Molecular Detection of Enteric Viruses in Under-Five Children with Diarrhea in Debre Tabore, Northwest Ethiopia

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Research Article

Keywords: diarrhea, under-children, enteric viruses, Debre Tabore, Ethiopia, vaccines

Posted Date: December 14th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-1157000/v1

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Abstract

Viral gastroenteritis belongs to the major public health problems of infant and children worldwide. The largest proportion of morbidity and mortality occurs in Sub-Saharan Africa. This preliminary study aimed to assess the burden and genetic diversity of enteric viruses among children with diarrhea in Debre Tabor. A cross-sectional study was undertaken from December 2015 to April 2016. A total of thirty-eight children, who presented with diarrhea at Debre Tabor health centers were included. Fecal samples were collected and screened for enteric viruses by RT-PCR. Data were analyzed by using SPSS statistical software. Descriptive statistical summary techniques were used to display the study findings. Out of the thirty-eight children screened, 52.6% were positive for at least one enteric virus. Six (30.0%) of the children had mixed enteric virus infections. Human adenovirus (HAdV) 7 (18.4%) was predominant followed by noroviruses 5 (13.2%), enterovirus 5 (13.2%), rotavirus 4 (10.5%), human astrovirus (HAstV) 2 (5.3%), and human parechovirus (HPeV) 1(2.6%). Overall nineteen different types of enteric virus genotypes were identified. Diverse adenovirus within species A (HAdV-12,-31), B (HAdV-3), C (HAdV-2), and F (HAdV-4) were detected. Norovirus II (GII.4 and GII.6) and norovirus I (GI.2, GI.3, and GI.5) genotypes were found. Sapovirus genotypes within genogroup II (GII.1, GII.5, and GII.6) were identified. Wild-type rotavirus G9[P8] genotype was detected in one of the rotavirus positive samples. Non-polio enteroviruses within species A (coxsackie A virus (CAV) 5, CAV6, and CAV14) and C (enterovirus (EV-C) 99) were also identified. HPeV-6 genotype was identified in one of the samples. In two of the fecal samples, classic HAstV-2 was detected. Phylogenetic analysis of these enteric viruses revealed that they have close phylogenetic relatedness with previous genotypes reported from Ethiopia. Diverse enteric viruses were detected in fecal samples from under-five children with diarrhea. The detection of heterogeneous enteric viruses in this small data set highlights the need for extended multicenter studies to describe the burden and genetic diversity.

1. Introduction

Diarrheal disease is one of the leading causes of morbidity and mortality worldwide. It is accounted for approximately 8% (499,000) of all deaths in under-five children worldwide in 2017, with the largest proportion of deaths in sub-Saharan Africa and Asia [1, 2]. Enteric viruses are the most common pathogens causing diarrhea in high-income as well as low-income countries [3, 4]. The most common agents are rotavirus (RV), human adenovirus (HAdV), noroviruses (NoV), sapovirus (SaV), and human astrovirus (HAstV). Human parechovirus (HPeV) and enterovirus (EV) have also been associated with gastroenteritis [5, 6].

