decease in digestive and absorptive capacity of the intestine, and is responsible for the malabsorption component of diarrhoea. Both cellular and viral proteins will induce immune cells to release variety of chemical mediators. The interaction between the immune cells and enteroendocrine systems lead to increased intracellular $Ca2^{+}$ and cAMP, these in turn will result in one or more of the following;

i) Activation of Cl^{-}channels causing increased Cl^{-} secretion into intestinal lumen

ii) Act on cytoskeleton and tight junctions resulting in weaker adherence between enterocytes leading to paracellular leakage and to increased permeability;

iii) Alterations in intestinal motility. This can also be a results of villus ischaemia

iv) Decreased enzyme expression and decreased nutrient and water absorption this is virus-induced down-regulation of the expression of absorptive enzymes [180].

Usually viruses cause diarrhoea by one or more of the above four mechanisms [180].

1.6 Management of diarrhoea

1.6.1 Treatment of viral diarrhoea

Rehydration is the mainstay of treatment for viral gastroenteritis and most cases resolve spontaneously without the need for additional interventions [181-183]. Rehydration can be done by administering oral rehydration solution (ORS) to prevent or correct dehydration which is a consequence of diarrhoea [181]. For children who are unable to tolerate ORS via the oral route (with persistent vomiting), nasogastric feeding can be used to administer ORS. Intravenous fluids may be given in cases with severe dehydration [181]. Currently there is a more effective, lower-osmolality ORS (with reduced concentrations of sodium and glucose, associated with less vomiting, less stool output, and a reduced need for intravenous infusions in comparison with standard ORS) has been developed for global use [184]. Table 1 shows the constituents of ORS solution.

Table 1. Constituents of Oral rehydration solution (ORS)

	mmol/L
Sodium	75
Chloride	65
Glucose, anhydrous	75
Potassium	20
Citrate	10
Total osmolality	245

1.6.2 Prevention of viral diarrhoea

The following are preventive measures for diarrhoea diseases:

i) Interruption of transmission of the causative agents include measures such as good infection control practices, these are important in hospitals and centres which care for small children in order to interrupt transmission and eventually prevent spread of infection [182, 185]. Improving practices related to the preparation and storage of weaning foods (to minimize microbial contamination and growth). Other measures are availability of safe water supply, washing hands (before and after preparing food or eating), safe disposing of faeces, including infant faeces.

ii) Exclusive breast feeding for 6 months and improved weaning practices are important measures to reduce the incidence of diarrhoea [182].

iii) Measures to improve host defences and thus diminish the risk of diarrhoea include: continuing to breast-feed for at least the first year of life [186], for rotavirus, two licenced vaccines in use have shown to be able to reduce morbidity and mortality [92, 93, 96, 98].

iv) Zinc deficiency is common among children in developing countries and it has been associated with infectious disease including diarrhoea [187-189]. A number of trials have supported zinc supplementation as an effective agent in treating and preventing diarrheal disease [190-193].

2.0. Aims of the thesis

2.1 General aim

Detection and molecular characterisation of viruses in hospitalised diarrhoeic children and asymptomatic controls in Dar es Salaam, Tanzania.

2.2 Specific aims

2.2.1. To determine the prevalence of rotavirus, norovirus and adenovirus in diarrhoeic children (Paper I, II and III).

2.2.2 To determine the prevalence of asymptomatic carriage of rotavirus, norovirus and adenovirus in non-diarrhoeic children (Paper I, II and III).

2.2.3. Molecular characterisation of rotavirus, norovirus and adenovirus in diarrhoeic children and non-diarrhoeic children (Paper I, II and III).

2.2.4 To explore potential associations between HIV, seasonality, clinical and demographic characteristics with viruses (rotavirus, norovirus and adenovirus) in hospitalised diarrhoeic children compared with asymptomatic controls (Paper I, II, III)

3.0 Rationale of the study

The information generated from this study will provide the prevalence of enteric viruses namely rotaviruses, noroviruses and adenoviruses in childhood diarrhoea in Tanzania. The study will also provide a comprehensive picture of genetic diversity of these viruses which is relevant information required for the development of efficacious vaccines. Furthermore, from early 2013, Tanzania started to implement a live attenuated monovalent vaccine (Rotarix) in the national childhood vaccination schedule. This study describes the molecular epidemiology of rotavirus prior to implementation of vaccination. The study also highlights seasonal variation, impact of HIV and clinical characteristics of viruses causing diarrhoea in children of Dar es Salaam, Tanzania.

4.0 Materials, Methods and methodological considerations

4.1 Study design and study sites

This study was a cross sectional case control study. The study was conducted between August 2010 and July 2011 in Dar es Salaam Tanzania. Dar es Salaam is a city with a population of about five million. The study was conducted at three major hospitals; Muhimbili National Hospital (MNH), Amana and Temeke Municipal Hospitals. MNH, with a bed capacity of 1200, is the largest hospital in the country and serve as a tertiary and national referral hospital. Amana and Temeke are Municipal district hospitals of Dar es Salaam.

4.2 Study population

The study included children less than two years of age. Cases were children admitted due to diarrhoea in the three hospitals studied. Controls were children without history of diarrhoea for one month prior to the study. They were either children attending child health clinics for routine immunisation and growth monitoring, hereafter referred to as community based controls (CBC) or children admitted to the same hospitals as the cases due to diseases other than diarrhoea, hereafter referred to as hospital based controls (HBC). Details of recruitment of study participants, collected

demographic and clinical information, case definition and exclusion criteria are described in paper I and II. HIV testing was done by HIV-DNA PCR as previously described [194]. HIV testing results were obtained from patient files. Weight and length measurements were performed as previous described [195]. Nutritional status of the study participants was assessed using Z-scores according to WHO criteria [2].

4.3 Specimen collection and storage

For diagnosis of viral pathogens causing diarrhoea, specimen can be whole stool or rectal swabs [196]. Whole stool specimens are preferred over rectal swabs because of the higher quantity of virus present in whole stool [197]. Furthermore specimens should be obtained during the acute phase of illness (within 48-72 hours after onset) while the stools are still liquid or semisolid and viral excretion is at its peak [196]. It is also recommended that collected stool specimens should be kept refrigerated at 4°C if testing occurs within 2-3 weeks or if testing is expected to occur over 3 weeks after collection or for archiving purposes, stool samples should be frozen at -20°C or -70°C [196]. At this temperature, specimens can be stored without compromising diagnostic yield. Norovirus RNA can be detected by using molecular techniques after at least 5 years when samples are stored under appropriate conditions[196, 198]. In this study we collected a single whole stool specimen from each child on the day the child was admitted due to diarrhoea or the day of enrolment for controls. A wide mouthed sterile plastic container was used for stool collection. The intention was to obtain at least 10g of stool; however, to be considered acceptable, the sample as an approximation resembles the size of 3 peas. The specimen was immediately placed into a Styrofoam container containing a cold pack and transported to the Central Pathology Laboratory (CPL). Upon arrival in the laboratory, two aliquots of the portion of stool specimen were frozen at -70^{0}C the same day as it was collected. At the end of data collection stool specimens were shipped on dry ice to Bergen, Norway for further analysis.

4.4 Study subjects for paper I, II and III

Sample collection was performed during two seasons, starting in August 2010 and in March 2011, aiming for minimum 300 cases and 300 controls in each period. The

target for cases was reached in January 2011 and in June 2011, while enrolment of controls continued in February 2011 and July 2011, thus enrolment continued for one complete year.

The study included a total of 1303 stool samples from cases and controls. Of these, 1266 samples had sufficient material to extract total nucleic acid and all these samples (705 cases and 561 controls) were used for analysis of Paper II.

A total of 1235 samples were adequate for detection of rotavirus and adenovirus using ELISA (690 cases and 545controls) and therefore were used for analysis of paper I and III.

For paper I and III the number in each group of controls i.e. CBC and HBC were 310 and 235, respectively. For paper II the number of CBC and HBC were 312 and 249 respectively.

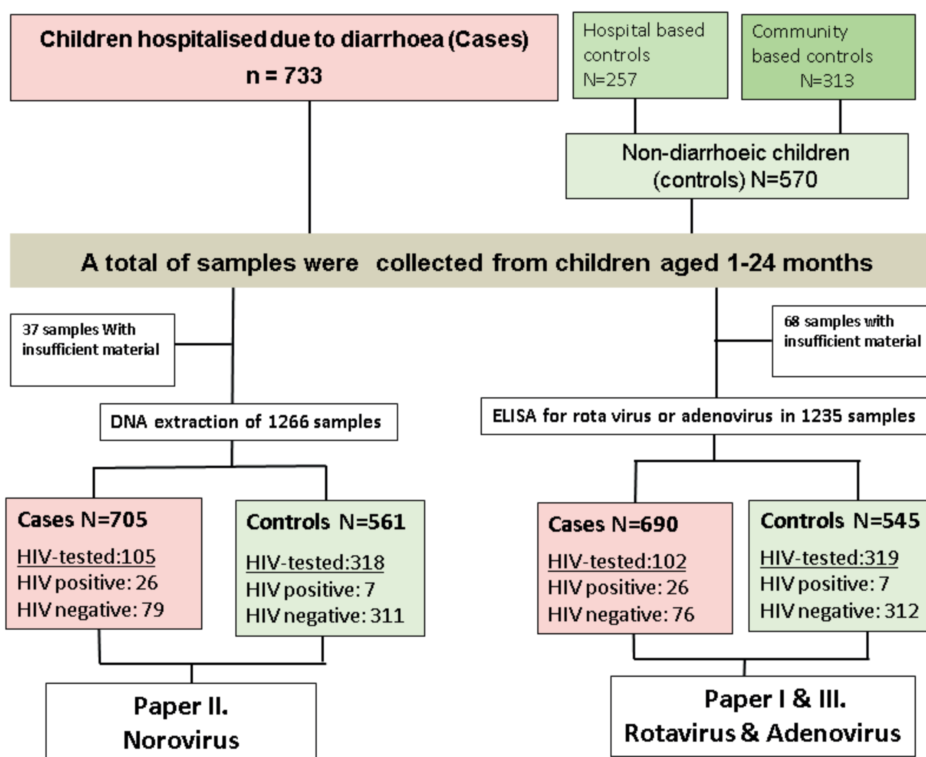

Figure 5: The flow chart of the number of samples used for each study

4.5 Laboratory methods

4.5.1 Extraction of total nucleic acid (RNA/DNA)

This study used PCR for detection and typing of norovirus (paper II) and typing of rotavirus and adenovirus (Paper I and III). The reliability of PCR depends on the efficient isolation of nucleic acids together with sufficient removal of inhibitory substances from the sample material [126, 145, 199]. Therefore the method used for nucleic acid extraction has to be well considered. Several commercial assays for the extraction of nucleic acids are available and used in routine diagnostics. The number of samples to be tested affects the choice of the method to be chosen. When dealing with large number of samples, automated method of nucleic acid extraction will be the right choice since it will decreases the hands-on time per sample and improves assay performance including precision [199]. A study by Verheyen *et al.* evaluated the performance of five automated methods of nucleic acid extraction from stool, these methods were 1; easyMAG, bioMerieux, 2; m2000sp, Abbott, 3; MagNA Pure LC 2.0, Roche, 4; QiaSymphony, Qiagen and 5: sample preparation module of the VERSANT kPCR Molecular System, Siemens. The five automated extraction methods yielded comparable results [199].

In this study we used MagNA Pure LC 2.0, Roche for extraction of total nucleic acid. In order to extract both RNA and DNA from the same sample material we used Magna Pure LC High Performance Total Nucleic Acid Isolation Kit (Roche Applied Science, Mannheim, Germany). Details of the method can be found in paper I. We first tested the method ability to extract both RNA and DNA by testing known norovirus genogroup I and II and giardia positive samples. The testing results could detect all the known positive and negative samples. This method was preferred because of large number of samples if this study and also the instrument was made available for use in this study by Microbiology laboratory, Haukeland University hospital.

4.5.2 Detection of rotavirus (Paper I)

Initially detection of rotavirus was mainly done using electron microscope (EM) [100, 200-202]. This method has an advantage of high specificity because of the distinctive appearance of rotavirus [100]. Although EM is still useful it is limited to reference laboratories [203]. Cell culture is not considered to be useful for diagnostic purposes, as it is technically cumbersome and slow [203]. The latex agglutination (LA) technique although gives rapid results and does not require expensive instruments, but it is reported to have lower sensitivity than the enzyme-linked immunosorbent assay (ELISA) [204, 205]. In addition LA may not be appropriate choice when dealing with large number of samples. ELISA is now well established as a sensitive and specific technique for the detection of rotavirus antigen in stools and was adopted by WHO as the standard diagnostic technique for use in epidemiological studies in developing countries [206]. There are several commercial available ELISA kits for detection of rotavirus which has been evaluated [207]. Some of the ELISA tests are monoclonal antibody-based, these are more costly but have the advantage of highest sensitivity compared to polyclonal antibody-based tests which are the least expensive but may be less sensitive [208]. However, a recent evaluation study found no significant difference in sensitivity of two monoclonal vs. one polyclonal ELISA kits [207]. These ELISA kits are RIDASCREEN® Rotavirus (R-Biopharm AG, Darmstadt, Germany) and the Premier™ Rotaclone® kit both uses monoclonal antibodies raised against rotavirus structural protein VP6. The third ELISA kit is ProSpecT™ Rotavirus, this kit uses polyclonal antibodies directed against rotavirus structural proteins. ProSpecT™ Rotavirus kit was reported by manufacture (Oxoid, Ltd) to have 100% and 99.2% sensitivity and specificity respectively when compared to EM and a commercial EIA kit as gold standard. We therefore chose to use the ProSpecT™ Rotavirus kit for detection of rotavirus in paper I. This ELISA kit was also reported to have 75% and 100% sensitivity and specificity, respectively, when compared to RT-PCR as a gold standard [207]. The ProSpecT™ Rotavirus kit we used is a replacement kit for the widely used rotavirus IDEIA™ Rotavirus EIA (Dako Diagnostics Ltd., Ely, UK), which was discontinued in March 2009. IDEIA™ Rotavirus kit was used in our

previous study [195]. We preferred this kit not only because of good reported sensitivity but also to get comparable results from our previous study.

4.5.3 Detection of human adenoviruses (Paper III)

Enteric adenovirus (type 40 and 41) are fastidious; they cannot be cultivated in cell lines which have traditionally been used for isolation of adenovirus. However, they can be isolated in Graham 293, a human embryonic kidney cell line transformed by adenovirus type 5-sheared DNA [158]. Therefore, cell culture is not routinely used for detection of adenovirus from stool samples. Antigen detection tests such as latex agglutination and ELISA are now widely used for detection of adenoviruses from stool samples. Furthermore, nested PCR which detects all described adenovirus types have recently been used in epidemiological studies. Studies which have used PCR for detection of adenovirus have shown high sensitivity by reporting high prevalence of adenovirus from stool samples [165, 166, 209] compared to studies which have used either LA or ELISA techniques [164, 195, 210-213]. However PCR is relatively expensive compared to ELISA especially when the expected prevalence is low. Among ELISA test kits available, some detect group or species specific adenovirus F 40 and F41 and others are designed to detect all the types of adenovirus. Due to the fact that other types of adenovirus than F40 and F41 have been implicated in diarrhoea cases [166, 179], the use of tests which detect other types than enteric adenovirus is important in order not to underestimate the prevalence of adenoviruses. In paper II we used ELISA test kit ProSpecT™ Adenovirus kit. According to the manufacturer (Oxoid, Hants, UK), this kit has sensitivity and specificity of 90.1% and 99.0% respectively. The test utilizes a genus-specific monoclonal antibody designed to detect epitopes common to 51 human adenovirus serotypes (Oxoid, Hants, UK). The testing was performed according to the manufacturer's instructions.

4.5.4 Detection of norovirus (Paper II)

Isolation of norovirus in cell cultures has not yet been achieved [126]. Although in some laboratories EM is used for detection of noroviruses, the sensitivity of EM is

low, requiring at least 10^6 viral particles per ml of stool [214]. Therefore direct examination of norovirus using EM is not useful, firstly because of the shedding of low concentration of the virus in stool and secondly it is difficult to differentiate norovirus from other caliciviruses [145]. Immunoassays such as ELISA kits are available and in use for detection of norovirus in stool, however, these test kits are less sensitive compared to RT-PCR for detection of norovirus [215, 216]. Furthermore the sensitivity of ELISA kits is low, less than 70%, therefore results need to be interpreted with caution [142]. Therefore, currently RT-PCR is widely used for detection of noroviruses in stool specimens. There is an increase in the use of real time RT- PCR because it allows rapid detection of the virus [214]. When choosing the RT-PCR for norovirus, there is a need to consider the choice of appropriate primers to be used because of existence of genetic diversity among circulating strains [145]. Several primer pairs from two different regions of the RNA i.e. ORF1, Polymerase region and ORF2, capsid region, have been described [217-225]. In paper II we used single-plex, one step, real time PCR for the detection of norovirus genogroups GI and GII as described in paper II. Primers used in this PCR were previously described [214, 226]. These primers are from the junction of ORF1-ORF2 which is regarded as the most conserved region in the norovirus genome [214]. This method was also chosen because it has already been established and used in the routine microbiology laboratory where the work was performed. Taking into consideration the large number of samples in this study one step real time RT-PCR was the appropriate choice. The details of the PCR parameters can be found in paper II. The advantage of the method chosen used was its ability to detect and differentiate norovirus genogroup I and II at the same time.

