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Enterovirus detection in stool samples from Mozambican children with acute gastroenteritis

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ABSTRACT

Enteroviruses (EV) are predominantly enteric viruses, present in all parts of the world causing disease in humans with a broad spectrum of clinical presentations. The purpose of this study was to identify non-polio enteroviruses (NPEV) in stool samples collected from children with acute gastroenteritis (AGE) symptoms of unknown etiology in four provinces (Maputo, Nampula, Sofala and Zambézia) of Mozambique. From June 2014 to March 2018, 327 stool samples were collected from children hospitalized with AGE in health care units. NPEVs were detected in 52 samples (52/327; 15.9%) and were more frequent in children under 5 years of age. The age group from 12 to 23 months was the most affected and showed more severity of disease. We also identified 26 different EV-types with the following detection pattern EV-*B*>EV-C>EV-A. The major EV-types were EV-A119 (9/52; 17.3%) and EV-C99 (8/52; 15.4%), accounting for 32.7% of the total. In addition to EV-A119, other uncommon EV-types were also identified, such as EV-B75, EV-B97 and EV-C113. The current study shows a high heterogeneity of EV types circulating in children with AGE in Mozambique as well as the identification of rarely described enteroviruses.

Acute gastroenteritis (AGE) is a significant cause of morbidity and mortality among children in developing countries (Kotloff, 2017). Diarrheal infections are the third leading cause of morbidity and mortality in children under 10 years of age worldwide (Abbafati et al., 2020). Despite this, about 40% of cases still remain of unknown etiology (Finkbeiner et al., 2008). In Mozambique, even with the strategies implemented to reduce diarrhea cases in the last two decades (1997–2017), it remains a great public health problem, being ranked as the third leading cause of death in children under 5 years of age being responsible for 7% of deaths in 2019 (Chissaque et al., 2018; UNICEF, 2020).

Enteric pathogens have been implicated in diarrheal diseases among which are bacterial, protozoan and viral (GBD 2015a, 2016; Kotloff, 2017). Among the viral pathogens rotavirus, calicivirus, astrovirus and adenovirus have been demonstrated as principal agents related to AGE cases in children (GBD 2015a, 2016; Kotloff, 2017). Other viruses have also been reported sporadically as etiological agents such as Aichi virus, Parechovirus, Enterovirus (EV) and Human bocaviruses (Pham et al., 2010).

The *Picornaviridae* family includes enteroviruses, which are small, icosahedral-shaped, non-enveloped viruses having single-stranded positive-sense RNA, whose capsid is composed of four structural proteins (VP1-VP4). They have been divided into 15 species based on their genetic and biological characteristics, including human rhinoviruses A to C

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and EVs A to L. However, only the EV A to D species and rhinoviruses are known to cause human infection (Simmonds et al., 2020; Zell et al., 2017) (Simmonds et al., 2020; Zell et al., 2017). These viruses are commonly transmitted by the fecal–oral or respiratory routes (Zell et al., 2017). Although some studies have demonstrated the detection of certain types of non-polio enterovirus (NPEV) in patients with AGE, the role of EVs in the etiology of AGE still remains unclear (Chia and Chia, 2010; Nagabushana et al., 2014; Rao et al., 2013).

In Mozambique, some studies have reported an association of rotavirus with AGE (de Deus et al., 2018; Kotloff et al., 2013). However, the information about NPEV circulation and its association with AGE cases is limited (Bero et al., 2015). In the country, a National Surveillance of Diarrhea (ViNaDia) has been implemented since 2014; the platform aims to determine the burden of diarrhea cases in children from 0 to 14 years of age and etiological agents associated with AGE in Health Units from four provinces (Maputo, Sofala, Zambézia and Nampula) (de Deus et al., 2018).

The molecular epidemiology of EVs in stool samples from children with AGE in sub-Saharan Africa remains largely unknown. Within this context, the purpose of this study was providing further insights into the prevalence of EV-types in children with AGE symptoms in four provinces of Mozambique from June 2014 to March 2018.

