



Isolation and full genome sequencing of two human Astroviruses isolated from children in Cairo, Egypt

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Abstract

Astroviridae is a diverse family of viruses that are circulating in a wide range of hosts. Diversity of these viruses plays a major role in the emergence of zoonotic diseases. Limited studies were conducted on the prevalence and genetic proprieties of human Astroviruses in Egypt. Despite the molecular and serological evidences of the existence human astroviruses in several regions of Egypt, attempts to isolate these viruses have been largely unsuccessful. The aims of the current study were to isolate and make full genome sequencing of two human Astroviruses genotype 1 and 4, from infected children in Cairo, Egypt. Currently, we report the isolation and full-genome sequencing of two human isolates based on our novel designed overlapped primers. The original stool samples were collected from children suffering from diarrhea, residing in Abo-El-Reesh Hospital, Cairo, Egypt, and were well characterized as having astrovirus infections. The phylogenetic relationship of the obtained sequences of our isolates collected in 2016, revealed that they are related to human astrovirus genotype 1 (HAstV1) and human astrovirus genotype 4 (HAstV4) isolates recovered from Italy and Russia; respectively, during this decade. The genetic diversity of the other HAstVs circulating in Egypt needs to be fully identified, as a first step toward the control strategy of this neglected virus.

Keywords: Astroviruses, Egypt, Isolation, Sequencing, Phylogenetic analysis

1. Introduction

Astroviruses (AstVs) were firstly detected by the Electron microscopy (EM) in stool samples collected from children suffering from diarrhea and vomiting in 1975 (Madeley and Cosgrove, 1975). Under EM,

AstVs appeared as small, round viruses with distinctive five or six-pointed star-like appearance, of about 28-30 nm in diameter. Of this point, AstVs were named according to the star-shape appearance on the

surface of the virus particles. According to ICTV. (2012), Astroviruses belong to Astroviridae family that includes two main genera, Mamastrovirus (MAstV) and Avastroviruses (AvAstVs). Finkbeiner *et al.*, (2008); Blomstrom *et al.*, (2010); Quan *et al.*, (2010), reported that using next generation sequencing (NGS) and metagenomic analysis, novel strains of AstVs from different mammals and avian species were characterized.

In previous studies of Monroe *et al.*, (1993); Willcocks and Carter, (1993), Astrovirus genome was reported as a positive-sense, single-stranded, non-segmented RNA of about 6.2-7.7 kbp that lacks a 5' cap but contains a 3' poly(A) tail. The genome has 5' and 3' untranslated regions (UTRs) at both ends of the viral genome. M'endez *et al.*, (2012) added that the genome is divided into three open reading frames (ORFs), where ORF1a encodes for a serine protease, ORF1b encodes the RNA-dependent RNA polymerase (RdRp), and ORF2 encodes for the structural proteins. The lengths of the ORFs are variable, depending on the species tropism of the virus, the virus subtype, and whether the virus has been adapted for cell culture (Willcocks *et al.*, 1994; De Benedictis *et al.*, 2011; Medici *et al.*, 2014).

Several classical genotypes of AstVs have been detected in human (1-8), and recently novel AstVs have been characterized (MLB1, MLB2, MLB3, and VA1 to VA5) (Bosch *et al.*, 2014). According to Gabbay *et al.*, (2007), HAstV-1 is most usually identified in children, followed by HAstV-2 and HAstV-5, whereas HAstV-6 and HAstV 8 have been infrequently recorded. On the other hand, Silva *et al.*, (2006); Wunderli *et al.*, (2011) reported that HAstV-4 and HAstV-8 are related with infections of mature children, and infants with fatal meningo-encephalitis. The rate of prevalence of human astrovirus (HAstV) infections among hospitalized children ranged from 2-26 % in children suffering from diarrhea (Appleton and Higgins, 1975; Bosch *et al.*, 2014; Vu *et al.*, 2016). Up till now, no data is available for full genome of the circulating AstVs in Egypt. In this context, the objectives of this study is to present a full genome

sequencing of the most common isolates of human AstVs genotype 1 and 4, based on our designed primers.

2. Material and methods

2.1. Virus isolation

Vero cells were cultured in 25 ml cell culture flask (10^5 cells/ ml) in 5 ml of Dulbecco's Modified Eagle's Medium (DMEM) with 5% Fetal bovine serum albumin (FBSA), and 1% of antimycotic antibiotic, and then incubated at 37°C, under 5% of CO₂ pressure, according to Molberg *et al.*, (1998); Brinker *et al.*, (2000); Jones *et al.*, (2010). Cells were microscopically monitored till reaching 90-100 % confluent monolayer. Positive stool samples for human astroviruses genotype 1 and 4 obtained from children residing in Abo-El-Reesh Hospital, Cairo, Egypt, were inoculated into Vero cells. Viruses were grown for 5 d. To allow virus adsorption, cells were incubated at 37°C for 1.5h followed by adding 3 ml of maintenance medium with trypsin in 25 ml flask, and then incubated at 37°C under 5% CO₂. During incubation, a daily microscopic observation was conducted for watching appearance of possible cytopathic effects (CPE) due to viral infection. The propagated virus at the 5th day was examined using RT-PCR for detection of RdRp gene (Chu *et al.*, 2008). Upon appearance of 50-100% CPE, cells containing the propagated virus were harvested. Harvested cells from the first passage were frozen and thawed 3 successive times, to allow rupture of these cells and release of the virus particles into the medium. The medium were centrifuged for 5 min. at 3000 rpm to remove cell debris.