4.5.5 PCRs for typing of rotavirus and adenovirus

Several methods can be used for typing of rotavirus. ELISA using serotype specific monoclonal antibodies or polyclonal antibodies has previously used to detect specific G and or P types from stool samples [227-230]. Although useful for typing rotavirus, ELISA may be cumbersome when dealing with a large number of specimens and continuous designing of new monoclonal/polyclonal antibodies against new strains is

required. Since the 1990s multiplexed semi-nested RT-PCR have been developed and used in most countries for typing of rotavirus [231-233]. The European rotavirus surveillance network has developed a multiplex semi-nested RT-PCR for rotavirus and the methods are available online. Primers designed in this method have been widely used in developing as well as developed countries with good results [195, 234-236]. For paper I, we therefore adopted and used the same method which we previously used, multiplex semi-nested RT-PCR for G and P typing [237].

Adenovirus serotyping can be done by neutralization or hemagglutination inhibition or by restriction endonuclease analysis of full-length adenovirus DNA. Both methods involve cell culture which is time consuming and also does not favour fastidious adenoviruses [238, 239]. PCR is useful for fastidious adenoviruses i.e. F40 and F41 to be typed. The choice of method depends on the need to type some or all types of adenovirus. In paper II, real time multiplex PCR was used to type 51 types of adenoviruses with pprimers and TaqMan probes from the conserved region of the hexon-coding gene of adenoviruses [240]. The details of the PCR parameters have been described in paper II.

4.5.6 PCRs for genotyping norovirus

Dual nomenclature systems exist for norovirus genotypes, RNA polymerase region in ORF1 and VP1 sequences [241]. PCR followed by nucleotide sequencing of small regions of ORF1 or ORF2 (CAP) of the norovirus genome are used to genotype norovirus strains [142]. For paper II genotyping of norovirus was done by conventional RT-PCR followed by sequencing of the polymerase region to genotype all norovirus positive samples. This method was chosen because it was previously established, published and used by our collaborators [242]. Randomly selected, 33 samples also underwent real time PCR followed by sequencing of the capsid region using the primers described by Kojima *et al.* [243]. This was done to confirm and compare the results from PCR of the polymerase region.

4.5.7 Sequencing and phylogenetic analysis of rotavirus, norovirus and adenovirus

Sanger sequencing was used for rotavirus, norovirus and adenovirus. This was followed by phylogenetic analysis using the RipSeq interpretation software (Isentio AS., Bergen, Norway) or nucleotide BLAST service (NCBI) for paper I and III. For paper II, genotyping of norovirus and the characterization of norovirus GII.4 variants were done by the norovirus automated genotyping tool [244].

4.6 *Statistical analysis*

Analysis of weight-for age, length for age and weight for length Z-scores was done using EPI Info (USD, Inc., Stone Mountain, GA, USA). The Statistical Package for the Social Sciences (SPSS for IBM-PC, release 20.0; SPSS Inc., Chicago, IL, USA) was used for data analysis, and a p-value of < 0.05 was considered significant. Pearson's Chi-squared test or Fishers exact test were used to compare differences in prevalence of rotavirus/ norovirus and adenovirus infection in cases and control. For paper I, II and III, the age distribution of children in cases and control was compared using Mann-Whitney U test.

Univariate analysis using cross tabulations was performed to assess relationship of norovirus (paper II) and adenovirus positivity (paper III) with season, demographic and clinical characteristic.

In paper I, we assessed the association between rotavirus infection and clinical\demographic characteristics using odds ratio (OR) in a logistic regression model. Because of small number of HIV tested participants, we performed a second regression analysis which included HIV as a factor; this was a manual, backwards, stepwise regression analysis by elimination of non-significant factors with p value of > 0.05.

4.7 Ethical considerations

This study was conducted in accordance with existing ethical guidelines of Muhimbili University of Health and Allied Sciences in Dar es Salaam, Tanzania and the Regional Committee for Medical and Health Research Ethics (REK) in Bergen, Norway. Ethical clearance was obtained from both locations. The permission to conduct the

study was also obtained from the respective Hospital authorities where recruitment of study participants took place. Parent/guardian of the child signed written informed consent form.

Table 2: Demographic and clinical characteristics of the study population

	Paper I and III		Paper II	
Demographic/clinical characteristic	Cases N=690 n (%)	Controls N=545 n (%)	Cases N=705 n (%)	Controls N=561 n (%)
Sex				
Male	422 (61.2)	296 (54.3)	436 (61.8)	306 (54.5)
Female	268 (38.8)	249 (45.7)	269 (38.2)	255 (45.5)
Age groups in months				
0-6	191 (27.7)	120 (22.0)	192 (27.2)	129 (23.0)
7-12	343 (49.7)	211 (38.7)	348 (49.4)	215 (38.3)
13-18	92 (13.3)	159 (29.2)	90 (12.8)	161 (28.7)
19-24	64 (9.3)	55 (10.1)	75 (10.6)	56 (10.0)
Place of residence (District)				
Kinondoni	202 (29.3)	158 (29.0)	211 (29.9)	162 (28.9)
Ilala	338 (49.0)	296 (54.3)	341 (48.4)	305 (54.4)
Temeke	150 (21.7)	91 (16.7)	153 (21.7)	94 (16.8)
Parent level of education				
Primary education	531 (77.0)	413 (75.8)	543 (77.0)	424 (75.6)
Secondary education	137 (19.9)	127 (23.3)	139 (19.7)	130 (23.2)
Higher education	22 (3.2)	5 (0.9)	23 (3.3)	7 (1.2)
Type of diarrhoea				
Acute diarrhoea	611 (88.8)	NA	627 (88.9)	NA
Persistent diarrhoea	79 (11.4)	NA	78 (11.1)	NA
Hydration status				
Dehydration	521 (75.5)	NA	533 (75.6)	NA
Nutrition status				
i)Underweight				
Malnourished	390 (56.5)	217 (39.8)	402 (57.0)	222 (39.6)
ii)Stunting (HAZ)				
Malnourished	469 (68.0)	301 (55.2)	480 (68.1)	306 (54.5%)
iii)Wasting (WLZ)				
Malnourished	200 (28.9)	111 (20.4)	212 (30.1)	117 (20.9)

NA: not applicable

5 Summary of results

5.1 *Characteristics of the study population*

Table 2 shows demographic and clinical characteristics of study population for paper I, II and III. HIV testing results were available on 423 children, of whom 33 and 390 children tested positive and negative, respectively.

5.2 *Overall prevalence of viruses*

At least one of the three viruses was detected in 352 cases, which is 48.8% of the case samples tested. Out of these 352 cases, 326 were infected with only one virus (92.9%) whereas 26 (7.4%) were infected with more than one virus. Among controls at least one virus was detected in 99 (17.6%) of the samples tested, of these 90 (90.9%) had single virus infection and 8 (8.1%) had mixed viral infection.

5.3 *Prevalence of enteric viruses in different age groups*

Among cases there was significant difference in prevalence of viruses in different age groups. Figure 6 shows the prevalences of viruses in different age groups. The peak prevalence of virus infection was seen in the age group 7-12 months (n=183, 52.0%, P= 0.004, OR=2.15, 95% CI: 1.275-3.63). The prevalence of at least one virus was also significantly higher in the age group of 0-6 months compared to older children

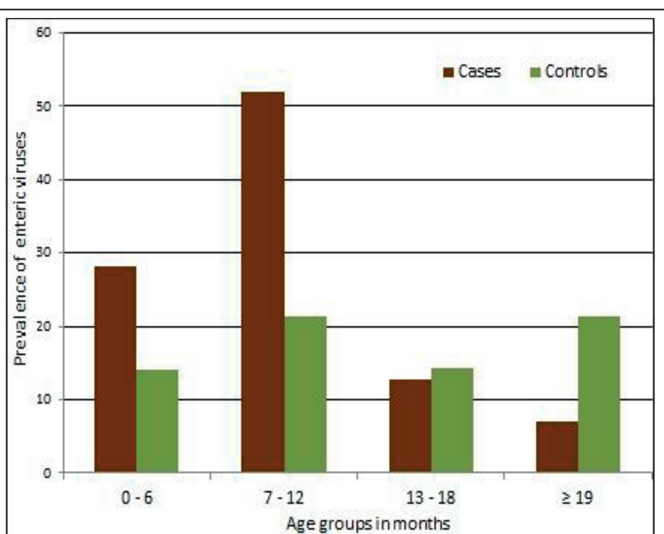

Figure 6: Prevalence of enteric viruses detected in different age groups among cases and controls.

aged 19 months and above (P=0.016, OR=1.98, 95% CI: 1.14-3.45). Overall prevalence of viruses did not differ significantly among controls.

5.4 Paper I

Genetic diversity of circulating rotavirus strains in Tanzania prior to the introduction of vaccination.

Moyo SJ, Blomberg B, Hanevik K, Kommedal O, Vainio K, Maselle SY, Langeland N. *PLoS One.* 2014; **20**;9(5):

In this study, we included 690 children admitted to hospital due to diarrhoea (cases) and 545 children without diarrhoea (controls). The prevalence of rotavirus was significantly higher in cases (32.5%) than in controls (7.7%, P<0.001). The prevalence of rotavirus did not differ significantly between community based controls (7.7%, 24/310) and hospital based controls (7.7%, 18/235, P>0.05).

G-genotypes: Sequencing results detected rotavirus G8 in 33 cases, which were misallocated to G12 when G genotype was assessed by PCR alone. Rotavirus G-typing based on sequencing results of 211 (190 cases and 21 controls) samples which were successfully sequenced, showed that genotype G1 (68.6%), was most common followed by G8 (17.3%), G12 (11.5%) and G4 (2.1%) in cases. Rotavirus G1 also predominated in controls and accounted for 66.7%, followed by G12 (19.0%) and G8 (14.3%). Genotype G2, G3 and G10 were not detected. The Tanzanian G1 variants displayed 94% similarity with the Rotarix vaccine G1 variant.

P-genotypes: Rotavirus P genotypes were detected in 236 samples (211 cases and 25 controls) using RT-PCR. The commonest P genotypes were P[8] 66.8%, P[4] 19.0%, and P[6] 14.2%. We did not detect rotavirus P[9], P[10]and P[11].

Rotavirus G/P combinations were found in 211 samples that were successfully typed for both G and P genotypes (among the 236 P-typed samples, 25 could not be G-typed). The commonest G/P combination in cases was G1P[8] accounting for 123 samples, followed by G8P[4] (n=27) and G12P[6] (n=21).

Rotavirus prevalence was significantly higher in cool (23.9%) than hot months (17.1%) of the year (P=0.012). Rotavirus was most frequently found in the age group of four to six months. The prevalence of rotavirus in cases was lower in stunted children (28.9%) than in non-stunted children (40.1%, P=0.003) and significantly lower in HIV-infected (15.4%, 4/26) than in HIV-uninfected children (55.3%, 42/76, P<0.001).

5.5 Paper II

Genetic diversity of norovirus in hospitalised diarrhoeic children and asymptomatic controls in Dar es Salaam, Tanzania

Moyo S, Hanevik K, Blomberg B, Kommedal O, Vainio K, Maselle S, Langeland N. *Infect Genet Evol*. 2014; 26:340-7.

This study included 705 cases and 561 controls. Norovirus was detected in 14.3%, 181/1266 children. The prevalence of norovirus was significantly higher in cases (18.3%, 129/705) than in controls, (9.2%, 52/561, P < 0.02, OR = 2.2, 95% CI: 1.56–3.09*)*. The prevalence of norovirus did not differ among the two control groups; community based controls (9.3%, 29/312) and hospital based controls (9.2%, 23/249).

Genogroups: Except for one child who had double infection with GI and GII all 129 cases had norovirus GII. Among norovirus positive controls, 23.1% had GI and 76.9% had GII. The proportion of norovirus GI was significantly higher in CBC 34.5%, (10/29) than in HBC 8.7%, (2/23), whereas GII was significantly higher in HBC 91.3% (21/23) than in CBC (65.5%, 19/29, P = 0.028, OR = 5.5, 95% CI: 1.1–28.5).

Genotypes: For cases, 108 GII samples were genotyped and for controls 23 and four samples were GII and GI genotyped respectively. Norovirus GII.4 predominated, both in cases and controls, but it was significantly more prevalent in cases than in controls (87.9% vs. 56.5%, P = 0.001).

Other genotypes detected among cases were GII.16 (4.6%), GII.21 (3.7%), GII.g (2.8%) and GII.13 (0.9%). Apart from GII.4, other genotypes detected in controls were GII.21 (26.1%), GII.g (8.7%) and one sample of each of GII.16 and GII.e

The prevalence of norovirus was significantly higher during the first than the second year of life (109/540, 20.2% vs. 20/165, 12.1% P = 0.02, OR 1.81, 95% CI: 1.08–3.02). In controls, there was no significant difference in the prevalence of norovirus in children below and above one year.

The highest numbers of norovirus were detected in the month of April 2011 which is the month of long rains and the beginning of cold season. However, we did not find significant differences in number of norovirus detected between dry and rainy season in cases (109/580, 18.8%, vs. 20/125, 16.0%, P = 0.46) nor in controls (7.9%, 19/239 vs. 10.2%, 33/323, P = 0.36).

The prevalence of norovirus in HIV-positive and negative children was (21.2%, 7/33) and (10.3%, 40/390, P = 0.05) respectively, regardless of diarrhoea symptoms. No significant difference in gender, parent's level of education or nutritional status with norovirus infection was observed within cases or controls.

5.6 Paper III

Detection and molecular characterisation of human adenovirus in diarrhoeic children of Dar es Salaam, Tanzania; a case control study

Moyo S, Kurt Hanevik, Bjørn Blomberg, Oyvind Kommedal, Svein Arne Nordbø, Samuel Maselle, Nina Langeland.(submitted)

This study included 690 children admitted to hospital due to diarrhoea (cases) and 545 children without diarrhoea (controls). Human adenovirus (HAdV) was detected in 37 children, corresponding to a prevalence of 3.5% (24/690) in diarrhoeic and 2.4% (13/545) in non-diarrhoeic children (P>0.05). There was no significant difference in the prevalence's of HAdV in the two control groups CBC (3.2%, 10/310) and HBC (1.3%, 3/235, P=0.14).

HAdV types: The proportions of enteric adenoviruses (type 40 and 41) were not significantly different in diarrhoeic and non-diarrhoeic children (50%, 12/24 vs. 46%, 6/13, P=0.82).

Other HAdV types detected were; 1, 2, 7, 18, 19 and 31.

Among HAdV-infected children, the median age was significantly lower in diarrhoeic than in non-diarrhoeic children (10 vs. 14 months, p<0.001). More than half of HAdV infected (54.2%) were dehydrated as compared to diarrhoeic children without HAdV (45.8%, P=0.01).

The prevalence of adenovirus was not significantly different between rainy and dry seasons. HAdV was not detected in the 33 known HIV positive children. There was no significant association between HAdV infection and gender, nutritional status of the child and parent educational level.

6 Discussion

6.1 Discussions of the main findings

6.1.1 Prevalence of enteric viruses

Diarrhoea is one of the most common diseases and a significant cause of morbidity and mortality worldwide. Viruses are recognized as important causes of diarrhoea especially in children during their first years of life. A number of viral agents are associated with diarrheal disease in children with three of these being most common, namely; rotavirus, norovirus and human adenovirus. This study was conducted in order to better understand the clinical and molecular epidemiology of enteric viruses causing diarrhoea in young children. The present study used real time PCR and ELISA to detect the viruses causing diarrhoea in children. One or more viruses were detected in nearly half of the samples tested, 48.8% among children admitted to hospital due to diarrhoea. This is higher compared to our previous study which found at least one virus in 32% of children. Prevalences reported in other studies also differ with regard to presence of viruses, ranging from 30% to 67% in Tunisia, Venezuela, Turkey, southeast China, and China [161, 213, 245-247]. Several factors may explain

the observed discrepancies in reported prevalences of enteric viruses. One is the detection methods used, some methods are more sensitive than others, such as PCR vs ELISA for norovirus [248-250]. The second reason is different seasons when the studies were conducted. Some viruses such as rotavirus shows peak prevalence in certain months of the year, therefore studies conducted during the peak season report higher prevalences [247] compared to studies conducted during non-peak season [195]. The other reasons are the number of enteric viruses detected. If the study has included the most common viruses it is likely to find higher prevalence of viruses than studies which have omitted some very common viruses. For example a study conducted in Tunisia [213] did not search for norovirus, whereas norovirus is known to be the second common to rotavirus in causing diarrhoea[251]. Different age groups or different inclusion criteria of the study population included and geographical variations may also be reasons for the observed variations. Of the three viruses detected in the present studies, rotavirus was the most frequent followed by norovirus and adenovirus. Children admitted with diarrhoea were six times more likely to be infected with rotavirus than those without diarrhoea. The study confirms findings from other studies twenty years ago in the same location and elsewhere [252, 253] indicating that rotavirus is still a major pathogen causing diarrhoea in children in Tanzania. The presence of rotavirus among controls may represent reservoirs for transmission in the community. Norovirus was the second common virus detected, which is similar to our previous study [195]. Despite using the same method for detection of norovirus i.e. RT-PCR, there is much variation in the reported prevalence in African countries ranging from 6.5% to 25.5% in Malawi, Tunisia, South Africa, Ghana, Ethiopia, Libya, Botswana and Nigeria [75, 254-259]. This could be due to different age groups included in these studies or geographical variation. The prevalence of norovirus in children without diarrhoea, both community controls and hospital controls, was significantly lower compared to children with diarrhoea as reported in most other studies [260-264]. The findings from these studies, and results from the present study, confirm the role of norovirus in causation of diarrhoeal disease, although in some countries the prevalence of norovirus infection has been found to be similar in children with and without diarrhoea [258, 265]. Human

adenovirus was the least frequent virus detected in our study. When we compared adenovirus prevalence in this study with reports from other African countries, we observe large variations. Studies detecting human adenovirus by PCR, which is known to be more sensitive, have reported higher prevalences [165, 166, 209] compared to studies employing EIA [212, 213]. The finding of an almost similar prevalence of human adenovirus in diarrhoeic and non-diarrhoeic children, but at higher median age of infection in non-diarrhoeic children, suggest prolonged shedding of adenovirus in stool after previous infection more than one month prior to the study. Alternatively, it could be asymptomatic adenovirus infections in children who may have acquired immunity from previous infections.