From June 2014 to March 2018, 327 stool specimens were collected from children (<5 years) diagnosed with AGE that attended the sentinel sites (Maputo, Sofala, Zambézia and Nampula) according to ViNaDia protocols (Bauhofer et al., 2020; de Deus et al., 2018). The patients were examined for fever, number of episodes and duration of vomiting and diarrhea, and dehydration. In order to ascertain the severity of diarrheal disease, we used the Ruuska and Vesikari scale (0 to 5 points - Slight, 6 to 10 points – Moderate, 11 to 15 points – Severe, 16 to 20 points high severity) (Ruuska and Vesikari, 1990). Viral RNA was extracted (QIAmp-Qiagen, Hilden, Germany) from stool samples and subjected to a broad-reactive real time RT-PCR (rRT-PCR) for human enteroviruses as previously described (Sousa et al., 2019; WHO, 2015). In the rRT-PCR, EV-positive samples (52/327; 15.9%) were subjected to virus isolation in both cell lines (RD and HEp2-C), which were cultured at 37°C and examined daily for development of cytopathic effect (CPE) (Fig. 1).

EV was isolated from 34 samples (34/52; 65.4%), in RD or HEp-2C cell culture. Of these 34, 14 (41.2%) were successfully isolated in RD cells, 6 (17.6%) in HEp-2C and 14 (41.2%) in both cell lines (Table 1). Additionally, Hep2-C cell culture was more effective at isolating EV-C species in line with previous results (Sousa et al., 2020).

After inoculation, the specimens that showed CPE were harvested and stored at −20°C until typing. Total viral nucleic acid was extracted from 140 µl of culture supernatants that showed CPE as aforementioned and used as template for cDNA synthesis using random primers (Promega, Madison) and SuperScript II Reverse Transcriptase (Invitrogen)