Despite the availability of effective vaccines, species A RV (RVA) is still the leading cause of morbidity and mortality worldwide [7]. Rotaviruses are double stranded (ds) RNA viruses belonging to the family Reoviridae. To date, more than nine species of RV have been recognized [8]. Species RVA is accountable for more than 90% of the infections. The RVA is further classified into P and G-types based on the VP4 and VP7 segments, respectively [9]. As per the WHO recommendation, Ethiopia has introduced the Rotarix vaccine since November 2013 [10, 11]. NoVs and SaVs are also recognized as the major viral pathogens among patients with diarrhea. They belong to the family of Caliciviridae, naked viruses with positive-
sense single-stranded (SS) RNA genome [12, 13]. Both of them affect humans and animals and show extensive genetic diversity. Based on the VP1 gene, SaVs and NoVs are classified into 15 (GI–GXV) and ten (GI–GX) genogroups, respectively [14, 15]. NoV genogroup GI, GII, GIV, GVIII, and GIX infect humans, with GII and GI predominance [14, 16, 17]. Human pathogenic SaV strains are assigned to GI, GII, GIV, and GV genogroups [14]. Adenoviruses are non-enveloped, dsDNA viruses with more than 90 recognized serotypes. They are associated with a wide spectrum of symptoms, including gastroenteritis, respiratory infections, conjunctivitis, hemorrhagic cystitis, and meningoencephalitis [18, 19]. Types 40 and 41 are the most frequently reported cause of HAdV-associated gastroenteritis [19–22]. HAstV are non-enveloped and contain a positive sense of ssRNA [24]. The HAstVs are categorized into classical and novel or divergent groups. The classic group consists of eight genotypes, which are responsible for 2 to 9% of acute gastroenteritis cases in children worldwide [25, 23]. Enterovirus and HPeV are belonging to the family Picornaviridae. The family contains extensively diverse genotypes. They are associated with a wide array of clinical manifestations ranging from respiratory or gastrointestinal symptoms to neonatal sepsis and infections of the central nervous system [26].

In Ethiopia, investigation of diarrheal samples is largely limited to light microscopic and limited bacterial culture. Investigation of enteric viruses as causes for diarrhea is uncommon. Moreover, epidemiological studies on viral infections are rare. Those few available studies focus on RVs and NoVs. Hence, the contributions of other enteric viruses in case of diarrhea remain unknown. Therefore, this study was aimed to provide preliminary information on the burden and genetic diversity of enteric viruses in Debre Tabore, Northwest Ethiopia.

2. Material And Methods

2.1. Study area and period

This cross-sectional study was conducted at the outpatient health institutions in Debre Tabore town, from December 1, 2015, to April 30, 2016. The town is an administrative center of South Gondar Zone, Amhara National Reginal State, Northwest Ethiopia. Debre Tabore has situated 50 km to the East of Lake Tana. There are a referral hospital and two health centers in the town. The study was approved by the Ethical Review Board of the University of Gondar (O/V/RC/05/1180/2016). Parents or guardians of children provided their informed consent to participate in the study.

2.2. Specimen collection

A total of thirty-eight fecal samples were collected from infants and under-five children with diarrhea attending the outpatient departments. Clinical information and demographic data such as age, sex, and residential area were recorded. Parents or guardians of children were instructed on how to collect fecal samples from their children. About 1-3 g of the fecal specimen was saved. The samples were stored at -20°C at the site of collection, before being transported to the Institute of Virology, Leipzig University, Germany, for further viral screening and molecular characterization.
2.3. Nucleic acid extraction

For enteric virus screening and genotyping, 10% suspensions of fecal samples were prepared using phosphate-buffered saline. Viral nucleic acids were extracted from 200 µl of the concentrated sample using MagNA Pure 96 Instrument (Roche, Germany) as per the instruction of the manufacturer. The extracted nucleic was stored at -80°C until further use.

2.4. Detection and quantification

The samples were screened for RVA, NoV, SaV, HAdV, HAstV, EV, and HPeV by using real-time RT-qPCR. Screening and quantification of each of the enteric viruses were carried out using published primers and probes [27–34].

2.5. Genotyping and sequencing of enteric viruses

Rotavirus strain was G and P genotyped by amplification of the VP7 and VP4 segments, respectively [28]. For SaV and NoV capsid genotyping, a portion of the VP1 gene was amplified by using previously published protocols [14, 35]. Likewise, HAdV genotyping was performed by amplification of a portion of the hexon loop one region as previously described [36]. For the classic HAstV genotyping, a partial region of the 5’ end of the open reading frame (ORF) 2 gene coding for the capsid protein precursor was amplified using primers and probes as described previously [32]. Samples positive for EVs were further amplified on the portion of the VP1 region using nested RT-PCR according to previously published primers [33]. The HPeV genotyping was performed by amplification and sequencing of the VP3/ VP1 transition region as described previously [32].