6.1.2 *Mixed viral infections*

In this study we observed a relatively high percentage (7.4%) of dual viral infections among positive samples in cases, most were a combination of rotavirus and norovirus (18/26), and followed by norovirus and adenovirus (6/26) and two were rotavirus and adenovirus. The prevalence of dual infection was not statistically different from what we reported in the previous study (8.0%) in the same study setting [195]. Other studies have reported almost similar rate of dual viral infections in children [213, 247, 266]. Whether a single virus is the main reason for the diarrheal illness or whether they potentiate each other still remains unclear. Mixed infections with viruses, bacteria and/or parasites may also occur, but was not investigated in this study.

6.1.3 *Distribution of enteric viruses by age groups*

We observed the peak age of viral infection in infants aged 7-12 months in cases (52.0%). This could be due to decreased protection from maternal antibodies which starts to decline at this age. The relatively high prevalence of enteric viruses in infants less than 6 months in cases may suggest lack of complete protection from maternal antibodies. There was a decline of viral infection in children aged above 13 months. The low prevalence of viruses in these older children could be partly due to immunity acquired through previous exposures. The mean age of rotavirus infection in this study compares with the mean age of rotavirus diarrhoea in studies from other developing

countries, which ranges from 6–9 months [267]. The median age of symptomatic norovirus infection was 9 months, which is in agreement with results from Libya [255]. The high prevalence of norovirus infection in children below 1year has also been documented in many other developing countries, suggesting that children tend to become infected at a young age [255, 258, 268, 269]. For adenovirus, the median age was significantly higher in adenovirus-infected children than adenovirus non-infected children in our study (12 vs. 10 months).

6.1.4 *Molecular epidemiology of viral infections*

This is the first report to present comprehensive molecular diversity for three important viruses causing diarrhoea in children in Tanzania. PCR and sequencing were used to characterise the viruses in this study. We found wide genetic diversity of all the three viruses examined.

Rotavirus

Among rotavirus, we detected five G and three P genotypes. The most common G/P combination was G1[P8]. This genotype combination is responsible for 50–65% of rotavirus infections in children worldwide [270]. In this study we found more than 60% of the study participants were affected by G1[P8] which is the genotype combination included in the current vaccine introduced in Tanzania (Rotarix). We therefore assume that the vaccine will be protective, given that the circulating genotype is stable. The second common genotype detected was G8. We report for the first time the presence of rotavirus genotype G8 and G12 in Tanzania. G12 genotype was previously documented only in three African countries, namely Malawi, South Africa and Nigeria where it was detected by using sequencing [73-75]. Rotavirus genotype G9 was the commonest genotype in our previous study which was conducted in Dar es Salaam eight years earlier [195]. However, in the present study only one strain of G9 in combination with P[8] was found. This emphasizes the need for continuous rotavirus strain surveillance.

Through phylogenetic analysis, the results of this study showed that the rotavirus G1P[8] strains from the studied population are distantly related to G1P[8] of the

vaccine strains in the Rotarix and RotaTeq vaccines. This suggests that circulating G1P[8] strains may have changed over time through accumulated mutations making them different from original vaccine strains which were isolated over twenty-six years ago [271, 272]. In this study we observed an increase in the prevalence of P[6] and P[4] rotavirus positive samples compared to the previous study in the same study setting [195]. Our results showed that genotype P[6] in this study is closely related to P[6] from other African countries such as Malawi and South Africa. Lower vaccine efficacy has been reported in developing countries [273]. This may partly be due high prevalence of circulating genotypes which are not included in the vaccine such as G8, G12, P[4] and P[6]. However, other possible contributing factors such as maternal antibodies, chronic enteropathy, and malnutrition need to be investigated [274, 275].

Norovirus

Among detected noroviruses, seven different norovirus genotypes were found. The presence of norovirus diversity has also been observed in other African countries such as South Africa, Tunisia and Malawi which shows that several different genotypes can co-exist at the same time [256, 258, 259]. The phylogenetic analysis of the norovirus, based on both RdRp and capsid region in the current study, showed predominance of norovirus GII.4 genotype, this is in agreement with previous studies conducted in the African region [134, 255, 256, 258, 259, 276-278]. This genotype has been circulating worldwide from the mid-1990s to recent years [279-286], confirming that it plays a major role in causing diarrhoea in children. Throughout the study period all detected norovirus GII.4 clustered with strain New Orleans 2009. We are reporting presence of this strain in Tanzania 2 years after it was first detected in winter 2009 in the USA [287] where it caused outbreaks. Since its emergence, the New Orleans strain has spread widely and has been reported in other countries such as Finland, Brazil and Vietnam etc.[280, 288, 289]. This is the first report of norovirus genotype GII.21 in an African region. The genotype was detected in children with and without diarrhoea. Norovirus GII.21 was first detected in humans in 2002 in Japan from patients with gastroenteritis and was reported as norovirus GII.16 [226, 290]. This genotype has also been reported recently in Brazil [291].

Adenovirus

Sequence analysis showed that a wide variety of human adenovirus species (five) and types (nine) circulate among diarrhoeic and non-diarrhoeic children in Dar es Salaam. Half of the adenovirus types were enteric adenovirus 40 and 41 in both diarrhoeic and non-diarrhoeic children. In the current cross sectional study, adenovirus type 40 and 41 occurred at equal frequency in diarrhoeic children. This observation is consistent with what has been reported in other studies [292, 293]. In non-diarrhoeic children, adenovirus type 40 was more prevalent than type 41. Some studies have reported antigenic drift of adenovirus type 41, whereby an increase of adenovirus type 41 at the expense of adenovirus type 40 has been observed. In order to detect serotype drift in the study setting, future studies are needed over a prolonged period of time, as reported elsewhere [36, 294, 295]. Human adenovirus types 1, 2, 3, and 7 which are associated with respiratory infections [173, 296], are also shown to be associated with diarrhoeal disease [179]. In the present study these adenovirus types were detected from both diarrhoeic and non-diarrhoeic children. When these HAdV types infect respiratory sites, they can be shed in the faeces of an infected person for months [173, 296]. Hence findings of this study partly support the theory of prolonged shedding of these human adenovirus species in faeces. However, we cannot rule out their role in diarrhoea aetiology because these types have also been reported to cause diarrhoea [179].

6.1.2 Seasonality of viral infections

Seasonality is an important feature of viral diarrhoea; most studies have demonstrated that viral diarrhoea peak during winter season. In paper I, we observed a high prevalence of rotavirus infection during the cooler months of the year. This is in agreement with studies conducted during cool months of the year [253, 297] which found high prevalences of rotavirus infection compared to studies conducted during hot months of the year [195, 298] in Dar es Salaam, Tanzania. In paper II, we observed a high number of norovirus infections during the month of April, which is regarded as a month of long rains and beginning of the cool months of the year in our

study setting. Therefore this finding partly supports winter month seasonality of norovirus associated diarrhoea, as documented by several studies in the past [247, 299, 300]. However, there were no significant differences in number of norovirus positive children during the rainy season compared to the dry season in this study. In paper III, human adenoviruses were found to be more prevalent during the short rain months of the study period, but not for the two rainy seasons combined. However, other studies have reported no specific seasonal pattern of adenovirus infection [36, 37]. Proper understanding of seasonality of viral infections may provide not only some insights into the possible modes of transmission of viruses but also is useful when considering the appropriate timing of immunization booster programs. This will be applicable in regions which have reported low efficacy of rotavirus vaccine and have demonstrated strong seasonality. Rotavirus vaccine is administered at the age of 6, 10 and 14 weeks in developing countries. However, if booster vaccination programs were to be considered for older children lacking immunity, vaccination during the pre-rotavirus season has been recommended [30].

6.1.3 Association between enteric viruses and HIV

Infection with HIV is common among children of sub-Saharan Africa countries, and diarrhoeal disease is a leading cause of morbidity and mortality in HIV-infected children in these areas [22, 23]. In paper I, we found significantly lower incidence of rotavirus in HIV infected children compared to HIV negative among cases. This is in agreement with findings of previous studies in Tanzania and other developing countries where rotavirus is not found at all or found at low prevalence among HIV-positive children with diarrhea [22, 234, 301-304]. In paper II, we found a higher prevalence of norovirus infection in HIV infected children than HIV negative children, but the association was not statistically significant. Our finding is different from a previous study in the same study setting [301] which reported a significant association of HIV infection and norovirus. The lack of significant association in the current study can partly be explained by the inclusion of acute/persistent diarrhoea, while the previous study included only children with chronic diarrhoea which is

reported to be common in HIV infected individuals [146]. In paper III, adenovirus was not detected in HIV infected children, which is in agreement with studies conducted in the neighbouring country Kenya and elsewhere [166, 302]. These studies suggest that adenovirus is not an important cause of diarrhoea in young HIV positive children. Understanding the causative agents of diarrhoea in HIV infected children needs further investigation.

6.2 Methodological discussion

6.2.1 Study design, study population and study participants

Although this was a case control study, it was not matched by age and sex, and hence may not an ideal study design. However, the large samples size of our study enabled us to do stratification of data by age and sex during analysis to take care of confounding, and multivariate analysis was also used for paper I. The source population used for cases was from secondary and tertiary hospitals and as a result the study used a specialized study population. This could be a limiting factor because we dealt with only severe cases admitted in these hospitals. However, this study was designed for analysis of the viruses in hospitalized diarrhoeic children, these are severe cases of gastroenteritis and hence make the source population appropriate for this purpose.

The two control groups used in this study were from child health clinics or children admitted due to diseases other than diarrhoea. Although the prevalence of viruses was significantly lower in these controls compared to cases, the control groups may not be appropriate, because they may not represent the same source population from which cases arose. An alternative way was to obtain controls from a well-defined source population where cases reside and make the study a population based study.

6.2.2 Laboratory methods

Extraction of nucleic acids is one of the crucial stages to get optimal PCR results [145]. Extracted RNA or DNA should be free from inhibitors of the PCR reaction. To avoid getting a false-negative result because of interference from inhibitors, an internal control must be incorporated in every molecular assay [199, 305]. In the present study MagNA Pure LC 2.0 (Roche) with total nucleic acid extraction kit was used. The method may not be able to remove all inhibitory substances present in stool. This insufficient removal of stool inhibitors is also expected in other extraction methods available [199]. We did not include internal controls in our PCR methods;

hence our results could have been affected by false negatives from stool inhibitors. This was especially seen when typing rotavirus, not all rotavirus ELISA positive samples were PCR positive. However all adenovirus ELISA positive samples were positive in PCR analysis during typing. If indeed stool inhibitors were present in the extracted nucleic acids, this may have caused underestimation of norovirus prevalence which was detected using direct RT-PCR. The study could thus have benefited from inclusion of internal controls in each PCR run.

ELISA was used for detection of rotavirus and adenovirus. Studies have indicated that ELISA is cheaper, easier to perform and relatively cheap compared to PCR [207, 306, 307]. At present, ELISA is most widely used for detection of these viruses, and we found prevalences of these viruses comparable to other studies in developing countries

	Sequence	Binding site base positions
Primer_G8-F	5'-TTRTCGCACCATTTGTGAAAT-3'	176-198
Our G8 variant A	5'-TTGTCACACCATTCGTAAACT-3'	176-198
Our G8 variant B	5'-TTGTCATACCATTCGTAAACT-3'	176-198
Primer_G12-F	5'-GGTTATGTAATCCGATGGACG-3'	548-567
Our G8 variant A+B	5'-GGTTATGCAATCCAATGGACA-3'	534-543

Figure 7: R otavirus G8 primers used and mismatching on during sequencing
Red underscored letters indicate positions with a mismatch against the above primer. Without performing sequencing, we could not only have reported wrong molecular epidemiology of rotavirus but also underestimated the presence of rare emerging genotypes such as G8 in Tanzania.

[212, 213, 253, 298, 308, 309]. Although PCR is reported to be more sensitive compared to ELISA for detection of these viruses, it may not be relevant for epidemiological studies, as well as routine diagnostics in developing countries, because of shedding of large amounts of viral particles in stool.

Semi nested RT-PCR is widely used for typing of rotaviruses both in developing and developed countries. Mistyping of rotavirus using PCR can result, due to constant accumulation of point mutations through genetic drift; emergence of novel genotypes; and possibly zoonotic transmission and subsequent assortment. In paper I we observed mistyping of rotavirus using RT-PCR, this was also reported by other studies [75, 310-312]. The strength of this paper was that sequencing was also used to confirm PCR

typing results. In paper I, we first used semi nested multiplex RT-PCR for G and P typing of all ELISA positive rotavirus samples. But sequencing of 43 randomly selected samples revealed that eight rotavirus G8 samples were mistyped as rotavirus G12. This necessitated re -analysing all G types by using sequencing. We then found that 33 G8 samples were mistyped as G12 using RT-PCR. Figure 7 below shows mismatch of primes of G8 used for RT-PCR. In the present study we observed two variants of rotavirus G8 A and B through sequencing, variant A was the most common G8 sequence variant. The Tanzanian rotavirus G8 variants displayed 3-4 mismatches against the G8-primer whereof 3 mismatches are located in the 3'-end portion. The 3'-terminal mismatch will be removed by the proof-reading polymerase leaving only 2 effective mismatches in the mid-part of the rotavirus G12-primer. This unintended binding site lies close to the expected binding-site on true rotavirus G12 isolates producing amplicons indistinguishable from true G12 amplicons. Without performing sequencing, we could not only have reported wrong molecular epidemiology of rotavirus but also underestimated the presence of rare emerging genotypes such as G8 in Tanzania. Figure 8 shows molecular epidemiology or rotavirus in this study using PCR and sequencing results. A different and a wrong molecular epidemiology could have been reported in this study if sequencing was not used.

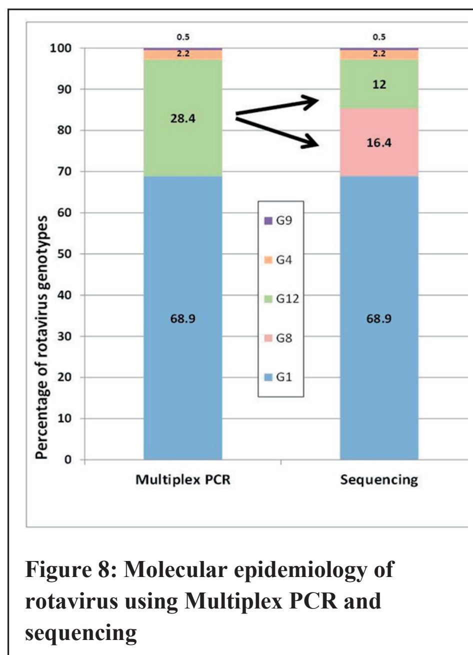

Figure 8: Molecular epidemiology of rotavirus using Multiplex PCR and sequencing

7 Study limitations

This study dealt with only three viruses known to be common in the setting. Other viruses such as sapoviruses, astroviruses and coronaviruses were not investigated. This may have underestimated the burden of viral diarrhoea in children in the study setting. In order to fully understand the aetiology of viral diarrhoea, a study which will search for more viruses is needed.

Acute or chronic diarrhoea can be caused by a combination of viruses, bacteria or parasites. As this study dealt with viruses only, it is difficult to ascertain if diarrhoea was caused by viruses or not. This is especially important when trying to associate a causative agent with clinical features such as dehydration and the type of diarrhoea, acute vs. persistent diarrhoea.

There were missing data in some months during the study period. This could have affected the assessment of seasonality of viral infections. This was especially observed in paper III were a clear seasonality was not found. Despite this limitation of missing data in some months during the study, in paper I we were able to show a clear pattern of seasonality for rotavirus.

This study was conducted in one region of Tanzania only, Dar es Salaam. In order to better understand the strain distribution of rotavirus which has been reported to show geographical variations even within the same country [313], a comprehensive study involving other regions of Tanzania may be required. This is especially important for viruses for which vaccines are available such as rotavirus.

Data on breast feeding is missing, this could have given additional information on how breast feeding protect against viral infections.

HIV testing results was not available for all study participants. A relatively small number of children with diarrhoea were tested for HIV. The small sample size for tested HIV participants lowered the power of the study.

Unsuccessful sequencing of a number of positive samples for both rotavirus and norovirus may have underestimated presence of other genotypes which could have been detected if sequencing was successful.

8 Conclusions

Based on the objectives of this study, the following conclusions were reached:

1. Viruses contribute a high burden of diarrhoea in hospitalised children of Dar e Salaam Tanzania. Children aged 7-12 months were especially affected.
2. There is high asymptomatic carriage of diarrhoea-associated viruses in children (paper I, II and III), which may be explained by prolonged shedding of the virus from previous infection, or new viral infection without symptoms because of low infective dose. These children may serve as a source of infection in the community.
3. Pre-rotavirus vaccination data in Tanzania (Paper I) showed predominance of rotavirus genotype G1, which is phylogenetically distantly related to the vaccine strains. We also observed the emergence of rotavirus genotype G8 and G12.
4. Genetic analysis of norovirus causing infection in Tanzania (Paper II) showed predominance of norovirus GII.4 both in diarrhoeic cases and among controls.
5. We showed high genetic diversity of human adenovirus types that circulate in the study setting, with equal proportion of enteric types and non-enteric types in cases and controls.
6. Rotavirus seasonality, as seen in Paper I, provides insights important for vaccination strategies, including potential shifts in seasonal peaks and duration of outbreaks. No clear pattern of seasonality was seen in norovirus infection (Paper II) and adenovirus infection (Paper III).
7. Our data suggests that rotavirus may not be an opportunistic pathogen in children infected with HIV (Paper I). We did not see an association between HIV and norovirus infection (Paper II) or adenovirus infection (Paper III).

