

## Full Length Research Paper

# Natural circulation of human enterovirus in Maputo city, Mozambique

Diocreciano M. Bero<sup>1\*</sup>, Nilsa de Deus<sup>1</sup>, Eliane V. da Costa<sup>2</sup>, Fernanda M. Burlandy<sup>2</sup>, Ilesh V. Jani<sup>1</sup> and Edson E. da Silva<sup>2</sup>

<sup>1</sup>Instituto Nacional de Saúde de Moçambique, Ministério da Saúde, Maputo – Moçambique.

<sup>2</sup>Enterovirus Laboratory, Oswaldo Cruz Institute/Oswaldo Cruz Foundation, Avenida Brazil 4365, Manguinhos, 21040-360, Rio de Janeiro, RJ, Brazil.

Received 13 April, 2015; Accepted 7 May, 2015

The human enteroviruses (HEV) are responsible for a large diversity of infections affecting humans. Most infections are asymptomatic, but these viruses can cause a wide spectrum of diseases, including severe cases involving the central nervous system. The aim of this study was to isolate and identify human enteroviruses in natural circulation in children less than 15 years of age admitted at the Mavalane General Hospital in Maputo City, Mozambique. In this study, 178 stool samples were processed, obtained during November 2011 to February 2012. Samples were inoculated onto cell culture and the isolates were identified as enterovirus by conventional RT-PCR in the 5' non-coding region followed by partial VP1 sequence. Twenty-six (26) out of the 45 cell-culture positive samples were constituted by *Enterovirus* (14.6% of the total 178 samples). EV-29 was the serotype most prevalent. The results show the importance of maintaining the cell line Hep2C in the diagnosis and *Enterovirus* circulating in the Maputo city, Mozambique.

**Key words:** Enterovirus, Stool, Cell culture, cell line Hep2C, Hospital Geral de Mavalane.

## INTRODUCTION

The human enteroviruses (HEV) belong to the genus *Enterovirus* and *Picornaviridae* family. These agents infect millions of people worldwide each year, resulting in a wide variety of clinical conditions ranging from unapparent infection, undifferentiated fevers, common cold to serious diseases such as aseptic meningitis, hand-foot-mouth disease, acute hemorrhagic conjunctivitis, myocarditis, encephalitis and paralytic poliomyelitis (Pallansch and Roos, 2001). Children are more susceptible to infection and transmission occurs either by the fecal-oral or respiratory tract. The virus can

be excreted in the feces for several weeks (Pallansch and Roos, 2001).

There are more than 80 species of HEV divided into four groups, designated HEV- A to D based on the genetic characteristics of the VP1 capsid gene (Oberste et al., 2006; Knowles et al., 2011). In general, the 5' and 3' noncoding regions (NCRs) are highly conserved, and the most variable region of the genome lies within the genes encoding the capsid proteins exposed on the virus surface, VP1, VP2, VP3, and VP4 (Hogle et al., 1985; Racaniello, 1988; Oxman, 1999).

\*Corresponding author. E-mail: dmbero@gmail.com. Tel: 00 258 21 42 71 31/4.

Molecular epidemiology of enteroviruses (EVs) is poorly known in Southern Africa, particularly in Mozambique. Therefore, the aim was to isolate and identify human enteroviruses from stool samples of children less than 15 years of age receiving medical attention in Mavalane General Hospital in Maputo city, Mozambique and to understand the natural circulation of HEV.

## MATERIALS AND METHODS

### Ethics statement

National Bioethics Committee on Health of Mozambique approved the study with the reference: 280/CNBS/11. All study participants were under 15 years old and parents or guardians authorized written consent for their participation by signing an informed consent form where each party stayed with a copy.

### Patients and specimens

A cross-sectional study involves enrolling a total of 178 stool specimens from children under 15 years old. Clinical specimens were collected from pediatric patients admitted in general hospital in Maputo City, Mozambique in the period from November 2011 to February 2012, with at least three or more of these signs and symptoms: fever, vomiting/nausea, difficulty breathing, cough, diarrhea, headaches and joint pain. Patient information's were recorded on a standard questionnaire including demographic and clinical details. Specimens were transported in dry ice to the Enterovirus Laboratory, Oswaldo Cruz Institute/Fundação Oswaldo Cruz/Rio de Janeiro, Brazil.