The PCR products for each of the enteric viruses were subjected to gel electrophoresis in a 2% agarose gel and purified using a Wizard® SV Gel and PCR Clean-Up System (Promega, Mannheim, Germany). Sequencing reactions were performed using BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Corporation, Foster City, CA, USA). Finally, sequencing was performed on ABI PRISM3500 Genetic Analyzer as per the manufacturer's instructions (Applied Biosystems). Phylogenetic trees were constructed using MEGA software (version 7). The Neighbor-Joining (NJ) method with Kimura’s two-parameter correction was selected to construct the trees. Bootstrap analysis was performed with 1,000 replicates. The nucleotide sequences of each of the enteric viruses were submitted and deposited to GenBank under the accession numbers: HAstV (OK483335-OK483336), NoV (OK493612-OK493616), and the other enteric viruses (OK500274-OK500289).

2.6. Statistical analyses

Data were statistically analyzed using the Statistical Package for the Social Sciences (SPSS) version 16 software. Discrete variables were expressed as numbers and percentages. Data were compared using chi-square and fisher’s exact test. The level of statistical significance was set at a P-value of 0.05.

3. Results
3.1. Enteric viruses stool positivity rate

During the preliminary assessment period, thirty-eight infants and children with diarrhea were enrolled and tested for RV, NoV, SaV, HAdV, HAstV, EV, and HPeV using RT-PCR. More than half (52.6%) of the children were male. The majority, 27 (71.1%) of the children were from rural villages. The mean (SD) age of the children was 31.5 (+/- 17.7) months (Table 1). The positivity rate of children at least for one of the enteric viruses was 52.6%. There was significant difference in positivity rate of children younger than 1 year (60%) and 1-<5 years old (50%) (P= 0.719) and the diversity of enteric viruses was similar. The EV and HPeV were exclusively detected in children older than 2 years. The detection rates of HAdV, NoV, EV, RV, SaV, HAstV and HPeV were 7 (18.4%), 5 (13.2%), 5 (13.2%), 4 (10.5%), 2 (5.3%), and 1 (2.6%), respectively (Table 2). Mixed infection was detected in 6 of the children (SaV + HAdV, SaV + EV, HAdV + EV (n = 2) and NoV + HAdV + HAstV).
Table 1
The positivity rate of enteric virus by demographic and clinical characteristics of the study participants

| Characteristics        | Number (%) | Enteric virus status |          |          |
|------------------------|------------|----------------------|----------|----------|
|                        |            | Positive (%)         | Negative (%) |          |          |
| **Sex**                |            |                      |          |          |
| Male                   | 20 (52.6)  | 12 (60.0)            | 8 (40.0) | P = 0.782 |
| Female                 | 18 (47.4)  | 8 (44.4)             | 10 (55.6) |          |          |
| **Age category**       |            |                      |          |          |
| 0 to <24 months        | 15 (39.5)  | 8 (53.3)             | 7 (46.7) | P = 0.944 |
| 24 to <60 months       | 23 (60.5)  | 12 (52.2)            | 11 (47.8) |          |          |
| **Residence**          |            |                      |          |          |
| Urban                  | 11 (28.9)  | 8 (72.7)             | 3 (27.3) | P = 0.113 |
| Rural                  | 27 (71.1)  | 12 (44.4)            | 15 (55.6) |          |          |
| **Vomiting**           |            |                      |          |          |
| Yes                    | 18 (55.4)  | 10 (55.6)            | 8 (44.4) | P = 0.732 |
| No                     | 20 (52.6)  | 10 (50.0)            | 10 (50.0) |          |          |
| **Fever**              |            |                      |          |          |
| Yes                    | 30 (78.9)  | 17 (56.7)            | 13 (43.3) | P = 0.355 |
| No                     | 8 (21.1)   | 3 (37.5)             | 5 (62.5)  |          |          |
| **Dehydration**        |            |                      |          |          |
| None                   | 35 (92.1)  | 19 (54.3)            | 16 (45.7) | P = 0.485 |
| Some                   | 3 (7.9)    | 1 (33.3)             | 2 (66.7)  |          |
Table 2  
Genotypic distribution of enteric viruses in under-five children with diarrhea