9 Recommendations

Due to our findings of mistyping of rotavirus when using PCR to identify strains, due to higher mutation-rates in viral genomes, we recommend to interpret rotavirus typing results with caution when G typing is based on multiplex PCR. Furthermore, our

results emphasize the higher robustness for typing of rotaviruses obtained by sequencing.

This study and pervious ones have shown that norovirus is second to rotavirus in causing diarrhoea in children hospitalised for diarrhoeal disease. There is no licenced vaccine for norovirus, the efforts to reduce disease burden have focused on effective disease surveillance and limiting disease transmission. There is no routine diagnosis of norovirus in our study setting, without which diagnosis and detection of norovirus in diarrhoeic children is difficult. This will hinder detection of an outbreak due to norovirus, in which the virus has been reported to have severe outcomes and death [122, 314]. We therefore recommend routine testing of norovirus to be established in the study setting for proper prevention and control measures.

10 Future perspectives

Diarrhoea continues to be a major health problem in children worldwide. Detection of enteric viruses using proper diagnostic methods will add important knowledge on the contribution of each virus in diarrhoea and hence proper preventive measures to be put in place. Due to the possibility of change in pattern of circulating serotypes/genotypes of viruses causing diarrhoea, it will be important to continue rotavirus surveillance in the study setting. This phenomenon was observed in paper I when compared to our previous study [195]. Rotavirus vaccination was started in Tanzania in 2013, and it will be important to conduct studies post rotavirus vaccination period, to evaluate the efficacy of the vaccine or if there is reduced incidence of hospitalization due to rotavirus diarrhoea or if this is overtaken by other viruses such as norovirus.

Further studies need to be conducted for longer periods of time, more than a year with equal number of samples every month, in order to better understand seasonality of these viruses, as well as to detect any shift of serotypes in virus infection such as adenovirus. Secondly, it will help to confirm consistency of the seasonal trends observed in this study.

Although this study used control groups, these were not necessarily children living in the same community as cases, future studies need to be done to include controls from

the community population in order to differentiate new infection or reinfection of the virus from prolonged carriage of the virus.

Adding analysis of bacterial, as well as parasitic pathogens will be useful to give a clearer picture of the role mixed infections in diarrhoea.

Further studies which will include a higher number of HIV infected participants is required to understand the burden of norovirus and adenovirus in HIV infected children.

References

1. Bern C, Martines J, de Zoysa I, Glass RI: **The magnitude of the global problem of diarrhoeal disease: a ten-year update**. *Bulletin of the World Health Organization* 1992, **70**(6):705-714.

2. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, Jha P, Campbell H, Walker CF, Cibulskis R *et al*: **Global, regional, and national causes of child mortality in 2008: a systematic analysis**. *Lancet* 2010, **375**(9730):1969-1987.

3. Kosek M, Bern C, Guerrant RL: **The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000**. *Bulletin of the World Health Organization* 2003, **81**(3):197-204.

4. Snyder JD, Merson MH: **The magnitude of the global problem of acute diarrhoeal disease: a review of active surveillance data**. *Bulletin of the World Health Organization* 1982, **60**(4):605-613.

5. Walker CL, Rudan I, Liu L, Nair H, Theodoratou E, Bhutta ZA, O'Brien KL, Campbell H, Black RE: **Global burden of childhood pneumonia and diarrhoea**. *Lancet* 2013, **381**(9875):1405-1416.

6. Rohde JE, Northrup RS: **Taking science where the diarrhoea is**. *Ciba Foundation symposium* 1976(42):339-366.

7. O'Ryan M, Prado V, Pickering LK: **A millennium update on pediatric diarrheal illness in the developing world**. *Seminars in pediatric infectious diseases* 2005, **16**(2):125-136.

8. WHO, UNICEF: **Model IMCI handbook: Integrated management of childhood illness.** , vol. Document no WHO/FCH/CAH/00.12; 2005.

9. Luby SP, Agboatwalla M, Painter J, Altaf A, Billhimer WL, Hoekstra RM: **Effect of intensive handwashing promotion on childhood diarrhea in high-risk communities in Pakistan: a randomized controlled trial**. *JAMA : the journal of the American Medical Association* 2004, **291**(21):2547-2554.

10. Checkley W, Gilman RH, Black RE, Epstein LD, Cabrera L, Sterling CR, Moulton LH: **Effect of water and sanitation on childhood health in a poor Peruvian peri-urban community**. *Lancet* 2004, **363**(9403):112-118.

11. Motarjemi Y, Kaferstein F, Moy G, Quevedo F: **Contaminated weaning food: a major risk factor for diarrhoea and associated malnutrition**. *Bulletin of the World Health Organization* 1993, **71**(1):79-92.

12. Huffman SL, Combest C: **Role of breast-feeding in the prevention and treatment of diarrhoea**. *Journal of diarrhoeal diseases research* 1990, **8**(3):68-81.

13. Ip S, Chung M, Raman G, Chew P, Magula N, DeVine D, Trikalinos T, Lau J: **Breastfeeding and maternal and infant health outcomes in developed countries**. *Evidence report/technology assessment* 2007(153):1-186.

14. Duijts L, Jaddoe VW, Hofman A, Moll HA: **Prolonged and exclusive breastfeeding reduces the risk of infectious diseases in infancy**. *Pediatrics* 2010, **126**(1):e18-25.

15. Maponga BA, Chirundu D, Gombe NT, Tshimanga M, Shambira G, Takundwa L: **Risk factors for contracting watery diarrhoea in Kadoma City, Zimbabwe, 2011: a case control study**. *BMC infectious diseases* 2013, **13**:567.

16. Arifeen S, Black RE, Antelman G, Baqui A, Caulfield L, Becker S: **Exclusive breastfeeding reduces acute respiratory infection and diarrhea deaths among infants in Dhaka slums**. *Pediatrics* 2001, **108**(4):E67.

17. Quigley MA, Kelly YJ, Sacker A: **Breastfeeding and hospitalization for diarrheal and respiratory infection in the United Kingdom Millennium Cohort Study**. *Pediatrics* 2007, **119**(4):e837-842.

18. Feachem RG, Koblinsky MA: **Interventions for the control of diarrhoeal diseases among young children: promotion of breast-feeding**. *Bulletin of the World Health Organization* 1984, **62**(2):271-291.

19. Teka T, Faruque AS, Fuchs GJ: **Risk factors for deaths in under-age-five children attending a diarrhoea treatment centre**. *Acta paediatrica* 1996, **85**(9):1070-1075.

20. Yoon PW, Black RE, Moulton LH, Becker S: **The effect of malnutrition on the risk of diarrheal and respiratory mortality in children < 2 y of age in Cebu, Philippines**. *The American journal of clinical nutrition* 1997, **65**(4):1070-1077.

21. Kotloff KL, Johnson JP, Nair P, Hickman D, Lippincott P, Wilson PD, Clemens JD: **Diarrheal morbidity during the first 2 years of life among HIV-infected infants**. *JAMA : the journal of the American Medical Association* 1994, **271**(6):448-452.

22. Pavia AT, Long EG, Ryder RW, Nsa W, Puhr ND, Wells JG, Martin P, Tauxe RV, Griffin PM: **Diarrhea among African children born to human immunodeficiency virus 1-infected mothers: clinical, microbiologic and epidemiologic features**. *The Pediatric infectious disease journal* 1992, **11**(12):996-1003.

23. Thea DM, St Louis ME, Atido U, Kanjinga K, Kembo B, Matondo M, Tshiamala T, Kamenga C, Davachi F, Brown C *et al*: **A prospective study of diarrhea and HIV-1 infection among 429 Zairian infants**. *The New England journal of medicine* 1993, **329**(23):1696-1702.

24. Rohayem J: **Norovirus seasonality and the potential impact of climate change**. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2009, **15**(6):524-527.

25. Cook SM, Glass RI, LeBaron CW, Ho MS: **Global seasonality of rotavirus infections**. *Bulletin of the World Health Organization* 1990, **68**(2):171-177.

26. Levy K, Hubbard AE, Eisenberg JN: **Seasonality of rotavirus disease in the tropics: a systematic review and meta-analysis**. *Int J Epidemiol* 2009, **38**(6):1487-1496.

27. Pitzer VE, Viboud C, Simonsen L, Steiner C, Panozzo CA, Alonso WJ, Miller MA, Glass RI, Glasser JW, Parashar UD *et al*: **Demographic variability, vaccination, and the spatiotemporal dynamics of rotavirus epidemics**. *Science* 2009, **325**(5938):290-294.

28. Atchison CJ, Tam CC, Hajat S, van Pelt W, Cowden JM, Lopman BA: **Temperature-dependent transmission of rotavirus in Great Britain and The Netherlands**. *Proc Biol Sci* 2010, **277**(1683):933-942.

29. D'Souza RM, Hall G, Becker NG: **Climatic factors associated with hospitalizations for rotavirus diarrhoea in children under 5 years of age**. *Epidemiol Infect* 2008, **136**(1):56-64.

30. Jagai JS, Sarkar R, Castronovo D, Kattula D, McEntee J, Ward H, Kang G, Naumova EN: **Seasonality of rotavirus in South Asia: a meta-analysis approach assessing associations with temperature, precipitation, and vegetation index**. *PLoS One* 2012, **7**(5):e38168.

31. Verhoef L, Depoortere E, Boxman I, Duizer E, van Duynhoven Y, Harris J, Johnsen C, Kroneman A, Le Guyader S, Lim W *et al*: **Emergence of new norovirus variants on spring cruise ships and prediction of winter epidemics**. *Emerging infectious diseases* 2008, **14**(2):238-243.

32. Fretz R, Herrmann L, Christen A, Svoboda P, Dubuis O, Viollier EH, Tanner M, Baumgartner A: **Frequency of Norovirus in stool samples from patients with gastrointestinal symptoms in Switzerland**. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 2005, **24**(3):214-216.

33. de Jong JC: **Epidemiology of enteric adenoviruses 40 and 41 and other adenoviruses in immunocompetent and immunodeficient individuals**. In: *Viral gastronenteritis.* edn. Edited by Desselberger U, Gray J. Amtserdam, The Netherlands: Elsevier Science B.V.; 2003: 407-445.

34. Dey SK, Shimizu H, Phan TG, Hayakawa Y, Islam A, Salim AF, Khan AR, Mizuguchi M, Okitsu S, Ushijima H: **Molecular epidemiology of adenovirus infection among infants and children with acute gastroenteritis in Dhaka City, Bangladesh**. *Infect Genet Evol* 2009, **9**(4):518-522.

35. Barnes GL, Uren E, Stevens KB, Bishop RF: **Etiology of acute gastroenteritis in hospitalized children in Melbourne, Australia, from April 1980 to March 1993**. *Journal of clinical microbiology* 1998, **36**(1):133-138.

36. de Jong JC, Bijlsma K, Wermenbol AG, Verweij-Uijterwaal MW, van der Avoort HG, Wood DJ, Bailey AS, Osterhaus AD: **Detection, typing, and subtyping of enteric adenoviruses 40 and 41 from fecal samples and observation of changing incidences of infections with these types and subtypes**. *Journal of clinical microbiology* 1993, **31**(6):1562-1569.

37. Soares CC, Volotão EM, Albuquerque MCM, da Silva FM, de Carvalho TRB, Nozawa CM, Linhares RE, Santos N: **Prevalence of enteric adenoviruses among children with diarrhea in four Brazilian cities**. *Journal of Clinical Virology* 2002, **23**(3):171-177.

38. O'Ryan M, Prado V, Pickering LK: **A millennium update on pediatric diarrheal illness in the developing world**. *Seminars in pediatric infectious diseases* 2005, **16**(2):125-136.

39. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, Wu Y, Sow SO, Sur D, Breiman RF *et al*: **Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric Multicenter Study, GEMS): a prospective, case-control study**. *Lancet* 2013, **382**(9888):209-222.

40. Moyo SJ, Gro N, Matee MI, Kitundu J, Myrmel H, Mylvaganam H, Maselle SY, Langeland N: **Age specific aetiological agents of diarrhoea in hospitalized children aged less than five years in Dar es Salaam, Tanzania**. *BMC Pediatr* 2011, **11**:19.

41. Kapikian AZ: **Overview of viral gastroenteritis**. *Archives of virology Supplementum* 1996, **12**:7-19.

42. Parashar UD, Bresee JS, Gentsch JR, Glass RI: **Rotavirus**. *Emerging infectious diseases* 1998, **4**(4):561-570.

43. Kapikian AZ, Wyatt RG, Dolin R, Thornhill TS, Kalica AR, Chanock RM: **Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis**. *Journal of virology* 1972, **10**(5):1075-1081.

44. Bishop RF, Davidson GP, Holmes IH, Ruck BJ: **Virus particles in epithelial cells of duodenal mucosa from children with acute non-bacterial gastroenteritis**. *Lancet* 1973, **2**(7841):1281-1283.

45. Flewett TH, Bryden AS, Davies H: **Letter: Virus particles in gastroenteritis**. *Lancet* 1973, **2**(7844):1497.

46. Madeley CR, Cosgrove BP: **Letter: 28 nm particles in faeces in infantile gastroenteritis**. *Lancet* 1975, **2**(7932):451-452.

47. Madeley CR, Cosgrove BP: **Letter: Viruses in infantile gastroenteritis**. *Lancet* 1975, **2**(7925):124.

48. Morris CA, Flewett TH, Bryden AS, Davies H: **Epidemic viral enteritis in a long-stay children's ward**. *Lancet* 1975, **1**(7897):4-5.

49. Chiba S, Nakata S, Numata-Kinoshita K, Honma S: **Sapporo virus: history and recent findings**. *The Journal of infectious diseases* 2000, **181 Suppl 2**:S303-308.

50. Caul EO, Paver WK, Clarke SK: **Letter: Coronavirus particles in faeces from patients with gastroenteritis**. *Lancet* 1975, **1**(7917):1192.

51. Yamashita T, Kobayashi S, Sakae K, Nakata S, Chiba S, Ishihara Y, Isomura S: **Isolation of cytopathic small round viruses with BS-C-1 cells from patients with gastroenteritis**. *The Journal of infectious diseases* 1991, **164**(5):954-957.

52. Chandra R: **Picobirnavirus, a novel group of undescribed viruses of mammals and birds: a minireview**. *Acta virologica* 1997, **41**(1):59-62.

53. Grohmann GS, Glass RI, Pereira HG, Monroe SS, Hightower AW, Weber R, Bryan RT: **Enteric viruses and diarrhea in HIV-infected patients. Enteric Opportunistic Infections Working Group**. *The New England journal of medicine* 1993, **329**(1):14-20.
54. Ludert JE, Liprandi F: **Identification of viruses with bi- and trisegmented double-stranded RNA genome in faeces of children with gastroenteritis**. *Research in virology* 1993, **144**(3):219-224.
55. Yolken R, Dubovi E, Leister F, Reid R, Almeido-Hill J, Santosham M: **Infantile gastroenteritis associated with excretion of pestivirus antigens**. *Lancet* 1989, **1**(8637):517-520.
56. Beards GM, Brown DW, Green J, Flewett TH: **Preliminary characterisation of torovirus-like particles of humans: comparison with Berne virus of horses and Breda virus of calves**. *Journal of medical virology* 1986, **20**(1):67-78.
57. Jamieson FB, Wang EE, Bain C, Good J, Duckmanton L, Petric M: **Human torovirus: a new nosocomial gastrointestinal pathogen**. *The Journal of infectious diseases* 1998, **178**(5):1263-1269.
58. Kapikian AZ: **viral infections of humans: epidemiology and control**. In: *Viral gastroenteritis.* edn. Edited by Evans AS, Kaslow RA. New York: Plenum Medical Book Company; 1997: 285-343.
59. kilgore PE, Glass RI: **Gastrointestinal syndromes**. In: *Clinical virology.* edn. Edited by Rchman DD, Whitley RJ, Hayden FG. New York: Churchhill Livingstone; 1997: 55-67.
60. Parashar UD, Glass RI: **Viral causes of gastroenteritis**. In: *Viral gastroenteritis.* edn. Edited by Desselberger U, Gray J. Amsterdam: Elsevier; 2003: 9-21.
61. Centers for Disease C, Prevention: **Rotavirus surveillance --- worldwide, 2009**. *MMWR Morbidity and mortality weekly report* 2011, **60**(16):514-516.
62. Tate JE, Burton AH, Boschi-Pinto C, Steele AD, Duque J, Parashar UD, Network WH-cGRS: **2008 estimate of worldwide rotavirus-associated mortality in children younger than 5 years before the introduction of universal rotavirus vaccination programmes: a systematic review and meta-analysis**. *The Lancet Infectious diseases* 2012, **12**(2):136-141.
63. Martella V, Banyai K, Matthijnssens J, Buonavoglia C, Ciarlet M: **Zoonotic aspects of rotaviruses**. *Veterinary microbiology* 2010, **140**(3-4):246-255.
64. Kapikian AZ, Hoshino Y, M. CR: **Rotaviruses**. In: *Fields virology Volume 2*, 4th edn. Edited by Howley PM. Philadelphia, Pa.: Lippincott Williams & Wilkins; 2001: 1787-1833.
65. Greenberg HB, Estes MK: **Rotaviruses: from pathogenesis to vaccination**. *Gastroenterology* 2009, **136**(6):1939-1951.
66. Hoshino Y, Sereno MM, Midthun K, Flores J, Kapikian AZ, Chanock RM: **Independent segregation of two antigenic specificities (VP3 and VP7) involved in neutralization of rotavirus infectivity**. *Proceedings of the National Academy of Sciences of the United States of America* 1985, **82**(24):8701-8704.