### Virus isolation

Fecal suspensions were treated with chloroform as previously described (WHO, 2004). Each specimen extract (0.2 ml) was inoculated in duplicate tubes of the three cell culture lineages, RD, L20B and Hep2C, as recommended (WHO, 2004). The tubes were incubated at 37°C and examined daily for evidence of cytopathic effect (CPE) and these were accompanied by negative control. Positive cultures were harvested and kept frozen (-20°C) until further analysis.

### Molecular characterization and Enterovirus genotyping

Isolates showing characteristic enterovirus CPE were subject to *in vitro* amplification by RT-PCR using enterovirus primers as previously described (Dos Santos et al., 2012). The Pan-EV pair of primers (PanEVF and PanEVR) used in one-step conventional RT-PCR, are broader reactive for the human enterovirus genus targeting conserved nucleotide sequences at the 5'-NCR of the EV genomes. Briefly 1.0 µl of sample was added to 3.2 µl reaction mixture A (containing 2.5 µl of 10x PCR buffer and 0.7 µl of reverse primer at 50 pmol/µl). The reaction mixture was first incubated at 95°C for 5 min and then chilled in ice for 5 min. The reaction mixture B containing 0.5 µl of each dNTP's 10 mM, 0.15 µl of RNASin at 5 U/µl, 0.7 µl of forward primer, 0.2 µl of transcriptase reverse at 20 U/µl, 0.2 µl of Taq DNA polymerase at 5 U/µl, 19.05 µl of water were added to mixture A. The tubes were placed in thermo cycler (Veriti™ 96-well Thermal Cycler) and cycled as follows: 42°C for 30 min, 30 cycles at 95°C for 45 s, 55°C for 45 s and 72°C for 45 s, and these were accompanied by negative and positive control

of the reaction, respectively.

All positive samples in Pan-EV were subjected to Pan-PV to test for the presence of polioviruses, as previously described (WHO, 2004). All isolates was identify by partial VP1 sequence (Oberste et al., 1999; Oberste et al., 2003). After edition, sequences were compared with those located at GenBank using the Blast Program 2.2.27 for confirmation of the viral identity and species determination (Altschul et al. 1990).

A phylogenetic tree was constructed based on MEGA 4.0.2 program (Kumar et al., 2001) using the Neighbor-Joining reconstruction method (Saitou and Nei, 1987). The genetic distances were estimated to using Kimura-Two parameter model (Kimura, 1980). The robustness of the branches in the phylogenetic tree was statistically evaluated by 1,000 bootstrap replicates (Felsenstein, 1985).

All samples negative by RT-PCR PAN EV were subjected to PCR to search Adenovirus members because in cell line Hep2C showed a highly suggestive adenovirus cytopathic effect for virus isolation in cell culture. The Adenoviruses were screened through a PCR reaction using specific primers previous described (Xu et al., 2000).

## RESULTS

A total of 45 (25.3%) samples had viral isolation in RD, L20B or Hep2C cell lines although it was not homogenous. The EV was identified in 14.6% of the samples (26/178). Molecular typing based on partial VP1 sequence showed a wide diversity of human enterovirus with 16 types detected (Table 1). The HEV-B was the most prevalent. Echovirus 29 (E29) serotype was presented in 5/26 (19.2%) of the EV samples, followed by EC99 in 3/26 (11.5%). The male and female ratio for enterovirus positivity was 1.8:1. Among the 26 participants who had enterovirus positive samples, 18 were from the age group of 0-24 months. The reported signs and symptoms (fever and vomiting/nausea, coughing) of participants show that the clinical manifestations were similar and occurred irrespective of the enterovirus group, HEV-A, B or C, detected (data not shown).

Two samples (32 and 163) were identified Poliovirus sabin like. The complete sequence of the gene encoding the protein VP1 of the two samples identified as Poliovirus Sabin like was performed to detect mutations that may be associated with reversion to virulence of the vaccine strains poliovirus. The sample 32 was identified as Poliovirus Sabin like 2 while sample 163 Poliovirus Sabin like 3 with 99.7% identity with the vaccine strain. Both samples were collected from children at one month of life, the first of which had record of having received polio vaccine and the second had no history of having received the vaccine.

The enteroviruses were distributed along all months of sample collection as follows: November (n=9, 34.6%); December (n=5, 19.2%); January (n=4, 15.4%); February (n=8, 30.8%) and E-29 was the only virus detected in all months (data not shown).