| Type of enteric viruses | Children (n=38) |
|-------------------------|----------------|
| Adenovirus              | 7 (18.4%)      |
| AdV-2                   | 2              |
| AdV-3                   | 1              |
| AdV-12                  | 1              |
| AdV-31                  | 2              |
| AdV-41                  | 1              |
| Enterovirus             | 5 (13.2%)      |
| CV-A5                   | 1              |
| CV-A6                   | 1              |
| CV-A14                  | 1              |
| EV-C99                  | 1              |
| Untypable               | 1              |
| Norovirus I and II      | 5 (13.2%)      |
| GII.4                   | 1              |
| GII.6                   | 1              |
| GI.2                    | 1              |
| GI.3                    | 1              |
| GI.5                    | 1              |
| Rotavirus A             | 4 (10.5%)      |
| G9P[8]                  | 1              |
| Untypable               | 3              |
| Sapovirus               | 3 (7.9%)       |
| GII.1                   | 1              |
| GII.5                   | 1              |
| GII.6                   | 1              |
| Human astrovirus, HastV-2 | 2 (5.3%)    |
| Type of enteric viruses                  | Children (n=38) |
|----------------------------------------|----------------|
| Human parechovirus, HpeV-6             | 1 (2.6%)       |
| Total                                  | 20 (52.6%)     |

### 3.2. Genotypic distribution and phylogenetic analysis of enteric viruses

All the fecal samples positive for the enteric viruses by RT-PCR were further amplified for the purpose of genotypic and phylogenetic analysis.

#### 3.2.1. Rotavirus

Rotavirus was detected in four (10.5%) of the fecal samples. Of these, only one of the samples was successfully genotyped and three could not be genotyped. Phylogenetic and sequence analyses revealed the detection of G9P[8] RV genotype. Both the VP7 and VP4 sequences showed close phylogenetic relatedness with other similar sequences reported from Ethiopia in 2012 and 2016 (Fig. 1A and Fig. 1B).

#### 3.2.2. Norovirus I and II

Norovirus was detected in five samples (13.2%). Amplification and sequencing of a portion of the VP1 region were successful in all of the positive samples. The phylogenetic and BLAST search analysis showed the presence of NoV II (GII.6 and GII.4) and NoV I (GI.2, G.3, and GI.5) genotypes. The present GII.4 sequence was identified as GII.4 Sydney 2012 variants by the online Norovirus Typing Tool. The GII.4 and GII.6 genotypes showed clustering with previously published global sequences (Fig. 1C).

#### 3.2.3. Astrovirus

This study revealed that 2 (5.3%) of the participants had HAstV infection. Phylogenetic analysis of the two positive cases from a partial region of the ORF2 gene confirmed the detection of HAstV-2 genotypes. The current Ethiopian HAstV strains showed close phylogenetic relatedness with a similar HAstV-2 strain reported from Kenya in 1999 (Fig. 1D).

#### 3.2.4. Sapovirus

For characterization of SaV strains, the major capsid coding region (VP1) was phylogenetically analyzed (Fig. 1E). All SaV positive samples were successfully genotyped and assigned into a single genogroup II. Three different GII genotypes (GII.1, GII.5, and GII.6) were found.

#### 3.2.5. Adenoviruses

In this study, seven of the samples were positive for HAdV. To characterize the HAdV strains the hexon loop one coding region was further amplified and sequenced. The phylogenetic analysis revealed that the
current Ethiopian strains belonged to the species HAdV-A (HAdV-12 and HAdV-31), HAdV- B (HAdV-3), HAdV-C (AdV-2), and HAdV-F (HAdV-41) species (Fig. 1F).