67. Laird AR, Gentsch JR, Nakagomi T, Nakagomi O, Glass RI: **Characterization of serotype G9 rotavirus strains isolated in the United States and India from 1993 to 2001**. *Journal of clinical microbiology* 2003, **41**(7):3100-3111.

68. Offit PA, Clark HF, Blavat G, Greenberg HB: **Reassortant rotaviruses containing structural proteins vp3 and vp7 from different parents induce antibodies protective against each parental serotype**. *Journal of virology* 1986, **60**(2):491-496.

69. Desselberger U, Gray J: **Rotaviruses**. In: *Viral gatsroenteritis.* edn. Edited by Desselberger U, Gray J. Amsterdam, The Netherlands: Elsevier Science B.V.; 2003: 105-115.

70. Matthijnssens J, Ciarlet M, McDonald SM, Attoui H, Banyai K, Brister JR, Buesa J, Esona MD, Estes MK, Gentsch JR *et al*: **Uniformity of rotavirus strain nomenclature proposed by the Rotavirus Classification Working Group (RCWG)**. *Archives of virology* 2011, **156**(8):1397-1413.

71. Santos N, Hoshino Y: **Global distribution of rotavirus serotypes/genotypes and its implication for the development and implementation of an effective rotavirus vaccine**. *Reviews in medical virology* 2005, **15**(1):29-56.

72. Trimis G, Koutsoumbari I, Kottaridi C, Palaiologou N, Assimakopoulou E, Spathis A, Lebessi E, Konstantopoulos A, Kafetzis D, Karakitsos P *et al*: **Hospital-based surveillance of rotavirus gastroenteritis in the era of limited vaccine uptake through the private sector**. *Vaccine* 2011, **29**(43):7292-7295.

73. Cunliffe NA, Ngwira BM, Dove W, Nakagomi O, Nakagomi T, Perez A, Hart CA, Kazembe PN, Mwansambo CC: **Serotype g12 rotaviruses, Lilongwe, Malawi**. *Emerging infectious diseases* 2009, **15**(1):87-90.

74. Page NA, de Beer MC, Seheri LM, Dewar JB, Steele AD: **The detection and molecular characterization of human G12 genotypes in South Africa**. *Journal of medical virology* 2009, **81**(1):106-113.

75. Oluwatoyin Japhet M, Adeyemi Adesina O, Famurewa O, Svensson L, Nordgren J: **Molecular epidemiology of rotavirus and norovirus in Ile-Ife, Nigeria: high prevalence of G12P[8] rotavirus strains and detection of a rare norovirus genotype**. *Journal of medical virology* 2012, **84**(9):1489-1496.

76. Chandrahasen C, Grimwood K, Redshaw N, Rich FJ, Wood C, Stanley J, Kirman JR, New Zealand Rotavirus Study G: **Geographical differences in the proportion of human group A rotavirus strains within New Zealand during one epidemic season**. *Journal of medical virology* 2010, **82**(5):897-902.

77. Esona MD, Geyer A, Page N, Trabelsi A, Fodha I, Aminu M, Agbaya VA, Tsion B, Kerin TK, Armah GE *et al*: **Genomic characterization of human rotavirus G8 strains from the African rotavirus network: relationship to animal rotaviruses**. *Journal of medical virology* 2009, **81**(5):937-951.

78. Esona MD, Steele D, Kerin T, Armah G, Peenze I, Geyer A, Page N, Nyangao J, Agbaya VA, Trabelsi A *et al*: **Determination of the G and P Types of Previously Nontypeable Rotavirus Strains from the African Rotavirus Network, 1996–2004: Identification of Unusual G Types**. *Journal of Infectious Diseases* 2010, **202**(Supplement 1):S49-S54.

79. Gentsch JR, Laird AR, Bielfelt B, Griffin DD, Banyai K, Ramachandran M, Jain V, Cunliffe NA, Nakagomi O, Kirkwood CD *et al*: **Serotype diversity and reassortment between human and animal rotavirus strains: implications for rotavirus vaccine programs**. *The Journal of infectious diseases* 2005, **192 Suppl 1**:S146-159.

80. Kiulia NM, Kamenwa R, Irimu G, Nyangao JO, Gatheru Z, Nyachieo A, Steele AD, Mwenda JM: **The epidemiology of human rotavirus associated with diarrhoea in Kenyan children: a review**. *Journal of tropical pediatrics* 2008, **54**(6):401-405.

81. Matthijnssens J, Rahman M, Yang X, Delbeke T, Arijs I, Kabue J-P, Muyembe J-JT, Van Ranst M: **G8 Rotavirus Strains Isolated in the Democratic Republic of Congo Belong to the DS-1-Like Genogroup**. *Journal of clinical microbiology* 2006, **44**(5):1801-1809.

82. Pietsch C, Petersen L, Patzer L, Liebert UG: **Molecular Characteristics of German G8P[4] Rotavirus Strain GER1H-09 Suggest that a Genotyping and Subclassification Update Is Required for G8**. *Journal of clinical microbiology* 2009, **47**(11):3569-3576.

83. Steyer A, Poljsak-Prijatelj M, Bufon TL, Marcun-Varda N, Marin J: **Rotavirus genotypes in Slovenia: unexpected detection of G8P[8] and G12P[8] genotypes**. *Journal of medical virology* 2007, **79**(5):626-632.

84. Midthun K, Kapikian AZ: **Rotavirus vaccines: an overview**. *Clinical microbiology reviews* 1996, **9**(3):423-434.

85. Georges-Courbot MC, Monges J, Siopathis MR, Roungou JB, Gresenguet G, Bellec L, Bouquety JC, Lanckriet C, Cadoz M, Hessel L *et al*: **Evaluation of the efficacy of a low-passage bovine rotavirus (strain WC3) vaccine in children in Central Africa**. *Research in virology* 1991, **142**(5):405-411.

86. Hanlon P, Hanlon L, Marsh V, Byass P, Shenton F, Hassan-King M, Jobe O, Sillah H, Hayes R, M'Boge BH *et al*: **Trial of an attenuated bovine rotavirus vaccine (RIT 4237) in Gambian infants**. *Lancet* 1987, **1**(8546):1342-1345.

87. Kapikian AZ, Hoshino Y, Chanock RM, Perez-Schael I: **Efficacy of a quadrivalent rhesus rotavirus-based human rotavirus vaccine aimed at preventing severe rotavirus diarrhea in infants and young children**. *The Journal of infectious diseases* 1996, **174 Suppl 1**:S65-72.

88. Centers for Disease C, Prevention: **Withdrawal of rotavirus vaccine recommendation**. *MMWR Morbidity and mortality weekly report* 1999, **48**(43):1007.

89. Heaton PM, Goveia MG, Miller JM, Offit P, Clark HF: **Development of a pentavalent rotavirus vaccine against prevalent serotypes of rotavirus gastroenteritis**. *The Journal of infectious diseases* 2005, **192 Suppl 1**:S17-21.

90. De Vos B, Vesikari T, Linhares AC, Salinas B, Perez-Schael I, Ruiz-Palacios GM, Guerrero Mde L, Phua KB, Delem A, Hardt K: **A rotavirus vaccine for prophylaxis of infants against rotavirus gastroenteritis**. *The Pediatric infectious disease journal* 2004, **23**(10 Suppl):S179-182.

91. Salinas B, Perez Schael I, Linhares AC, Ruiz Palacios GM, Guerrero ML, Yarzabal JP, Cervantes Y, Costa Clemens S, Damaso S, Hardt K *et al*: **Evaluation of safety, immunogenicity and efficacy of an attenuated rotavirus vaccine, RIX4414: A randomized, placebo-controlled trial in Latin American infants**. *The Pediatric infectious disease journal* 2005, **24**(9):807-816.

92. Curns AT, Steiner CA, Barrett M, Hunter K, Wilson E, Parashar UD: **Reduction in acute gastroenteritis hospitalizations among US children after introduction of rotavirus vaccine: analysis of hospital discharge data from 18 US states**. *The Journal of infectious diseases* 2010, **201**(11):1617-1624.

93. Richardson V, Hernandez-Pichardo J, Quintanar-Solares M, Esparza-Aguilar M, Johnson B, Gomez-Altamirano CM, Parashar U, Patel M: **Effect of rotavirus vaccination on death from childhood diarrhea in Mexico**. *The New England journal of medicine* 2010, **362**(4):299-305.

94. Quintanar-Solares M, Yen C, Richardson V, Esparza-Aguilar M, Parashar UD, Patel MM: **Impact of rotavirus vaccination on diarrhea-related hospitalizations among children < 5 years of age in Mexico**. *The Pediatric infectious disease journal* 2011, **30**(1 Suppl):S11-15.

95. Yen C, Armero Guardado JA, Alberto P, Rodriguez Araujo DS, Mena C, Cuellar E, Nolasco JB, De Oliveira LH, Pastor D, Tate JE *et al*: **Decline in rotavirus hospitalizations and health care visits for childhood diarrhea following rotavirus vaccination in El Salvador**. *The Pediatric infectious disease journal* 2011, **30**(1 Suppl):S6-S10.

96. Braeckman T, Van Herck K, Raes M, Vergison A, Sabbe M, Van Damme P: **Rotavirus vaccines in Belgium: policy and impact**. *The Pediatric infectious disease journal* 2011, **30**(1 Suppl):S21-24.

97. Molto Y, Cortes JE, De Oliveira LH, Mike A, Solis I, Suman O, Coronado L, Patel MM, Parashar UD, Cortese MM: **Reduction of diarrhea-associated hospitalizations among children aged < 5 Years in Panama following the introduction of rotavirus vaccine**. *The Pediatric infectious disease journal* 2011, **30**(1 Suppl):S16-20.

98. Buttery JP, Lambert SB, Grimwood K, Nissen MD, Field EJ, Macartney KK, Akikusa JD, Kelly JJ, Kirkwood CD: **Reduction in rotavirus-associated acute gastroenteritis following introduction of rotavirus vaccine into Australia's National Childhood vaccine schedule**. *The Pediatric infectious disease journal* 2011, **30**(1 Suppl):S25-29.

99. Fernandes EG, Sato HK, Leshem E, Flannery B, Konstantyner TC, Veras MA, Patel MM: **Impact of rotavirus vaccination on diarrhea-related hospitalizations in Sao Paulo State, Brazil**. *Vaccine* 2014, **32**(27):3402-3408.

100. Estes MK, Kapikian AZ: **Rotaviruses**. In: *Fields virology. Volume 2*, edn. Edited by Knipe DM, Howley PM. Philadelsphia, USA: Wolters Kluwer and Lippincott Williams & Wilkins; 2007: 1917-1974.

101. Gordon AG: **Rotavirus infections and the sompe syndrome**. *The Journal of infectious diseases* 1982, **146**(1):117-118.

102. Gurwith M, Wenman W, Hinde D, Feltham S, Greenberg H: **A prospective study of rotavirus infection in infants and young children**. *The Journal of infectious diseases* 1981, **144**(3):218-224.

103. Foster SO, Palmer EL, Gary GW, Jr., Martin ML, Herrmann KL, Beasley P, Sampson J: **Gastroenteritis due to rotavirus in an isolated Pacific island group: an epidemic of 3,439 cases**. *The Journal of infectious diseases* 1980, **141**(1):32-39.

104. Oishi I, Kimura T, Murakami T, Haruki K, Yamazaki K, Seto Y, Minekawa Y, Funamoto H: **Serial observations of chronic rotavirus infection in an immunodeficient child**. *Microbiology and immunology* 1991, **35**(11):953-961.

105. Konno T, Suzuki H, Kutsuzawa T, Imai A, Katsushima N, Sakamoto M, Kitaoka S, Tsuboi R, Adachi M: **Human rotavirus infection in infants and young children with intussusception**. *Journal of medical virology* 1978, **2**(3):265-269.

106. Nakagomi T: **Rotavirus infection and intussusception: a view from retrospect**. *Microbiology and immunology* 2000, **44**(8):619-628.

107. Richardson S, Grimwood K, Gorrell R, Palombo E, Barnes G, Bishop R: **Extended excretion of rotavirus after severe diarrhoea in young children**. *Lancet* 1998, **351**(9119):1844-1848.

108. Franco MA, Angel J, Greenberg HB: **Immunity and correlates of protection for rotavirus vaccines**. *Vaccine* 2006, **24**(15):2718-2731.

109. Velazquez FR, Matson DO, Calva JJ, Guerrero L, Morrow AL, Carter-Campbell S, Glass RI, Estes MK, Pickering LK, Ruiz-Palacios GM: **Rotavirus infections in infants as protection against subsequent infections**. *The New England journal of medicine* 1996, **335**(14):1022-1028.

110. Velazquez FR, Matson DO, Guerrero ML, Shults J, Calva JJ, Morrow AL, Glass RI, Pickering LK, Ruiz-Palacios GM: **Serum antibody as a marker of protection against natural rotavirus infection and disease**. *The Journal of infectious diseases* 2000, **182**(6):1602-1609.

111. Bishop RF, Bugg HC, Masendycz PJ, Lund JS, Gorrell RJ, Barnes GL: **Serum, fecal, and breast milk rotavirus antibodies as indices of infection in mother-infant pairs**. *The Journal of infectious diseases* 1996, **174 Suppl 1**:S22-29.

112. Colomina J, Raga J, Gil MT, Buesa J: **[Virus-specific serum and fecal antibodies response in children with acute rotavirus gastroenteritis]**. *Enfermedades infecciosas y microbiologia clinica* 1998, **16**(2):55-60.

113. Burns JW, Siadat-Pajouh M, Krishnaney AA, Greenberg HB: **Protective effect of rotavirus VP6-specific IgA monoclonal antibodies that lack neutralizing activity**. *Science* 1996, **272**(5258):104-107.

114. Hjelt K, Grauballe PC, Paerregaard A, Nielsen OH, Krasilnikoff PA: **Protective effect of preexisting rotavirus-specific immunoglobulin A against naturally acquired rotavirus infection in children**. *Journal of medical virology* 1987, **21**(1):39-47.

115. O'Ryan ML, Matson DO, Estes MK, Pickering LK: **Anti-rotavirus G type-specific and isotype-specific antibodies in children with natural rotavirus infections**. *The Journal of infectious diseases* 1994, **169**(3):504-511.

116. Gorrell RJ, Bishop RF: **Homotypic and heterotypic serum neutralizing antibody response to rotavirus proteins following natural primary infection and reinfection in children**. *Journal of medical virology* 1999, **57**(2):204-211.

117. Ward RL: **Mechanisms of protection against rotavirus in humans and mice**. *The Journal of infectious diseases* 1996, **174 Suppl 1**:S51-58.

118. Johansen K, Hinkula J, Espinoza F, Levi M, Zeng C, Ruden U, Vesikari T, Estes M, Svensson L: **Humoral and cell-mediated immune responses in humans to the NSP4 enterotoxin of rotavirus**. *Journal of medical virology* 1999, **59**(3):369-377.

119. de Wit MA, Koopmans MP, Kortbeek LM, Wannet WJ, Vinje J, van Leusden F, Bartelds AI, van Duynhoven YT: **Sensor, a population-based cohort study on gastroenteritis in the Netherlands: incidence and etiology**. *American journal of epidemiology* 2001, **154**(7):666-674.

120. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, Jones JL, Griffin PM: **Foodborne illness acquired in the United States--major pathogens**. *Emerging infectious diseases* 2011, **17**(1):7-15.

121. Fischer Walker CL, Perin J, Aryee MJ, Boschi-Pinto C, Black RE: **Diarrhea incidence in low- and middle-income countries in 1990 and 2010: a systematic review**. *BMC public health* 2012, **12**:220.

122. Ahmed SM, Hall AJ, Robinson AE, Verhoef L, Premkumar P, Parashar UD, Koopmans M, Lopman BA: **Global prevalence of norovirus in cases of gastroenteritis: a systematic review and meta-analysis**. *The Lancet Infectious diseases* 2014, **14**(8):725-730.

123. Patel MM, Widdowson MA, Glass RI, Akazawa K, Vinje J, Parashar UD: **Systematic literature review of role of noroviruses in sporadic gastroenteritis**. *Emerging infectious diseases* 2008, **14**(8):1224-1231.

124. Treanor JJ, Atmar RL, Frey SE, Gormley R, Chen WH, Ferreira J, Goodwin R, Borkowski A, Clemens R, Mendelman PM: **A Novel Intramuscular Bivalent Norovirus Virus-Like Particle Vaccine Candidate-Reactogenicity, Safety, and Immunogenicity in a Phase 1 Trial in Healthy Adults**. *The Journal of infectious diseases* 2014.

125. Prasad BV, Hardy ME, Dokland T, Bella J, Rossmann MG, Estes MK: **X-ray crystallographic structure of the Norwalk virus capsid**. *Science* 1999, **286**(5438):287-290.

126. Atmar RL, Estes MK: **Diagnosis of noncultivatable gastroenteritis viruses, the human caliciviruses**. *Clinical microbiology reviews* 2001, **14**(1):15-37.

127. Glass PJ, White LJ, Ball JM, Leparc-Goffart I, Hardy ME, Estes MK: **Norwalk virus open reading frame 3 encodes a minor structural protein**. *Journal of virology* 2000, **74**(14):6581-6591.

128. Bertolotti-Ciarlet A, Crawford SE, Hutson AM, Estes MK: **The 3' end of Norwalk virus mRNA contains determinants that regulate the expression and stability of the viral capsid protein VP1: a novel function for the VP2 protein**. *Journal of virology* 2003, **77**(21):11603-11615.