Adenovirus PCR reaction and run electrophoretic run gave a fragment of 134 pb in 20 of the 23 samples. The samples positive for adenovirus was 11.2% (20/178)

**Table 1.** Enterovirus serotypes identified, and socio-demographic data of children younger than 15 years old.

Species and type		Cell line			Sex	Type of feed	Reason for hospitalization
		RD	L20B	Hep2C			
<b>HEV-A</b>	3						
Coxsackievirus A5	2	CPE	NCPE	CPE	M	3	Malnutrition
Coxsackievirus A10	1	CPE	NCPE	NCPE	M	2	GEA
<b>HEV-B</b>	14						
Coxsackievirus B4	1	CPE	NCPE	CPE	M	3	Malaria
Coxsackievirus B6	1	CPE	NCPE	CPE	F	3	Malaria
Echovirus 7	1	CPE	NCPE	CPE	M	2	Malnutrition
Echovirus 11	2	CPE	NCPE	CPE, NCPE	M, F	3	Malnutrition, BPN
Echovirus 13	2	CPE	NCPE	NCPE	M	2, 3	Malnutrition, BPN and GEA
Echovirus 21	1	CPE	NCPE	CPE	M	3	Malaria
Echovirus 25	1	CPE	NCPE	CPE	F	3	Malaria
Echovirus 29	5	CPE	NCPE	NCPE	M, F	3	Malnutrition, Malaria
<b>HEV-C</b>	9						
Coxsackievirus A13	3	NCPE	NCPE	CPE	M, F	2, 3	Malaria, BPN
Coxsackievirus A20	1	NCPE	NCPE	CPE	M	2	BPN
Enterovirus C99	3	NCPE	NCPE	CPE	M	3	BPN
Poliovirus Sabin like 2	1	CPE	NCPE	NCPE	M	1	BPN
Poliovirus Sabin like 3	1	CPE	CPE	CPE	M		

F = Female; M = Male; Type of feeding: 1 = Only breast milk; 2 = Mixed (breast milk and other food; 3 = Diversified (not breast milk); Cell lines: RD= Human Rhabdomyosarcoma; L20B= Transgenic mouse cell lines (L20B and L<sub>o</sub>) expressing the human poliovirus surface receptor; Hep2C= Human larynx epidermoid carcinoma; CEP = cytophatic effect; NCEP= non cytophatic effect; BPN = Bronchopneumonia; AGE = Acute gastroenteritis.; HEV-A = Human Enterovirus Specie A; HEV-B= Human Enterovirus Specie B; HEV-C = Human Enterovirus Specie C; PV\* = *Poliovirus vaccine strain Sabin like*.

(data not shown) and only two samples (75 and 128) showed co-infection between enterovirus and adenovirus and both the species HEV-B.

## DISCUSSION

This is the first study that reports the natural circulation of *Enterovirus* in Mozambique. The isolation in cell culture shows a low permissive cell line RD for members of the species EV-C and confirms the interest of maintaining the cell line Hep2C in the diagnosis for monitoring the circulation of *Enterovirus* as evidenced by Bessaud and colleagues (2012).

The results of the molecular identification demonstrate the existence of serotypes; the species HEV-B (54%), and within this specie, E29 was most identified. In fact, the HEV-B was the most identified enterovirus in previous studies conducted in Taiwan and Central African Republic (Lo et al., 2010; Bessaud et al., 2012).

Overall, the frequency of human enteroviruses was 14.6%, and this data is also consistent with previous report, conducted in Brazil, Australia, Korea and Egypt that reported 15-17.6% (Santos et al., 2002; Kelly et al., 2006; Roh et al., 2009; Afifi et al., 2009). Nevertheless,

some studies have reported prevalence above 20% in England and Pakistan (Iturriza-Gomara et al., 2006; Saeed et al., 2007).

A higher frequency of enterovirus isolation in male children was observed although this finding was consistent with the larger number of male participants (1.8:1). Enterovirus infection has been reported more frequently in males compared to females (Moore and Morens, 1984; Gondo et al., 1995; Dechkum et al., 1998).

The phylogenetic analysis of the two samples Poliovirus Sabin like had 99.7% nucleotide identity with strain Poliovirus Sabin 2 and 3 isolated by Rezapkin and colleagues (2002). However, for the sample 32, although the participant had received dose of trivalent oral polio vaccine, this fact can be explained by more efficient replication of live attenuated poliovirus type 2 in the gastrointestinal tract, among serotypes that comprise the vaccine (Modlin, 1995).