### 3.2.6. Enterovirus and parechovirus

Five and one of the samples were positive for EV and HPeV, respectively. Sequence analysis based on the VP1 gene revealed that the current Ethiopian EV isolates grouped into EV-A (CAV5, CAV6, and CAV14) and EV-C (EV-C99). One of the positive samples was not successfully amplified. Phylogenetic analysis of the species A EVs indicated that CAV5 sequences clustered most closely to previous Ethiopian sequences isolated in Gondar in 2016. Likewise, the current CAV6 sequence showed close relatedness with a similar strain reported from China in 2017. The CAV14 sequence of this study should distinct clustering from the global and previous Ethiopia isolates (Fig. 1G). The EV-C99 sequence showed close phylogenetic relatedness to sequences isolated in Cameroon in 2009 (Fig. 1H). One of the samples was positive for HPeV and its sequence analysis revealed that the identified genotype was HPeV-6.

### 4. Discussion

Although the burden of enteric viruses has been well characterized for industrialized countries, there is little information in resource-limited countries. In this preliminary assessment, based on direct molecular detection and characterization, we confirmed the detection of seven different and diverse types of enteric viruses. To the best of our knowledge, this is one of the very few assessments which considered the screening of multiple enteric viruses in Ethiopian under-five children.

The burden and genotypic distribution of RV in Ethiopia has been reviewed by considering studies published before 2020 [37]. According to this review, the most common genotypes were G12P[8], G3P[6], G1P[8], and G3P[8]. The G3 and P[8] were the predominant G and P-types in Ethiopia [37]. In the present study, four of the samples were positive for RVA, with a positivity rate of 10.5%. The present low positivity rate of RV is consistent with a previous report from northwestern Ethiopia [38]. In our study, unfortunately, genotyping was not successful for three of the samples might be due to the low viral load observed during the RT-qPCR assay. Similar to other previous studies G9[P8] was detected [39, 40, 41]. Phylogenic analysis of both the VP7 and VP4 sequences demonstrated that the current rotavirus genotype is closely related to previously reported genotypes from Ethiopia in 2009 and 2012.

There is a paucity of data on the genetic diversity of caliciviruses in Ethiopia. In the present study, SaV was detected and sequenced. The phylogenetic analysis based on sequences of a portion of the VP1 region confirmed the detection of GII.1, GII.5, and GII.6 genotypes. Except for GII.5, the present SaV genotypes were reported from previous studies in Northwest Ethiopia [42, 43] and Addis Ababa [44]. The reporting of the GII.5 genotype in this study is a new addition to the data on the genetic diversity of SaV in Ethiopia. In most of the previous studies in Africa, SaV-GI.1 followed by GI.2 was the predominant genotype detected in clinical samples [45]. A similar proportion of NoVs to that of SaVs positive cases was observed in this study. Extensively diverse NoV genotypes within genogroup I and genogroup II were reported from previous studies in Ethiopia [40, 42–44]. The present study also confirms the circulation of
three genotypes in genogroup I (GI.2, GI.3, and G1.5) and two genotypes in genogroup II (GII.4, and GII.6) genotypes in Debre Tabore, Northwest Ethiopia. Except for G1.5, the other SaV genotypes were reported previously from Gondar [41].

In this study, HAdV was the most frequently detected enteric virus, with a detection rate of 18.4%. In this small data set, sequence analysis revealed that a wide variety of HAdV species (four) and types (five) were circulating in Debre Tabore, Northwest Ethiopia. The HAdV types were belonging to HAdV-A (HAdV-12 and -31), HAdV-B (HAdV-3), HAdV-C (HAdV-2), and HAdV-F (HAdV-41). This observation was consistent with a previous report in Ethiopia in 2016 (46). It has been documented that types 40 and 41 are the most frequently reported cause of adenovirus-associated gastroenteritis (21, 22). A single HAdV-41 was detected in the present study and the phylogenetic analysis showed that this genotype has close relatedness with the previous genotype reported from Bahir Dar in 2016 and other global reports. Adenovirus types are known to infect respiratory tracts were also detected. These HAdV types might be shed from the respiratory tract and detected in the feces of the children. However, their contribution to the causation of diarrhea cannot be excluded as they have also been reported to cause diarrhea [19, 20].