129. Hoffmann D, Seebach J, Foley BT, Frosner G, Nadas K, Protzer U, Schatzl HM: **Isolated norovirus GII.7 strain within an extended GII.4 outbreak**. *Journal of medical virology* 2010, **82**(6):1058-1064.

130. Wang QH, Costantini V, Saif LJ: **Porcine enteric caliciviruses: genetic and antigenic relatedness to human caliciviruses, diagnosis and epidemiology**. *Vaccine* 2007, **25**(30):5453-5466.

131. Hoa Tran TN, Trainor E, Nakagomi T, Cunliffe NA, Nakagomi O: **Molecular epidemiology of noroviruses associated with acute sporadic gastroenteritis in children: global distribution of genogroups, genotypes and GII.4 variants**. *J Clin Virol* 2013, **56**(3):185-193.

132. Ramani S, Atmar RL, Estes MK: **Epidemiology of human noroviruses and updates on vaccine development**. *Current opinion in gastroenterology* 2014, **30**(1):25-33.

133. Desai R, Hembree CD, Handel A, Matthews JE, Dickey BW, McDonald S, Hall AJ, Parashar UD, Leon JS, Lopman B: **Severe outcomes are associated with genogroup 2 genotype 4 norovirus outbreaks: a systematic literature review**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2012, **55**(2):189-193.

134. Papaventsis DC, Dove W, Cunliffe NA, Nakagomi O, Combe P, Grosjean P, Hart CA: **Norovirus infection in children with acute gastroenteritis, Madagascar, 2004-2005**. *Emerging infectious diseases* 2007, **13**(6):908-911.

135. Kamel AH, Ali MA, El-Nady HG, de Rougemont A, Pothier P, Belliot G: **Predominance and circulation of enteric viruses in the region of Greater Cairo, Egypt**. *Journal of clinical microbiology* 2009, **47**(4):1037-1045.

136. Hansman GS, Katayama K, Maneekarn N, Peerakome S, Khamrin P, Tonusin S, Okitsu S, Nishio O, Takeda N, Ushijima H: **Genetic diversity of norovirus and sapovirus in hospitalized infants with sporadic cases of acute gastroenteritis in Chiang Mai, Thailand**. *Journal of clinical microbiology* 2004, **42**(3):1305-1307.

137. El-Kamary SS, Pasetti MF, Mendelman PM, Frey SE, Bernstein DI, Treanor JJ, Ferreira J, Chen WH, Sublett R, Richardson C *et al*: **Adjuvanted intranasal Norwalk virus-like particle vaccine elicits antibodies and antibody-secreting cells that express homing receptors for mucosal and peripheral lymphoid tissues**. *The Journal of infectious diseases* 2010, **202**(11):1649-1658.

138. Tacket CO, Sztein MB, Losonsky GA, Wasserman SS, Estes MK: **Humoral, mucosal, and cellular immune responses to oral Norwalk virus-like particles in volunteers**. *Clinical immunology* 2003, **108**(3):241-247.

139. Atmar RL, Bernstein DI, Harro CD, Al-Ibrahim MS, Chen WH, Ferreira J, Estes MK, Graham DY, Opekun AR, Richardson C *et al*: **Norovirus vaccine against experimental human Norwalk Virus illness**. *The New England journal of medicine* 2011, **365**(23):2178-2187.

140. Hall AJ, Wikswo ME, Manikonda K, Roberts VA, Yoder JS, Gould LH: **Acute gastroenteritis surveillance through the National Outbreak Reporting System, United States**. *Emerging infectious diseases* 2013, **19**(8):1305-1309.

141. Thornton AC, Jennings-Conklin KS, McCormick MI: **Noroviruses: agents in outbreaks of acute gastroenteritis**. *Disaster management & response : DMR : an official publication of the Emergency Nurses Association* 2004, **2**(1):4-9.

142. Vinje J: **Advances in Laboratory Methods for Detection and Typing of Norovirus**. *Journal of clinical microbiology* 2014.

143. Schaub SA, Oshiro RK: **Public health concerns about caliciviruses as waterborne contaminants**. *The Journal of infectious diseases* 2000, **181 Suppl 2**:S374-380.

144. Rockx B, De Wit M, Vennema H, Vinje J, De Bruin E, Van Duynhoven Y, Koopmans M: **Natural history of human calicivirus infection: a prospective cohort study**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2002, **35**(3):246-253.

145. Green KY: **Caliciviridae:The Noroviruses**. In: *Field virology. Volume 1*, edn. Edited by Knipe DM, Howley PM. Philadelphia, USA.: Wolter Kluwer and Lippincott Williams & Wilkins; 2007: 949-980.

146. Bok K, Green KY: **Norovirus gastroenteritis in immunocompromised patients**. *The New England journal of medicine* 2012, **367**(22):2126-2132.

147. Parrino TA, Schreiber DS, Trier JS, Kapikian AZ, Blacklow NR: **Clinical immunity in acute gastroenteritis caused by Norwalk agent**. *The New England journal of medicine* 1977, **297**(2):86-89.

148. Johnson PC, Mathewson JJ, DuPont HL, Greenberg HB: **Multiple-challenge study of host susceptibility to Norwalk gastroenteritis in US adults**. *The Journal of infectious diseases* 1990, **161**(1):18-21.

149. Hutson AM, Atmar RL, Graham DY, Estes MK: **Norwalk virus infection and disease is associated with ABO histo-blood group type**. *The Journal of infectious diseases* 2002, **185**(9):1335-1337.

150. Lindesmith L, Moe C, Marionneau S, Ruvoen N, Jiang X, Lindblad L, Stewart P, LePendu J, Baric R: **Human susceptibility and resistance to Norwalk virus infection**. *Nature medicine* 2003, **9**(5):548-553.

151. Tan M, Jin M, Xie H, Duan Z, Jiang X, Fang Z: **Outbreak studies of a GII-3 and a GII-4 norovirus revealed an association between HBGA phenotypes and viral infection**. *Journal of medical virology* 2008, **80**(7):1296-1301.

152. Hutson AM, Atmar RL, Estes MK: **Norovirus disease: changing epidemiology and host susceptibility factors**. *Trends in microbiology* 2004, **12**(6):279-287.

153. Larsson MM, Rydell GE, Grahn A, Rodriguez-Diaz J, Akerlind B, Hutson AM, Estes MK, Larson G, Svensson L: **Antibody prevalence and titer to norovirus (genogroup II) correlate with secretor (FUT2) but not with ABO phenotype or Lewis (FUT3) genotype**. *The Journal of infectious diseases* 2006, **194**(10):1422-1427.

154. Lindesmith L, Moe C, Lependu J, Frelinger JA, Treanor J, Baric RS: **Cellular and humoral immunity following Snow Mountain virus challenge**. *Journal of virology* 2005, **79**(5):2900-2909.

155. Marionneau S, Ruvoen N, Le Moullac-Vaidye B, Clement M, Cailleau-Thomas A, Ruiz-Palacois G, Huang P, Jiang X, Le Pendu J: **Norwalk virus binds to histo-blood group antigens present on gastroduodenal epithelial cells of secretor individuals**. *Gastroenterology* 2002, **122**(7):1967-1977.

156. Cannon JL, Lindesmith LC, Donaldson EF, Saxe L, Baric RS, Vinje J: **Herd immunity to GII.4 noroviruses is supported by outbreak patient sera**. *Journal of virology* 2009, **83**(11):5363-5374.

157. Lindesmith LC, Donaldson EF, Lobue AD, Cannon JL, Zheng DP, Vinje J, Baric RS: **Mechanisms of GII.4 norovirus persistence in human populations**. *PLoS medicine* 2008, **5**(2):e31.

158. Berk AJ: **Adenoviridae**. In: *Fields virology. Volume 2*, edn. Edited by Knipe DM, Howley PM. Philadelphia, USA.: wolters Kluwer and Lippincott Williams &Wilkins; 2007: 2355-2394.

159. Hilmarsdottir I, Baldvinsdottir GE, Harethardottir H, Briem H, Sigurethsson SI: **Enteropathogens in acute diarrhea: a general practice-based study in a Nordic country**. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 2012, **31**(7):1501-1509.

160. McAuliffe GN, Anderson TP, Stevens M, Adams J, Coleman R, Mahagamasekera P, Young S, Henderson T, Hofmann M, Jennings LC *et al*: **Systematic application of multiplex PCR enhances the detection of bacteria, parasites, and viruses in stool samples**. *The Journal of infection* 2013, **67**(2):122-129.

161. Ozdemir S, Delialioglu N, Emekdas G: **[Investigation of rotavirus, adenovirus and astrovirus frequencies in children with acute gastroenteritis and evaluation of epidemiological features]**. *Mikrobiyoloji bulteni* 2010, **44**(4):571-578.

162. Bon F, Fascia P, Dauvergne M, Tenenbaum D, Planson H, Petion AM, Pothier P, Kohli E: **Prevalence of group A rotavirus, human calicivirus, astrovirus, and adenovirus type 40 and 41 infections among children with acute gastroenteritis in Dijon, France**. *Journal of clinical microbiology* 1999, **37**(9):3055-3058.

163. Andreasi MS, Cardoso D, Fernandes SM, Tozetti IA, Borges AM, Fiaccadori FS, Santos RA, Souza M: **Adenovirus, calicivirus and astrovirus detection in fecal samples of hospitalized children with acute gastroenteritis from Campo Grande, MS, Brazil**. *Memorias do Instituto Oswaldo Cruz* 2008, **103**(7):741-744.

164. Basu G, Rossouw J, Sebunya TK, Gashe BA, de Beer M, Dewar JB, Steele AD: **Prevalence of rotavirus, adenovirus and astrovirus infection in young children with gastroenteritis in Gaborone, Botswana**. *East African medical journal* 2003, **80**(12):652-655.

165. Reither K, Ignatius R, Weitzel T, Seidu-Korkor A, Anyidoho L, Saad E, Djie-Maletz A, Ziniel P, Amoo-Sakyi F, Danikuu F *et al*: **Acute childhood diarrhoea in northern Ghana: epidemiological, clinical and microbiological characteristics**. *BMC infectious diseases* 2007, **7**:104.

166. Magwalivha M, Wolfaardt M, Kiulia NM, van Zyl WB, Mwenda JM, Taylor MB: **High prevalence of species D human adenoviruses in fecal specimens from Urban Kenyan children with diarrhea**. *Journal of medical virology* 2010, **82**(1):77-84.

167. Sambe-Ba B, Espie E, Faye ME, Timbine LG, Sembene M, Gassama-Sow A: **Community-acquired diarrhea among children and adults in urban settings in Senegal: clinical, epidemiological and microbiological aspects**. *BMC infectious diseases* 2013, **13**:580.

168. Baum SG: **Adenovirus**. In: *Principles and practice of infectious diseases.* edn. Edited by Mandell GL, Bennet JE, Dolim R. New York: Churchhill Livingstone; 2000: 1624-1630.

169. Brown M, Grydsuk JD, Fortsas E, Petric M: **Structural features unique to enteric adenoviruses**. *Archives of virology Supplementum* 1996, **12**:301-307.

170. wadell G, Allard A, Hierholzer JC: **Adenoviruses**. In: *Manual of clinical microbiology.* edn. Edited by Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. Washington: ASM press; 1999: 970-982.

171. Benko M, Harrach B, Both GW, Russell WC, Adair BM, Adam E, de Jong JC, Hess M, Johnson M, Kajon A *et al*: **Adenoviruses**. In: *Virus taxonomy: Classification and nomenclature of viruses (8th report of the International Committee on the Taxonomy of Viruses).* edn. Edited by Fauquet CM, Mayo MA, Maniloff J, Desselberger U, Ball LA. New York: Elsevier Academic Press; 2005: 213-228.

172. Jones MS, 2nd, Harrach B, Ganac RD, Gozum MM, Dela Cruz WP, Riedel B, Pan C, Delwart EL, Schnurr DP: **New adenovirus species found in a patient presenting with gastroenteritis**. *Journal of virology* 2007, **81**(11):5978-5984.

173. Wold WSM, Horwitz MS: **Adenoviruses**. In: *Fields virology.* 5th edn. Edited by Knipe DM, Howley M, Griffin DE, Lamb RA, Matin MA, Roizman B, Straus SE. Philadelphia: Lippincott Williams and Wilkins; 2007: 2395-2428.

174. Lasaro MO, Ertl HC: **New insights on adenovirus as vaccine vectors**. *Molecular therapy : the journal of the American Society of Gene Therapy* 2009, **17**(8):1333-1339.

175. Stevenson F, Mautner V: **Aspects of the molecular biology of enteric adenoviurses**. In: *Viral gastroenteritis. Volume 9*, edn. Edited by Desselberger U, Gray J. Amsterdam, The Netherlands: Elsevier science B.V.; 2003: 389-406.

176. Richmond SJ, Caul EO, Dunn SM, Ashley CR, Clarke SK, Seymour NR: **An outbreak of gastroenteritis in young children caused by adenoviruses**. *Lancet* 1979, **1**(8127):1178-1181.

177. Clark B, McKendrick M: **A review of viral gastroenteritis**. *Current opinion in infectious diseases* 2004, **17**(5):461-469.

178. Hierholzer JC: **Adenoviruses in the immunocompromised host**. *Clinical microbiology reviews* 1992, **5**(3):262-274.

179. Li L, Phan TG, Nguyen TA, Kim KS, Seo JK, Shimizu H, Suzuki E, Okitsu S, Ushijima H: **Molecular epidemiology of adenovirus infection among pediatric population with diarrhea in Asia**. *Microbiology and immunology* 2005, **49**(2):121-128.

180. Michelangeli UD, Glass RI: **Physiology and pathopysiology of he gut in relation to diarrhoea**. In: *Viral gastroenteritis. Volume 9*, edn. Edited by Desselberger U, Gray J. Amsterdam, The Netherlands: Elsevier; 2003.

181. Bass D: **Treatmetn of viral gastroenteritis**. In: *Viral gastroenteritis. Volume 9*, edn. Edited by Desselberger U, Gray J. Amsterdam, The Netherlands: Elsevier; 2003: 93-104.

182. Ramani S, Kang G: **Viruses causing childhood diarrhoea in the developing world**. *Current opinion in infectious diseases* 2009, **22**(5):477-482.

183. Claeson M, Merson MH: **Global progress in the control of diarrheal diseases**. *The Pediatric infectious disease journal* 1990, **9**(5):345-355.

184. Guarino A, Dupont C, Gorelov AV, Gottrand F, Lee JK, Lin Z, Lo Vecchio A, Nguyen TD, Salazar-Lindo E: **The management of acute diarrhea in children in developed and developing areas: from evidence base to clinical practice**. *Expert opinion on pharmacotherapy* 2012, **13**(1):17-26.

185. Ramani S, Arumugam R, Gopalarathinam N, Mohanty I, Mathew S, Gladstone BP, Jana AK, Kuruvilla KA, Kang G: **Investigation of the environment and of mothers in transmission of rotavirus infections in the neonatal nursery**. *Journal of medical virology* 2008, **80**(6):1099-1105.

186. Prameela KK, Vijaya LR: **The importance of breastfeeding in rotaviral diarrhoeas**. *Malaysian journal of nutrition* 2012, **18**(1):103-111.

187. Liberato SC, Singh G, Mulholland K: **Zinc supplementation in young children: A review of the literature focusing on diarrhoea prevention and treatment**. *Clinical nutrition* 2014.

188. Saaka M, Oosthuizen J, Beatty S: **Effect of prenatal zinc supplementation on birthweight**. *Journal of health, population, and nutrition* 2009, **27**(5):619-631.

189. Black RE: **Zinc deficiency, infectious disease and mortality in the developing world**. *The Journal of nutrition* 2003, **133**(5 Suppl 1):1485S-1489S.

190. Santosham M, Chandran A, Fitzwater S, Fischer-Walker C, Baqui AH, Black R: **Progress and barriers for the control of diarrhoeal disease**. *Lancet* 2010, **376**(9734):63-67.
191. Baqui AH, Black RE, El Arifeen S, Yunus M, Chakraborty J, Ahmed S, Vaughan JP: **Effect of zinc supplementation started during diarrhoea on morbidity and mortality in Bangladeshi children: community randomised trial**. *Bmj* 2002, **325**(7372):1059.
192. Bhandari N, Mazumder S, Taneja S, Dube B, Agarwal RC, Mahalanabis D, Fontaine O, Black RE, Bhan MK: **Effectiveness of zinc supplementation plus oral rehydration salts compared with oral rehydration salts alone as a treatment for acute diarrhea in a primary care setting: a cluster randomized trial**. *Pediatrics* 2008, **121**(5):e1279-1285.
193. Bhutta ZA, Black RE, Brown KH, Gardner JM, Gore S, Hidayat A, Khatun F, Martorell R, Ninh NX, Penny ME *et al*: **Prevention of diarrhea and pneumonia by zinc supplementation in children in developing countries: pooled analysis of randomized controlled trials. Zinc Investigators' Collaborative Group**. *The Journal of pediatrics* 1999, **135**(6):689-697.
194. Ezeamama AE, Spiegelman D, Hertzmark E, Bosch RJ, Manji KP, Duggan C, Kupka R, Lo MW, Okuma JO, Kisenge R *et al*: **HIV infection and the incidence of malaria among HIV-exposed children from Tanzania**. *The Journal of infectious diseases* 2012, **205**(10):1486-1494.
195. Moyo SJ, Gro N, Kirsti V, Matee MI, Kitundu J, Maselle SY, Langeland N, Myrmel H: **Prevalence of enteropathogenic viruses and molecular characterization of group A rotavirus among children with diarrhea in Dar es Salaam Tanzania**. *BMC public health* 2007, **7**:359.
196. Division of Viral Diseases NCfI, Respiratory Diseases CfDC, Prevention: **Updated norovirus outbreak management and disease prevention guidelines**. *MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports / Centers for Disease Control* 2011, **60**(RR-3):1-18.
197. Brandt CD, Kim HW, Rodriguez WJ, Thomas L, Yolken RH, Arrobio JO, Kapikian AZ, Parrott RH, Chanock RM: **Comparison of direct electron microscopy, immune electron microscopy, and rotavirus enzyme-linked immunosorbent assay for detection of gastroenteritis viruses in children**. *Journal of clinical microbiology* 1981, **13**(5):976-981.
198. Zheng DP, Widdowson MA, Glass RI, Vinje J: **Molecular epidemiology of genogroup II-genotype 4 noroviruses in the United States between 1994 and 2006**. *Journal of clinical microbiology* 2010, **48**(1):168-177.
199. Verheyen J, Kaiser R, Bozic M, Timmen-Wego M, Maier BK, Kessler HH: **Extraction of viral nucleic acids: comparison of five automated nucleic acid extraction platforms**. *J Clin Virol* 2012, **54**(3):255-259.
200. Birch CJ, Lewis FA, Kennett ML, Homola M, Pritchard H, Gust ID: **A study of the prevalence of rotavirus infection in children with gastroenteritis admitted to an infectious diseases hospital**. *Journal of medical virology* 1977, **1**(1):69-77.