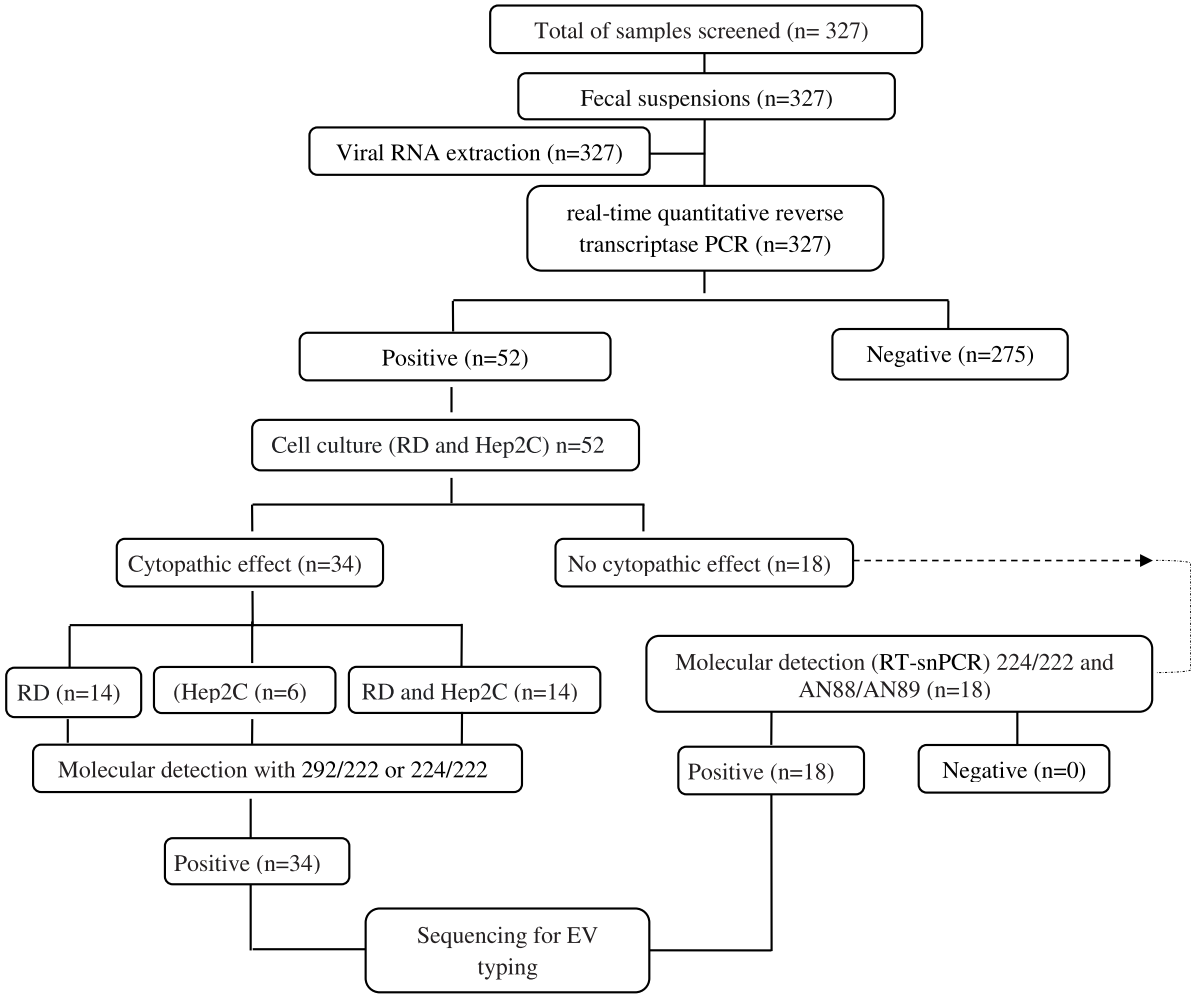

Fig. 1. Schematic representation of the enterovirus screening algorithm used in this study. Fecal suspensions were screened by rRT-PCR. Positive samples were inoculated into both cell lines (RD and HEp-2C). All isolates were subjected to the molecular detection using the primer pairs 292/222 or 224/222. Samples that did not yield CPE were subjected to the molecular detection from fecal suspensions through RT-snPCR with primer pairs 224/222 and AN88/AN89. The respective amplicons were sequenced and used for enterovirus typing.

Table 1
Frequency of cytopathic effect, according to cell line for enteroviruses, and laboratory results.

Cytopathic effect according to cell line			Results by: RT-PCR/RTsn-PCR	Enterovirus typed	EV-species
RD	**HEp2C**	**RD and Hep-2C**			
+	–	–	+/NA	CVA10 (1)	A (13)
+	–	+	+/NA	CVA16 (2)	
–	–	–	+/+ (9)	EV-A119 (9)	
–	–	–	+/+ (1)	EV-A76 (1)	
2	–	1	10		
–	–	+	+/NA	CVB3 (1)	B (21)
+	–	–	+/NA	CVB5 (1)	
+	–	–	+/NA	E1 (1)	
+	–	+	+/NA	E7 (2)	
–	–	+ (2)	+/NA	E11 (2)	
+	–	+ (2)	+/NA	E13 (3)	
+(2)	–	–	+/NA	E14 (2)	
–	–	+	+/NA	E20 (1)	
–	–	+	+/NA	E21 (1)	
–	–	+	+/NA	E25 (1)	
+	–	–	+/NA	E30 (1)	
+	–	–	+/NA	EV-B74 (1)	
+ (2)	–	–	+/+ (1)	EV-B75 (3)	
–	–	–	+/+ (1)	EV-B97 (1)	
10	–	9	2		
–	–	+	+/NA	CVA1 (1)	C (18)
+	–	–	+/NA	CVA11 (1)	
–	+	–	+/NA	CVA13 (1)	
–	+	–	+/NA	CVA19 (1)	
–	–	+	+/NA	CVA20 (1)	
+	–	–	+/NA	CVA24 (1)	
–	–	–	+/+ (4)	EV-C113 (4)	
–	+ (4)	+ (2)	+/+ (2)	EV-C99 (8)	
2	6	4	6		

Cell lines: RD-Human Rhabdomyosarcoma; Hep2C—Human larynx epidermoid carcinoma; rRT-PCR-real-time RT-PCR; sn-PCR-semi-nested PCR. NA- not applicable; EV-detected is indicated by the number in brackets.

according to the manufacturer's protocol. PCR was performed using primers 292/222 and 224/222 to amplify ~340 bp (within VP1) and ~750 bp (spanning the VP3/VP1 junction), respectively (Supplementary figure 1) (Oberste et al., 2003). The specimens that did not have produce CPE in cell culture (18/52; 34.6%) were subjected to a semi-nested PCR (RT-snPCR, Supplementary figure 1) amplification of partial VP1 gene according to previously described methods (Nix et al., 2006; WHO, 2015). All samples that successfully yielded amplicons of the expected size (27/52, using the primer pair 292/222 and 7/52, using the primer pair 224/222) and RT-snPCR (18/52, with the primer pair AN89/ AN88) were cycle-sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). To determine the EV-type, VP1 sequences were compared with those available in the GenBank. A suggested flowchart for this study is provided in Fig. 1. Mozambican partial VP1 sequences were deposited in GenBank under accession numbers: MT822164 - MT822174, MT876336 - MT876349 and MT943116 - MT943128.