In sample 163, according to the information collected during the survey, the participant did not receive the vaccine poliomyelitis. This event is predictable in countries or areas where the trivalent oral polio vaccine is used, which is one of the advantages of oral vaccination by enabling the expansion of vaccine virus in other subjects and the environment, conferring protection by

inducing production of antibodies not only serum but also an intestinal resistance and speed with which people vaccinated develop a long immunity (Melnick, 1996; da Silva et al., 2005).

However these findings reinforce the necessity of epidemiological surveillance of poliovirus in Mozambique. In 2011 there was two cases of virus circulation derived polio vaccine (cVDPV) serotype 1 in the region center of the country although the present study was conducted in the southern region of Mozambique (Conceição et al., 2011; MISAU, 2012).

The overall period of study and sample collection was short (November, 2011 to February, 2012), which did not allow a seasonal analysis, which would be important in future studies.

Most (70%) of the enterovirus cases were detected in children up to 24 months of age. In fact, these age groups (0-24 months/old) are subject to different susceptibility, disease severity and prognosis of infections and the peak of enterovirus infections occur in the first two years of age (Pallansch and Roos, 2001).

In summary, this finding shows the importance of maintaining the cell line Hep2C in the diagnosis and existence of serotypes of human enteroviruses in natural circulation in Maputo city.

### Conflict of interests

The authors did not declare any conflict of interest.

### ACKNOWLEDGEMENT

The entire participant's for having accepted to join the study, the Ministry of Health of Mozambique by financial support are acknowledged. Thanks go to all the Enterovirus Laboratory/FIOCRUZ staff who contributed to this research with excellent technical assistance and the Brazilian Research Council (CNPQ) for the financial support for laboratory supplies.