Enteric viruses including EV, HPeV, and HAstV are responsible for a wide range of clinical diseases in humans. Unfortunately, the burden and genetic diversity of these enteric viruses in Sub-Saharan Africa remain under-documented. In the current study, a considerable number of EV (CAV5, CAV6, CAV14, and EV-C99), HPeV-6) and HAstV-2 genotypes were detected from the stool samples of children with diarrhea. Similar to our observation, the present EV genotypes have been reported from previous studies in Ethiopia and other sub-Saharan African countries [42, 47–51]. The phylogenetic and individual sequence analysis showed the genotypes are closely related to previous isolates from Ethiopia, Cameroon, and China. Similar to the present study, HPeV-2 and HAstV-2 genotypes were reported from Gondar [46, 51].

The present study has the following limitations. Case number is very low but it is from a single center. The positivity rates and genetic diversities of the different enteric viruses observed in this smaller study might be enlightening but not conclusive. Longitudinal studies with large sample sizes are required to evaluate the precise prevalence, genetic diversity, and their contributions to diarrheal disease in children in Ethiopia.

In conclusion, enteric viruses were detected in 52.6% of the samples obtained from under-five children with diarrhea. Phylogenic analysis revealed the circulation of extensively diverse genotypes in the study area. The detection of heterogeneous enteric viruses in this small data set highlights the need for extended multicenter studies to describe the burden and genetic diversity.

**Abbreviations**

CAV: Coxsackie A virus

DNA: Deoxyribonucleic acid
dsRNA: double stranded DNA

dsRNA: double stranded RNA

EV: Enterovirus

HAdV: Human adenovirus

HAstV: Human astrovirus

HPeV: Human parechovirus

NoV: Noroviruses,

ORF: Open Reading Frame

PCR: Polymerase Chain Reaction

RNA: Ribonucleic acid

RT-PCR: Reverse Transcription Polymerase Chain Reaction

RT-qPCR: Reverse Transcription quantitative Polymerase Chain Reaction

RV: Rotavirus

SaV: Sapovirus

ssRNA: double stranded RNA

VP: Viral Protein

VP4: Viral Protein Four

VP7: Viral Protein Seven

Declarations

Acknowledgments

Our special thanks go to the study participants and the data collectors. We are also grateful to Sandra Bergs for the excellent technical assistance.

Author contributions

AG and UGL designed the study. AG carried out the laboratory experiments. AG performed the data analysis and drafted the manuscript. UGL revised it. Both authors read and approved the final
manuscript.

**Funding**

The authors received no specific fund for this work

**Availability of data and material**

The dataset used during this study is available and can be shared upon request from the corresponding author. The nucleotide sequences for genotypes reported in this study are deposited in GenBank

**Ethical approval and consent to participate**

This study was approved by the Ethical Review Board of the University of Gondar. Written informed consent was obtained from parents and guardian of the children.

**Competing interest**

Both authors declare they have no competing interests

**Consent for publication**

Not applicable

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Figures
Figure 1

Phylogenetic analysis of RVA (A and B), NoV (C), HAstV (D), SaV (E), HAdV (F), EVA (G) and EVC (H). A portion the VP7 and VP4 regions were used for RVA. Partial sequences of hexon loop one and ORF2 were used for HAdV and HAstV, respectively. Portions of the VP1 coding regions were used for NoV, SaV and EV. The trees were constructed by using Neighbor-Joining (NJ) method with a bootstrap analysis of 1000. Strains from the present study are labeled with black triangles and white triangles indicate previously reported strains from Ethiopia.