Most of the AGE clinical samples were from children younger than 2 years (275/327; 84.6%) (Table 2). The males had an incidence of 58.4% compared with females with an M/F ratio of approximately 1.40. These findings are very similar to studies performed in other countries (Gosert et al., 2018). The patients whose stool specimens were positive for EV were children aged from 0 to 23 months (43/52; 82.7%) with the highest prevalence (24/52; 46.2%) in children 12 to 23 months (Table 2). Similar results were obtained in other regions such as Japan and India, which reported 44% and 48.4% (Patil et al., 2014; Pham et al., 2018). It is worth mentioning that higher EV-detection (66.7%) was reported in Thailand (Chansaenroj et al., 2017). This variation might be due to difference cohort evaluated and climatic factors, as humidity and elevated temperature (Pons-salort et al., 2018). The association between sociodemographic characteristics and the presence of EV in the bivariate analysis is described in Table 2. Children between the ages of 12 and 23 months were more affected than those under 1 year old, with a prevalence of 20.9% (p=0.045). Similar data was reported in Brazil (Coutinho et al., 2019). The high detection in this group can be associated with the characteristic behavior of that age, such as taking objects to the mouth and diversified diet, which provides a greater probability of infection (Coutinho et al., 2019). Gender and provenance (province) were not related to enterovirus infection.

Table 2
Descriptive characteristics and bivariate analysis of the children with AGE.

Characteristics	*N* = 327	EV-Positive (%)	EV-positive rates (%)	P-value	Crude OR (95% CI)
Sex					
Male	191	30/191 (15.7)	30/52 (57.7)		Ref.
Female	136	22/136 (16.2)	22/52 (42.3)	0.909	1.036 (0.568 – 1.887)
Age group (months)					
0–11	160	19/160 (11.9)	19/52 (36.5)		Ref.
12–23	115	24/115 (20.9)	24/52 (46.2)	0.045	1.957 (1.015 – 3.776)
24–59	52	9/52 (17.3)	9/52 (17.3)	0.318	1.553 (0.655 – 3.683)
Province					
Maputo	106	11/106 (10.4)	11/52 (21.2)		Ref
Sofala	29	6/29 (20.7)	6/52 (11.5)	0.146	2.253 (0.754 – 6.728)
Zambézia	64	13/64 (20.3)	13/52 (25)	0.076	2.201 (0.920 – 5.265)
Nampula	128	22/128 (17.2)	22/52 (42.3)	0.14	1.792 (0.826 – 3.890)

N - total of patients; EV – Enterovirus; Ref: Reference categories.

In order to evaluate the disease severity, the patients were examined for fever, number of episodes and duration of vomiting and diarrhea,

and the degree of dehydration. The patients EV-positive were considered severe in the different age groups according to the Ruuska and Vesikari scale (Table 3). Similar results were reported in a study conducted by Patil et al. in India (Patil et al., 2014).

Molecular typing of enterovirus revealed the presence of three EV-species (*A, B* and *C*) and a high genetic diversity revealed by identification of 26 EV-types. In general, we were able to identify EV type in 15.9% (52/327) of the samples. These findings are consistent with previous studies that reported a frequency ranging from 16.6 to 22 percent (Chitambar et al., 2012; Patil et al., 2014; Rao et al., 2013). Conversely, investigations conducted in India and Brazil reported a high EV-detection rate (26 to 40%) (Chitambar et al., 2012; Machado et al., 2020). This difference can be attributed to viral detection methods as well as seasonal factors.

EV-B species was more prevalent EV-detected (40.4%; 21/52) followed by EV-C species (34.6%; 18/52) and EV-A species (25%; 13/52) (Table 1 and Table 4). These findings were similar to previous reports that showed EV-B species more frequently detected than EV-C and EV-A species in children with AGE (Delogu et al., 2018; Chansaenroj et al., 2017; Machado et al., 2020; Pham et al., 2018). EV-A119 (17.3%; 9/52) and EV-C99 (15.4%; 8/52) were the most frequent EV-types identified. Seventeen EV-types (CVA19, EV-A76, CVA10, CVB3, CVB5, E1, E14, E20, E21, E30, EV-B74, EV-B97, CVA1, CVA11, CVA20, CVA24 and EV-C113) were detected only once. Noteworthy that EV-A119 was identified during four years of this study (2015–2018) in three regions of country (Table 4).