### REFERENCES

- Afifi SS, Zaki SA, Mohamed AF, Hosseiny HE (2009). Isolation and identification of non-polio enteroviruses from children in different Egyptian governorates. *Aust. J. Basic Appl. Sci.* 3(4):3230-3238.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Bessaud M, Pillet S, Ibrahim W, Joffret ML, Pozzetto B, Delpeyroux F, et al. (2012). Molecular characterization of human enteroviruses in the Central African Republic: uncovering wide diversity and identification of a new human enterovirus A71 genogroup. *J. Clin. Microbiol.* 50(5):1650-1658.
- Conceição C, Cardoso N, Gujarl L, Cliff J, Novela M (2011). Investigação de Poliomielite por Poliovirus Derivado da Vacina em Moçambique, 2011. Short communication, 1º Congresso Nacional de Saúde Pública.
- da Silva EE, De Azevedo JRM, Costa EV (2005). Enterovirus de Importância Médica. In: Coura JR. *Dinâmica das Doenças Infecciosas e Parasitárias*. V 2. Rio de Janeiro: Guanabara & Koogan. pp. 1681-1700.
- Dechkum N, Pangsawan Y, Jayavasu C, Saguanwongse S (1998). Coxsackie B virus infection and myocarditis in Thailand, 1987-1989. *Southeast Asian J. Trop. Med. Public Health* 29:273-276.
- Dos Santos GPL, Burlandy FM, Da Costa EVV, da Silva EE (2012). Direct detection of Enterovirus genome in cell-culture negative cerebrospinal fluid from aseptic meningitis cases in Brasil. *Virus Res.* 17:1-2.
- Felsenstein J (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution* 39:783-791.
- Gondo K, Kusuhara K, Take H, Ueda K (1995). Echovirus type 9 epidemic in Kagoshima, southern Japan: Seroepidemiology and clinical observation of aseptic meningitis. *Pediatr. Infect. Dis. J.* 14:787-791.
- Hogle JM, Chow M, Filman DJ (1985). Three-dimensional structure of poliovirus at 29 Å resolution. *Science* 229: 1358-1365.
- Iturriza-Gómara M, Megson B, Gray J (2006). Molecular detection and characterization of human enteroviruses directly from clinical samples using RT-PCR and DNA sequencing. *J. Med. Virol.* 78: 243-253.
- Kelly H, Brussen KA, Lawrence A, Elliot E, Pearn J, Thorley B (2006). Polioviruses and other enteroviruses isolated from faecal samples of patients with acute flaccid paralysis in Australia, 1996-2004. *J. Paediatr. Child Health* 42: 370-376.
- Kimura M (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J. Mol. Evol.* 16:111-120.
- Knowles NJ, Hovi T, Hyypiä T, King AMQ, Lindberg M, Pallansch MA, et al. (2011). Picornaviridae. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz, EJ, editors. *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses*. San Diego: Elsevier. pp. 855-880.
- Kumar S, Tamura K, Jakobsen IB, Nei M (2001). MEGA 2: Molecular evolutionary genetics analysis software. *Bioinformatics* 17:1244-1245.
- Lo C, Wu K, Lin M, Chen C, Ho DM, Tang R, Chan Y (2010). Application of a Molecular Method for the Classification of Human Enteroviruses and its Correlation with Clinical Manifestations. *J. Microbiol. Immunol. Infect.* 43(5):354-359.
- Melnick JL (1996). Enterovirus: poliovirus, coxsackieviruses, echoviruses, and newer enteroviruses. In Knipe DM, Howley PM (eds), *Fields Virology*. 3a edição. Philadelphia: Lippincott - Raven Publishers. pp. 655-712.
- MISAU (2012). *Retroinformação sobre a vigilância das PFA em Moçambique, Janeiro a Dezembro de 2011*. Direcção Nacional de Saúde Pública. Maputo, Janeiro. pp. 1-6.
- Modlin JF (1995). Coxsackieviruses, echoviruses and newer enteroviruses. In: Mandell GL, Bennet JE & Dolin R, editors. *Principles and Practice of Infectious Diseases*, 4a edição. New York: Churchill Livingstone. pp. 1620-1632.
- Moore M, Morens DM (1984). Enteroviruses, including Polioviruses. In: Belshe RB. *Textbook of Human Virology*, USA PSG: Publishing. pp. 403-483.
- Oberste MS, Maher K, Kilpatrick DR, Flemister MR, Brown BA, Pallansch MA (1999). Typing of human Enteroviruses by partial sequencing of VP1. *J. Clin. Microbiol.* 37(5):1288-1293.
- Oberste MS, Maher K, Williams AJ, Dybdahl-Sissoko, Brown NBA, Gookin MS et al. (2006). Species-specific RT-PCR amplification of human enteroviruses: A tool for rapid species identification of uncharacterized enteroviruses. *J. Gen. Virol.* 87:119-128.
- Oberste MS, Nix WA, Maher K, Pallansch MA (2003). Improved molecular identification of enteroviruses by RT-PCR and amplicon sequencing. *J. Clin. Virol.* 26:375-377.
- Oxman M (1999). Enterovirus. In: OXMAN, M. *Tratado de Medicina Interna*, vol.1. 20a edição. Bennett & Plum. pp.1968-78.
- Pallansch MA, Roos RP (2001). Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Fields BN, Knipe DM, Howley PM, Chanock RM, Melnick JL, Monath TP, Roizman B & Straus SE. *Fields Virology*. 4a edição. Philadelphia: Lippincott-Williams & Wilkins. 2:723-767.
- Racaniello VR (1988). Poliovirus neurovirulence. *Adv. Virus Res.* 34:217-246.

- Rezapkin GV, Fan L, Asher DM, Fibi MR, Dragunsky EM, Chumakov KM (2002). Mutations in Sabin 2 strain of poliovirus and stability of attenuation phenotype. *J. Virol.* 258(1):152-160.
- Roh EJ, Jin YM, Chung EH, Chang YP, Park WS, Park K, Jee YM (2009). Molecular identification and clinical features of enteroviral infection in children of central Korea: An overview of enteroviral epidemiology between spring 2005 and autumn 2006. *Korean J. Pediatr.* 52:11.
- Saeed M, Zaidi SZ, Naeem A, Masroor M, Sharif S, Shaikat S, et al., (2007). Epidemiology and clinical findings associated with enteroviral acute flaccid paralysis in Pakistan. *BMC Infect. Dis.* 7:6.
- Saitou N, Nei M (1987). The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4:406-425.
- Santos AP, Costa EV, Oliveira SS, Souza MC, da Silva EE (2002). RT-PCR based analysis of cell culture negative stools samples from poliomyelitis suspected cases. *J. Clin. Virol.* 23:149-152.
- WHO (2004). World Health Organization. Polio laboratory manual, 4a edição. Geneva: Ed. WHO Document Production Services. pp. 87-121.
- Xu W, McDonough MC, Erdman DD (2000). Species-specific identification of Human adenoviruses by multiplex PCR Assay. *J. Clin. Microbiol.* 38:4114-20.