201. Nicolaieff A, Obert G, van Regenmortel MH: **Detection of rotavirus by serological trapping on antibody-coated electron microscope grids**. *Journal of clinical microbiology* 1980, **12**(1):101-104.

202. Paniker CK, Mathew S, Mathan M: **Rotavirus and acute diarrhoeal disease in children in a southern Indian coastal town**. *Bulletin of the World Health Organization* 1982, **60**(1):123-127.

203. Wilhelmi I, Roman E, Sanchez-Fauquier A: **Viruses causing gastroenteritis**. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* 2003, **9**(4):247-262.

204. Christensen ml: **Rotaviruses**. In: *Manual of clinical microbiology.* edn. Edited by Murray PR, Baron EJ, Pfaller MA, Tenover FC, Yolken RH. Washington: ASM Press; 1999: 999-1004.

205. Thomas EE, Puterman ML, Kawano E, Curran M: **Evaluation of seven immunoassays for detection of rotavirus in pediatric stool samples**. *Journal of clinical microbiology* 1988, **26**(6):1189-1193.

206. Flewett TH, Arias CF, Avendano LF, Ghafoor A, Mathan MM, Mendis L, Moe K, Bishop RF: **Comparative evaluation of the WHO and DAKOPATTS enzyme-linked immunoassay kits for rotavirus detection**. *Bulletin of the World Health Organization* 1989, **67**(4):369-374.

207. Gautam R, Lyde F, Esona MD, Quaye O, Bowen MD: **Comparison of Premier Rotaclone(R), ProSpecT, and RIDASCREEN(R) rotavirus enzyme immunoassay kits for detection of rotavirus antigen in stool specimens**. *J Clin Virol* 2013, **58**(1):292-294.

208. Dennehy PH, Gauntlett DR, Tente WE: **Comparison of nine commercial immunoassays for the detection of rotavirus in fecal specimens**. *Journal of clinical microbiology* 1988, **26**(9):1630-1634.

209. Aminu M, Ahmad AA, Umoh JU, de Beer MC, Esona MD, Steele AD: **Adenovirus infection in children with diarrhea disease in Northwestern Nigeria**. *Ann Afr Med* 2007, **6**(4):168-173.

210. Moore P, Steele AD, Lecatsas G, Alexander JJ: **Characterisation of gastro-enteritis-associated adenoviruses in South Africa**. *S Afr Med J* 1998, **88**(12):1587-1592.

211. Audu R, Omilabu SA, Peenze I, Steele D: **Viral diarrhoea in young children in two districts in Nigeria**. *Cent Afr J Med* 2002, **48**(5-6):59-63.

212. Dey RS, Ghosh S, Chawla-Sarkar M, Panchalingam S, Nataro JP, Sur D, Manna B, Ramamurthy T: **Circulation of a novel pattern of infections by enteric adenovirus serotype 41 among children below 5 years of age in Kolkata, India**. *Journal of clinical microbiology* 2011, **49**(2):500-505.

213. Fodha I, Chouikha A, Peenze I, De Beer M, Dewar J, Geyer A, Messaadi F, Trabelsi A, Boujaafar N, Taylor MB *et al*: **Identification of viral agents causing diarrhea among children in the Eastern Center of Tunisia**. *Journal of medical virology* 2006, **78**(9):1198-1203.

214. Kageyama T, Kojima S, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Takeda N, Katayama K: **Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-**

time quantitative reverse transcription-PCR. *Journal of clinical microbiology* 2003, **41**(4):1548-1557.

215. de Bruin E, Duizer E, Vennema H, Koopmans MP: **Diagnosis of Norovirus outbreaks by commercial ELISA or RT-PCR**. *J Virol Methods* 2006, **137**(2):259-264.

216. Kele B, Lengyel G, Deak J: **Comparison of an ELISA and two reverse transcription polymerase chain reaction methods for norovirus detection**. *Diagnostic microbiology and infectious disease* 2011, **70**(4):475-478.

217. Ando T, Noel JS, Fankhauser RL: **Genetic classification of "Norwalk-like viruses**. *The Journal of infectious diseases* 2000, **181 Suppl 2**:S336-348.

218. Green J, Gallimore CI, Norcott JP, Lewis D, Brown DW: **Broadly reactive reverse transcriptase polymerase chain reaction for the diagnosis of SRSV-associated gastroenteritis**. *Journal of medical virology* 1995, **47**(4):392-398.

219. Vinje J, Vennema H, Maunula L, von Bonsdorff CH, Hoehne M, Schreier E, Richards A, Green J, Brown D, Beard SS *et al*: **International collaborative study to compare reverse transcriptase PCR assays for detection and genotyping of noroviruses**. *Journal of clinical microbiology* 2003, **41**(4):1423-1433.

220. Jiang X, Huang PW, Zhong WM, Farkas T, Cubitt DW, Matson DO: **Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR**. *J Virol Methods* 1999, **83**(1-2):145-154.

221. Moe CL, Gentsch J, Ando T, Grohmann G, Monroe SS, Jiang X, Wang J, Estes MK, Seto Y, Humphrey C *et al*: **Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis**. *Journal of clinical microbiology* 1994, **32**(3):642-648.

222. Wang J, Jiang X, Madore HP, Gray J, Desselberger U, Ando T, Seto Y, Oishi I, Lew JF, Green KY *et al*: **Sequence diversity of small, round-structured viruses in the Norwalk virus group**. *Journal of virology* 1994, **68**(9):5982-5990.

223. Yamazaki K, Oseto M, Seto Y, Utagawa E, Kimoto T, Minekawa Y, Inouye S, Yamazaki S, Okuno Y, Oishi I: **Reverse transcription-polymerase chain reaction detection and sequence analysis of small round-structured viruses in Japan**. *Archives of virology Supplementum* 1996, **12**:271-276.

224. Hafliger D, Gilgen M, Luthy J, Hubner P: **Seminested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood**. *Int J Food Microbiol* 1997, **37**(1):27-36.

225. Noel JS, Ando T, Leite JP, Green KY, Dingle KE, Estes MK, Seto Y, Monroe SS, Glass RI: **Correlation of patient immune responses with genetically characterized small round-structured viruses involved in outbreaks of nonbacterial acute gastroenteritis in the United States, 1990 to 1995**. *Journal of medical virology* 1997, **53**(4):372-383.

226. Kageyama T, Shinohara M, Uchida K, Fukushi S, Hoshino FB, Kojima S, Takai R, Oka T, Takeda N, Katayama K: **Coexistence of multiple genotypes, including newly identified genotypes, in outbreaks of gastroenteritis due to Norovirus in Japan**. *Journal of clinical microbiology* 2004, **42**(7):2988-2995.

227. Coulson BS: **Typing of human rotavirus VP4 by an enzyme immunoassay using monoclonal antibodies**. *Journal of clinical microbiology* 1993, **31**(1):1-8.

228. Coulson BS: **VP4 and VP7 typing using monoclonal antibodies**. *Archives of virology Supplementum* 1996, **12**:113-118.

229. Masendycz PJ, Palombo EA, Gorrell RJ, Bishop RF: **Comparison of enzyme immunoassay, PCR, and type-specific cDNA probe techniques for identification of group A rotavirus gene 4 types (P types)**. *Journal of clinical microbiology* 1997, **35**(12):3104-3108.

230. Souza MB, Racz ML, Leite JP, Soares CM, Martins RM, Munford V, Cardoso DD: **Molecular and serological characterization of group a rotavirus isolates obtained from hospitalized children in Goiania, Brazil, 1998-2000**. *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* 2003, **22**(7):441-443.

231. Fischer TK, Page NA, Griffin DD, Eugen-Olsen J, Pedersen AG, Valentiner-Branth P, Molbak K, Sommerfelt H, Nielsen NM: **Characterization of incompletely typed rotavirus strains from Guinea-Bissau: identification of G8 and G9 types and a high frequency of mixed infections**. *Virology* 2003, **311**(1):125-133.

232. Gouvea V, Glass RI, Woods P, Taniguchi K, Clark HF, Forrester B, Fang ZY: **Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool specimens**. *Journal of clinical microbiology* 1990, **28**(2):276-282.

233. Das BK, Gentsch JR, Cicirello HG, Woods PA, Gupta A, Ramachandran M, Kumar R, Bhan MK, Glass RI: **Characterization of rotavirus strains from newborns in New Delhi, India**. *Journal of clinical microbiology* 1994, **32**(7):1820-1822.

234. Cunliffe NA, Gondwe JS, Kirkwood CD, Graham SM, Nhlane NM, Thindwa BD, Dove W, Broadhead RL, Molyneux ME, Hart CA: **Effect of concomitant HIV infection on presentation and outcome of rotavirus gastroenteritis in Malawian children**. *Lancet* 2001, **358**(9281):550-555.

235. Mwenda JM, Ntoto KM, Abebe A, Enweronu-Laryea C, Amina I, McHomvu J, Kisakye A, Mpabalwani EM, Pazvakavambwa I, Armah GE *et al*: **Burden and epidemiology of rotavirus diarrhea in selected African countries: preliminary results from the African Rotavirus Surveillance Network**. *The Journal of infectious diseases* 2010, **202 Suppl**:S5-S11.

236. Theamboonlers A, Bhattarakosol P, Chongsrisawat V, Sungkapalee T, Wutthirattanakowit N, Poovorawan Y: **Molecular characterization of group A human rotaviruses in Bangkok and Buriram, Thailand during 2004-2006 reveals the predominance of G1P[8], G9P[8] and a rare G3P[19] strain**. *Virus Genes* 2008, **36**(2):289-298.

237. EUROROTA: **European Rotavirus Detection and Typing Methods**. In.; 2009.

238. Adrian T, Wadell G, Hierholzer JC, Wigand R: **DNA restriction analysis of adenovirus prototypes 1 to 41**. *Archives of virology* 1986, **91**(3-4):277-290.

239. Wadell G, Hammarskjold ML, Winberg G, Varsanyi TM, Sundell G: **Genetic variability of adenoviruses**. *Ann N Y Acad Sci* 1980, **354**:16-42.

240. Allard A, Albinsson B, Wadell G: **Rapid typing of human adenoviruses by a general PCR combined with restriction endonuclease analysis**. *Journal of clinical microbiology* 2001, **39**(2):498-505.

241. Kroneman A, Vega E, Vennema H, Vinje J, White PA, Hansman G, Green K, Martella V, Katayama K, Koopmans M: **Proposal for a unified norovirus nomenclature and genotyping**. *Archives of virology* 2013, **158**(10):2059-2068.

242. Vainio K, Myrmel M: **Molecular epidemiology of norovirus outbreaks in Norway during 2000 to 2005 and comparison of four norovirus real-time reverse transcriptase PCR assays**. *Journal of clinical microbiology* 2006, **44**(10):3695-3702.

243. Kojima S, Kageyama T, Fukushi S, Hoshino FB, Shinohara M, Uchida K, Natori K, Takeda N, Katayama K: **Genogroup-specific PCR primers for detection of Norwalk-like viruses**. *J Virol Methods* 2002, **100**(1-2):107-114.

244. Kroneman A, Vennema H, Deforche K, v d Avoort H, Penaranda S, Oberste MS, Vinje J, Koopmans M: **An automated genotyping tool for enteroviruses and noroviruses**. *J Clin Virol* 2011, **51**(2):121-125.

245. Chen Y, Li Z, Han D, Cui D, Chen X, Zheng S, Yu F, Liu J, Lai S, Yan Y *et al*: **Viral agents associated with acute diarrhea among outpatient children in southeastern China**. *The Pediatric infectious disease journal* 2013, **32**(7):e285-290.

246. Gonzalez GG, Liprandi F, Ludert JE: **Molecular epidemiology of enteric viruses in children with sporadic gastroenteritis in Valencia, Venezuela**. *Journal of medical virology* 2011, **83**(11):1972-1982.

247. Liu C, Grillner L, Jonsson K, Linde A, Shen K, Lindell AT, Wirgart BZ, Johansen K: **Identification of viral agents associated with diarrhea in young children during a winter season in Beijing, China**. *J Clin Virol* 2006, **35**(1):69-72.

248. Dimitriadis A, Bruggink LD, Marshall JA: **Evaluation of the Dako IDEIA norovirus EIA assay for detection of norovirus using faecal specimens from Australian gastroenteritis outbreaks**. *Pathology* 2006, **38**(2):157-165.

249. Gunson RN, Miller J, Carman WF: **Comparison of real-time PCR and EIA for the detection of outbreaks of acute gastroenteritis caused by norovirus**. *Commun Dis Public Health* 2003, **6**(4):297-299.

250. Rohayem J, Berger S, Juretzek T, Herchenroder O, Mogel M, Poppe M, Henker J, Rethwilm A: **A simple and rapid single-step multiplex RT-PCR to detect Norovirus, Astrovirus and Adenovirus in clinical stool samples**. *J Virol Methods* 2004, **118**(1):49-59.

251. Glass RI, Parashar UD, Estes MK: **Norovirus gastroenteritis**. *The New England journal of medicine* 2009, **361**(18):1776-1785.

252. Aminu M, Page NA, Ahmad AA, Umoh JU, Dewar J, Steele AD: **Diversity of rotavirus VP7 and VP4 genotypes in Northwestern Nigeria**. *The Journal of infectious diseases* 2010, **202 Suppl**:S198-204.

253. Sam NE, Haukenes G, Szilvay AM, Mhalu F: **Rotavirus infection in Tanzania: a virological, epidemiological and clinical study among young children**. *APMIS* 1992, **100**(9):790-796.

254. Armah GE, Sow SO, Breiman RF, Dallas MJ, Tapia MD, Feikin DR, Binka FN, Steele AD, Laserson KF, Ansah NA *et al*: **Efficacy of pentavalent rotavirus vaccine against severe rotavirus gastroenteritis in infants in developing countries in sub-Saharan Africa: a randomised, double-blind, placebo-controlled trial**. *Lancet* 2010, **376**(9741):606-614.

255. Abugalia M, Cuevas L, Kirby A, Dove W, Nakagomi O, Nakagomi T, Kara M, Gweder R, Smeo M, Cunliffe N: **Clinical features and molecular epidemiology of rotavirus and norovirus infections in Libyan children**. *Journal of medical virology* 2011, **83**(10):1849-1856.

256. Sdiri-Loulizi K, Ambert-Balay K, Gharbi-Khelifi H, Sakly N, Hassine M, Chouchane S, Guediche MN, Pothier P, Aouni M: **Molecular epidemiology of norovirus gastroenteritis investigated using samples collected from children in Tunisia during a four-year period: detection of the norovirus variant GGII.4 Hunter as early as January 2003**. *Journal of clinical microbiology* 2009, **47**(2):421-429.

257. Dove W, Cunliffe NA, Gondwe JS, Broadhead RL, Molyneux ME, Nakagomi O, Hart CA: **Detection and characterization of human caliciviruses in hospitalized children with acute gastroenteritis in Blantyre, Malawi**. *Journal of medical virology* 2005, **77**(4):522-527.

258. Trainor E, Lopman B, Iturriza-Gomara M, Dove W, Ngwira B, Nakagomi O, Nakagomi T, Parashar U, Cunliffe N: **Detection and molecular characterisation of noroviruses in hospitalised children in Malawi, 1997-2007**. *Journal of medical virology* 2013, **85**(7):1299-1306.

259. Mans J, de Villiers JC, du Plessis NM, Avenant T, Taylor MB: **Emerging norovirus GII.4 2008 variant detected in hospitalised paediatric patients in South Africa**. *J Clin Virol* 2010, **49**(4):258-264.

260. de Wit MA, Koopmans MP, Kortbeek LM, van Leeuwen NJ, Bartelds AI, van Duynhoven YT: **Gastroenteritis in sentinel general practices,The Netherlands**. *Emerging infectious diseases* 2001, **7**(1):82-91.