The viral distribution by provinces in the three regions of the country was distinct, suggesting that viral circulation across communities and geographical distribution associated with climatic factors may have a role in the EV-circulation, as previously reported in Italy (Delogu et al., 2018). It's also worth noting the existence of rarely described EV in stool samples, such as EV-B75, EV-B97, EV-C113 and EV-A119. Additionally, the isolation efficiency of these viruses in different cell culture systems revealed a lower susceptibility to viral infection in both cell lines (RD and HEp2-C) (Table 1). EV-A119 and EV-C99 were the most commonly detected EV-types. Similar results were obtained in a study carried out in Brazil, which found a high detection rate of EV-C99 in AGE cases (Machado et al., 2020).

Another remarkable finding was the first description of EV-A119 in patients with AGE symptoms. EV-A119 has only been detected in healthy children in Cameroon and Côte d'Ivoire and in a child with acute flaccid paralysis in Nigeria (Adeniji et al., 2016; Ayukekbong et al., 2013; Cristanziano et al., 2015), even though a recent study has shown a high prevalence of EV-A119 in a cohort of Malawian children (Brouwer et al., 2018).

This study was conducted with increased coverage of sentinel posts in urban areas at the expense of rural areas, which may have underestimated the EV detection in rural regions, representing a study constraint. In addition, it is worth noting that due to lack of matched health controls the EV-detection from AGE patients must be evaluated cautiously.

In summary, this work provides an important information about the circulation and the identification of enteroviruses detected in AGE patients in Mozambique. Although, finding an EV in an individual does not proof an association between the virus and the disease, the detection of uncommon EV-types, highlights the need to improve the current surveillance strategies to monitor emergence/re-emergence of non-polio enteroviruses as well as poliovirus circulation (Sabin, vaccine derived poliovirus or wild type) in areas with low vaccine coverage and deficient acute flaccid paralysis surveillance.

Table 3
Severity of diarrheal disease between EV-positive samples.

Parameters	EV positive
Number of children	52
Mean diarrhea duration (days)	3.0 (2.2–4.0)
Mean diarrhea episodes (24 h)	4.4 (3. 6–5.4)
Mean vomiting duration (days)	1.9 (1.6–2.5)
Mean vomiting episodes (24 h)	3.3 (2.7–3.9)
Fever (°C)	37.3 (36.6–38.2)
Dehydration	1 a 5%
Ruuska and Vesikari score	12

Numbers between brackets are the 95% confidence limit value.

Table 4
Temporal distribution by province and EV-species.

Year	Province	Species and types *Enterovirus A* (*n* = 13)	*Enterovirus B* (*n* = 21)	*Enterovirus C* (*n* = 18)
2014	–	–	–	–
2015	Maputo	–	E20	–
	Nampula	CVA16	E7, E25, E30	EV-C99(2)
	Sofala	EV-A119	–	EV-C99
	Zambézia	–	CVB3	CVA19
2016	Maputo	–	CVB5, E14, E21, EV-B74, EV-B75	–
	Nampula	–	E7, EV-B75, EV-B97	EV-C99(2), CVA13 (2), CVA24
	Sofala	EV-A119	–	EV-C99, EV-C113, CVA13
	Zambézia	EV-A76, CVA16	E13(2)	–
2017	Maputo	EV-A119	–	EV-C99, CVA11
	Nampula	EV-A119	E11, E13	EV-C99, CVA1, CVA20
	Zambézia	EV-119(2), CVA10	E14, EV-B75	CVA13
	Sofala	–	–	–
2018	Maputo	EV-A119	E1	–
	Nampula	EV-A119	E11	–
	Sofala	EV-A119	–	–
	Zambézia	–	–	–

(-) No detection.

Declarations

Ethics approval and consent to participate

The study was reviewed and approved by the National Bioethics Committee for Health in Mozambique (IRB00002657, reference number 348/CNBS/13) and written informed consent was obtained from the guardians of eligible children.

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Author statement

We also declare that all authors have seen and approved this version of the manuscript.

Declaration of competing interest

All authors have declared no conflict of interest.

Data availability

Data will be made available on request.

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