261. O'Ryan ML, Mamani N, Gaggero A, Avendano LF, Prieto S, Pena A, Jiang X, Matson DO: **Human caliciviruses are a significant pathogen of acute sporadic diarrhea in children of Santiago, Chile**. *The Journal of infectious diseases* 2000, **182**(5):1519-1522.
262. Oh DY, Gaedicke G, Schreier E: **Viral agents of acute gastroenteritis in German children: prevalence and molecular diversity**. *Journal of medical virology* 2003, **71**(1):82-93.
263. Parashar UD, Li JF, Cama R, DeZalia M, Monroe SS, Taylor DN, Figueroa D, Gilman RH, Glass RI: **Human caliciviruses as a cause of severe gastroenteritis in Peruvian children**. *The Journal of infectious diseases* 2004, **190**(6):1088-1092.
264. Monica B, Ramani S, Banerjee I, Primrose B, Iturriza-Gomara M, Gallimore CI, Brown DW, M F, Moses PD, Gray JJ *et al*: **Human caliciviruses in symptomatic and asymptomatic infections in children in Vellore, South India**. *Journal of medical virology* 2007, **79**(5):544-551.
265. Bucardo F, Nordgren J, Carlsson B, Kindberg E, Paniagua M, Mollby R, Svensson L: **Asymptomatic norovirus infections in Nicaraguan children and its association with viral properties and histo-blood group antigens**. *The Pediatric infectious disease journal* 2010, **29**(10):934-939.
266. Huh JW, Kim WH, Moon SG, Lee JB, Lim YH: **Viral etiology and incidence associated with acute gastroenteritis in a 5-year survey in Gyeonggi province, South Korea**. *J Clin Virol* 2009, **44**(2):152-156.
267. Bresee JS, Glass RI, Ivanoff B, Gentsch JR: **Current status and future priorities for rotavirus vaccine development, evaluation and implementation in developing countries**. *Vaccine* 1999, **17**(18):2207-2222.
268. Bucardo F, Nordgren J, Carlsson B, Paniagua M, Lindgren PE, Espinoza F, Svensson L: **Pediatric norovirus diarrhea in Nicaragua**. *Journal of clinical microbiology* 2008, **46**(8):2573-2580.
269. Nitiema LW, Nordgren J, Ouermi D, Dianou D, Traore AS, Svensson L, Simpore J: **Burden of rotavirus and other enteropathogens among children with diarrhea in Burkina Faso**. *Int J Infect Dis* 2011, **15**(9):e646-652.
270. Arora R, Chitambar SD: **Full genomic analysis of Indian G1P[8] rotavirus strains**. *Infect Genet Evol* 2011, **11**(2):504-511.
271. Cortese MM, Parashar UD, Centers for Disease C, Prevention: **Prevention of rotavirus gastroenteritis among infants and children: recommendations of the Advisory Committee on Immunization Practices (ACIP)**. *MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports / Centers for Disease Control* 2009, **58**(RR-2):1-25.
272. Afrad MH, Hassan Z, Farjana S, Moni S, Barua S, Das SK, Faruque AS, Azim T, Rahman M: **Changing profile of rotavirus genotypes in Bangladesh, 2006-2012**. *BMC infectious diseases* 2013, **13**:320.

273. Madhi SA, Cunliffe NA, Steele D, Witte D, Kirsten M, Louw C, Ngwira B, Victor JC, Gillard PH, Cheuvart BB *et al*: **Effect of human rotavirus vaccine on severe diarrhea in African infants**. *The New England journal of medicine* 2010, **362**(4):289-298.

274. Parashar UD, Burton A, Lanata C, Boschi-Pinto C, Shibuya K, Steele D, Birmingham M, Glass RI: **Global mortality associated with rotavirus disease among children in 2004**. *The Journal of infectious diseases* 2009, **200 Suppl 1**:S9-S15.

275. Patel M, Shane AL, Parashar UD, Jiang B, Gentsch JR, Glass RI: **Oral rotavirus vaccines: how well will they work where they are needed most?** *The Journal of infectious diseases* 2009, **200 Suppl 1**:S39-48.

276. Mattison K, Sebunya TK, Shukla A, Noliwe LN, Bidawid S: **Molecular detection and characterization of noroviruses from children in Botswana**. *Journal of medical virology* 2010, **82**(2):321-324.

277. Silva PA, Stark K, Mockenhaupt FP, Reither K, Weitzel T, Ignatius R, Saad E, Seidu-Korkor A, Bienzle U, Schreier E: **Molecular characterization of enteric viral agents from children in northern region of Ghana**. *Journal of medical virology* 2008, **80**(10):1790-1798.

278. Ayukekbong J, Lindh M, Nenonen N, Tah F, Nkuo-Akenji T, Bergstrom T: **Enteric viruses in healthy children in Cameroon: viral load and genotyping of norovirus strains**. *Journal of medical virology* 2011, **83**(12):2135-2142.

279. Bull RA, Tu ET, McIver CJ, Rawlinson WD, White PA: **Emergence of a new norovirus genotype II.4 variant associated with global outbreaks of gastroenteritis**. *Journal of clinical microbiology* 2006, **44**(2):327-333.

280. Huhti L, Blazevic V, Puustinen L, Hemming M, Salminen M, Vesikari T: **Genetic analyses of norovirus GII.4 variants in Finnish children from 1998 to 2013**. *Infect Genet Evol* 2014.

281. Kroneman A, Vennema H, Harris J, Reuter G, von Bonsdorff CH, Hedlund KO, Vainio K, Jackson V, Pothier P, Koch J *et al*: **Increase in norovirus activity reported in Europe**. *Euro Surveill* 2006, **11**(12):E061214 061211.

282. Medici MC, Martinelli M, Abelli LA, Ruggeri FM, Di Bartolo I, Arcangeletti MC, Pinardi F, De Conto F, Izzi G, Bernasconi S *et al*: **Molecular epidemiology of norovirus infections in sporadic cases of viral gastroenteritis among children in Northern Italy**. *Journal of medical virology* 2006, **78**(11):1486-1492.

283. Noel JS, Fankhauser RL, Ando T, Monroe SS, Glass RI: **Identification of a distinct common strain of "Norwalk-like viruses" having a global distribution**. *The Journal of infectious diseases* 1999, **179**(6):1334-1344.

284. Siebenga JJ, Vennema H, Zheng DP, Vinje J, Lee BE, Pang XL, Ho EC, Lim W, Choudekar A, Broor S *et al*: **Norovirus illness is a global problem: emergence and spread of norovirus GII.4 variants, 2001-2007**. *The Journal of infectious diseases* 2009, **200**(5):802-812.

285. Tu ET, Bull RA, Greening GE, Hewitt J, Lyon MJ, Marshall JA, McIver CJ, Rawlinson WD, White PA: **Epidemics of gastroenteritis during 2006 were associated with the spread of norovirus GII.4 variants 2006a and 2006b**. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 2008, **46**(3):413-420.

286. Tu ET, Nguyen T, Lee P, Bull RA, Musto J, Hansman G, White PA, Rawlinson WD, McIver CJ: **Norovirus GII.4 strains and outbreaks, Australia**. *Emerging infectious diseases* 2007, **13**(7):1128-1130.

287. Vega E, Barclay L, Gregoricus N, Williams K, Lee D, Vinje J: **Novel surveillance network for norovirus gastroenteritis outbreaks, United States**. *Emerging infectious diseases* 2011, **17**(8):1389-1395.

288. Fioretti JM, Bello G, Rocha MS, Victoria M, Leite JP, Miagostovich MP: **Temporal dynamics of norovirus GII.4 variants in Brazil between 2004 and 2012**. *PLoS One* 2014, **9**(3):e92988.

289. Tra My PV, Lam HM, Thompson CN, Phuc HL, Tuyet PT, Vinh H, Hoang NV, Minh P, Vinh NT, Thuy CT *et al*: **The dynamics of GII.4 Norovirus in Ho Chi Minh City, Vietnam**. *Infect Genet Evol* 2013, **18**:335-343.

290. Okada M, Ogawa T, Kaiho I, Shinozaki K: **Genetic analysis of noroviruses in Chiba Prefecture, Japan, between 1999 and 2004**. *Journal of clinical microbiology* 2005, **43**(9):4391-4401.

291. Ferreira MS, Cubel Garcia Rde C, Xavier Mda P, Ribeiro RL, Assis RM, Mota Mdo C, Leite JP, Miagostovich MP, de Oliveira SA: **Genotyping of gastroenteric viruses in hospitalised children: first report of norovirus GII.21 in Brazil**. *Memorias do Instituto Oswaldo Cruz* 2012, **107**(8):1064-1067.

292. de Jong JC, Wigand R, Kidd AH, Wadell G, Kapsenberg JG, Muzerie CJ, Wermenbol AG, Firtzlaff RG: **Candidate adenoviruses 40 and 41: fastidious adenoviruses from human infant stool**. *Journal of medical virology* 1983, **11**(3):215-231.

293. Saderi H, Roustai MH, Sabahi F, Sadeghizadeh M, Owlia P, De Jong JC: **Incidence of enteric adenovirus gastroenteritis in Iranian children**. *J Clin Virol* 2002, **24**(1-2):1-5.

294. Li L, Shimizu H, Doan LT, Tung PG, Okitsu S, Nishio O, Suzuki E, Seo JK, Kim KS, Muller WE *et al*: **Characterizations of adenovirus type 41 isolates from children with acute gastroenteritis in Japan, Vietnam, and Korea**. *Journal of clinical microbiology* 2004, **42**(9):4032-4039.

295. Shimizu H, Phan TG, Nishimura S, Okitsu S, Maneekarn N, Ushijima H: **An outbreak of adenovirus serotype 41 infection in infants and children with acute gastroenteritis in Maizuru City, Japan**. *Infect Genet Evol* 2007, **7**(2):279-284.

296. Garnett CT, Erdman D, Xu W, Gooding LR: **Prevalence and quantitation of species C adenovirus DNA in human mucosal lymphocytes**. *Journal of virology* 2002, **76**(21):10608-10616.

297. Brookfield DSK, Cosgrove BP, Bell EJ, Madeley CR: **Viruses demonstrated in children in Tanzania: studies in diarrhoea and measles**. *Journal of Infection* 1979, **1**(3):249-255.

298. Mhalu FS, Myrmel H, Msengi A, Haukenes G: **Prevalence of infection with rotavirus and enteric adenoviruses among children in Tanzania**. *NIPH Ann* 1988, **11**(1):3-7.

299. Inouye S, Yamashita K, Yamadera S, Yoshikawa M, Kato N, Okabe N: **Surveillance of viral gastroenteritis in Japan: pediatric cases and outbreak incidents**. *The Journal of infectious diseases* 2000, **181 Suppl 2**:S270-274.

300. Xi JN, Graham DY, Wang KN, Estes MK: **Norwalk virus genome cloning and characterization**. *Science* 1990, **250**(4987):1580-1583.

301. Cegielski JP, Msengi AE, Miller SE: **Enteric viruses associated with HIV infection in Tanzanian children with chronic diarrhea**. *Pediatr AIDS HIV Infect* 1994, **5**(5):296-299.

302. Liste MB, Natera I, Suarez JA, Pujol FH, Liprandi F, Ludert JE: **Enteric virus infections and diarrhea in healthy and human immunodeficiency virus-infected children**. *Journal of clinical microbiology* 2000, **38**(8):2873-2877.

303. Oshitani H, Kasolo FC, Mpabalwani M, Luo NP, Matsubayashi N, Bhat GH, Suzuki H, Numazaki Y, Zumla A, DuPont HL: **Association of rotavirus and human immunodeficiency virus infection in children hospitalized with acute diarrhea, Lusaka, Zambia**. *The Journal of infectious diseases* 1994, **169**(4):897-900.

304. Rossit AR, de Almeida MT, Nogueira CA, da Costa Oliveira JG, Barbosa DM, Moscardini AC, Mascarenhas JD, Gabbay YB, Marques FR, Cardoso LV *et al*: **Bacterial, yeast, parasitic, and viral enteropathogens in HIV-infected children from Sao Paulo State, Southeastern Brazil**. *Diagnostic microbiology and infectious disease* 2007, **57**(1):59-66.

305. Rabenau HF, Kessler HH, Kortenbusch M, Steinhorst A, Raggam RB, Berger A: **Verification and validation of diagnostic laboratory tests in clinical virology**. *J Clin Virol* 2007, **40**(2):93-98.

306. Stroparo E, Cruz CR, Debur Mdo C, Vidal LR, Nogueira MB, Almeida SM, Pereira LA, Rotta I, Raboni SM: **Adenovirus respiratory infection: significant increase in diagnosis using PCR comparing with antigen detection and culture methods**. *Revista do Instituto de Medicina Tropical de Sao Paulo* 2010, **52**(6):317-321.

307. Rovida F, Campanini G, Sarasini A, Adzasehoun KM, Piralla A, Baldanti F: **Comparison of immunologic and molecular assays for the diagnosis of gastrointestinal viral infections**. *Diagnostic microbiology and infectious disease* 2013, **75**(1):110-111.

308. Kiulia NM, Nyaga MM, Seheri ML, Wolfaardt M, van Zyl WB, Esona MD, Irimu G, Inoti M, Gatinu BW, Njenga PK *et al*: **Rotavirus G and P types circulating in the eastern region of Kenya: predominance of G9 and emergence of G12 genotypes**. *The Pediatric infectious disease journal* 2014, **33 Suppl 1**:S85-88.

309. Page AL, Jusot V, Mamaty AA, Adamou L, Kaplon J, Pothier P, Djibo A, Manzo ML, Toure B, Langendorf C *et al*: **Rotavirus surveillance in urban and rural areas of Niger, April 2010-March 2012**. *Emerging infectious diseases* 2014, **20**(4):573-580.

310. Aladin F, Nawaz S, Iturriza-Gomara M, Gray J: **Identification of G8 rotavirus strains determined as G12 by rotavirus genotyping PCR: updating the current genotyping methods**. *J Clin Virol* 2010, **47**(4):340-344.

311. Iturriza-Gomara M, Kang G, Gray J: **Rotavirus genotyping: keeping up with an evolving population of human rotaviruses**. *J Clin Virol* 2004, **31**(4):259-265.

312. Nordgren J, Nitiema LW, Sharma S, Ouermi D, Traore AS, Simpore J, Svensson L: **Emergence of unusual G6P[6] rotaviruses in children, Burkina Faso, 2009-2010**. *Emerging infectious diseases* 2012, **18**(4):589-597.

313. Chandrahasen C, Grimwood K, Redshaw N, Rich FJ, Wood C, Stanley J, Kirman JR: **Geographical differences in the proportion of human group A rotavirus strains within New Zealand during one epidemic season**. *Journal of medical virology* 2010, **82**(5):897-902.

314. Godoy P, Ferrrus G, Torner N, Camps N, Sala MR, Guix S, Bartolome R, Martinez A, Simon MD, Dominguez A: **High incidence of norovirus GII.4 outbreaks in hospitals and nursing homes in Catalonia (Spain), 2010-2011**. *Epidemiol Infect* 2014:1-9.

APPENDIX I

QUESTIONNARE FOR CASES

MUHIMBILI UNIVERSITY OF HEALTJH AND ALLIED SCIENCES (MUHAS)

Title: Rotavirus infection in hospitalized children with diarrhoea and asymptomatic children below five years at Muhimbili National Hospital, Dar es Salaam Tanzania

1. Child ID Number □□□□□□
2. Name and initials of the child …………………………..
3. Informant (a) Parent (mother / father) □
   (b) Care taker □
4. Residential area/ place of stay ………………………..
5. Which district are you coming from?
   a) Ilala □
   b) Temeke □
   c) Kinondoni □
6. What is the parent/guardian’s highest level of education?
   a) Informal □
   b) Primary □
   c) Secondary □
   d) Post-secondary education □

Particulars of the child

7. Sex (a) male □ (b) Female □
8. Age …………………..
9. Date of birth (from MCH card) …………………..
10. Weight (in kilograms) ………………….
11. Height (in centimetres) ……………….
12. Mid Upper Arm Circumference (MUAC) (in centimetres) ………..

Symptoms and signs

13. What is the duration of Diarrhoea?
   (a) ≤ 13 days □
   (b) ≥ 14 days □

14. Is the stool blood stained?

    (a) Yes □ (b) No □

15. Has the child been treated with antibiotics the last 2 weeks?

    (a) Yes □ (b) No □

16. If so when/why/ name of drug?

    ……………………………………………….

17. Hydration status of the child

    a) no dehydration
    b) some dehydration
    c) severe dehydration

Breast feeding practice

18. Is the child on exclusive breast feeding (if below six months)

    (a) Yes □ (b) no □

19. Is the child still breast feeding (if the child is above (six- twelve months)

    (a) Yes □ (b) no □

APPENDIX II

QUESTIONNARE FOR CONTROLS

MUHIMBILI UNIVERSITY OF HEALTH AND ALLIED SCIENCES (MUHAS)

Title: Rotavirus infection in hospitalized children with diarrhoea and asymptomatic children below five years at Muhimbili National Hospital, Dar es Salaam Tanzania

1. Child ID Number □□□□□□
2. Name and initials of the child …………………………..
3. Informant (a) Parent (mother / father)

   (b) Care taker □
4. Residential area/ place of stay ………………………..
5. Which district are you coming from?

   a) Ilala □

   b) Temeke □

   c) Kinondoni □
6. What is the parent/guardian's highest level of education?

a) Informal □

b) Primary □

c) Secondary □

d) Post secondary □

Particulars of the child

7. Sex (a) male □ (b) Female □
8. Age …………………..
9. Date of birth (from MCH card) …………………..
10. Weight (in kilograms) ………………….
11. Height (in centimetres) ……………….
12. Mid Upper Arm Circumference (MUAC) (in centimetres) ………..
13. Has the child been treated with antibiotics the last 2 weeks?

    (a) Yes □ (b) No □
14. If so when/why/ name of drug?

    ……………………………………………….

Breast feeding practice

15. Is the child on exclusive breast feeding (if below six months)

    (a) Yes □ (b) no □

16. Is the child still breast feeding (if the child is above six- twelve months)

    (a) Yes □ (b) no □