2.2. Full genome sequencing of the human Astroviruses

For human astrovirus amplification, the following forward (F) and reverse (R) primers were designed based on all reference strains of human astroviruses present in Genbank, to amplify the three ORFs (open reading frame) of human astroviruses present in ten overlapped fragments (Table 1). Primers (200 mM HPLC grade) were purchased in lyophilized form

(Macrogen, South Korea). They were re-constituted in TE buffer (composed of; 10 mM Tris-HCl pH 7.4, 1 mM disodium salt of Ethylene-diamine tetraacetic acid (EDTA) pH 8.0, in nucleases free water), to a final concentration of 200 μ M, divided into aliquots and then stored at -20°C till being used. A volume of 140

μ l of each isolate was subjected to viral RNA extraction using QIAamp virus RNA mini kit (Qiagen). RNA was subjected to the synthesis of cDNA by using Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, Germany).

Table 1: List of primers used to amplify all genome fragment of ORF1a, ORF1b and ORF2[#] genes of Human Astrovirus isolates

Fragment	Primer name	Primer sequence (5' to 3')	Molecular Size
F1	F30 R868	CAAGATGGCACACGWTGAGCCAT GTTCTTGCTATTDGCACTGTCATG	838 bp
F2	F 732 R1451	ATAAGCTAGATGCTGTRAGGC GCCAACGACATGTGCTGCTGTTACTAT	719 bp
F3	F1269 R 2091	ATGGGAAGGTTGTGGCCACTGTACCAAC CCTACCCCTACCATGCTTGGTCT	822 bp
F4	F1960 R 2844	ACTAGAACAGAAAAGTGTTAGCG CCTTGGTCTTCTGGGGCCCTTTGTAGT	884 bp
F5	F 2708 R 3543	ATTATTGAAACAGCYATAAAGAC GCCTTCCATTGGTGACCACCCACAT	835 bp
F6	F 3357 R 4335	AGGAGACAAACCTGAAGTACTTTGG CCTCCCCTCCAAATGCGATGGAGT	978 bp
F7	F4174 R 4714	ACCTGATCTTGAATCACTCCATGGGAAGC GCCAAACTGAGTRCTYCCAGTAGCGT	540 bp
F8	F4562 R5399	ATTGTCAATAARCAACTCAGGAAACARGG CCACCAACCHCCTTTGACYAGCCAAT	837 bp
F9	F5240 R5963	ATGAATGTRCCWGAGSRSAGYCATTTTGC GCTKGWAACCTGSTGYTTGTT	723 bp
F10	F5755 R6759	AGGMCATKAYRMTGYKARRRTTG TCTAAACAGAGACAGAAAAGAAT	1004 bp

where; [#]ORF: Open reading frame

2.3. Amplification of the human viral genome using PCR

The Polymerase chain reactions (PCRs) were carried out using Phusion master mix kit (Thermo Scientific, Wilmington, USA). List of primers shown in Table (1) were used to amplify the full length of the ten fragments of the two HAstVs isolates according to manufacturer's protocol. Each fragment was amplified by using specific 1 µl F, 1 µl R primer, 2 µl of cDNA and 12.5 µl Phusion master mixes, and then completed to 25 µl using deionized water. The mixture was subjected to PCR by an initial denaturation step (98 °C, 30 sec), followed by 40 cycles each consisting of 98°C for 10 sec, 50°C for 30 sec, 68°C for 30 sec. The last cycle was linked to a final extension step at 72°C for 10 min. The PCR products were analyzed in 1% agarose gel. The PCR products were purified from agarose gels using a QIAquick gel extraction kit (Qiagen). Purity of the purified PCR products were spectrophotometrically measured at λ_{max} (260/280), and then 20 µg were subjected to automated sequencing from both directions using the F and R specific primers. Purified amplicons were Sanger sequenced according to the primer/amplicon combinations at the MacroGen sequencing facility (MacroGen, South Korea). Finally, nucleotides (nt) were assembled using SeqMan (DNASTAR, Madison, WI).

2.4. Phylogenetic analysis of the Astroviruses

The alignment of sequence was performed using the BioEdit 7.0 software. The phylogenetic tree was elaborated using MEGA7 program, by applying the neighbor-joining method with Kimura's two-parameter distance model and 1000 bootstrap replicates, in reference to Kumar *et al.*, (2016).

3. Results

3.1. Isolation of the human Astroviruses

Positive stool samples for human AstV were obtained from children suffering from gastroenteritis during 2016. Positive samples containing the two human isolates were inoculated into Vero cells. Due to virus propagation, cytopathic effects appeared on the infected cells 3 days post infection, as demonstrated by marked morphological changes including; rounding, clumping, darkness and complete destruction in the cell monolayer. This effect increased dramatically by the 5th day as shown in Fig. (1). The propagated virus at the 5th day was examined using RT-PCR for detection of RdRp gene as demonstrated in Fig. (2).

3.2. Sequencing of the full viral genome and phylogenetic analysis

We designed relatively conserved 10 primer sets (F and R) to generate overlapping amplicons covering the full-length of HAstV genome. The primers were designed based on alignments of all the published full-length HAstV sequences representing the major genotypes of HAstV. Although sequence variation was expected within the primers, the 3' ends from which the elongation takes place were fully conserved. Ten overlapping PCRs on cDNA for two isolates (Human astrovirus/Egypt/79/2017 and Human Astrovirus/Egypt/95/2017) were carried out. Using F and R primers, the expected 838, 719, 822, 884, 835, 978, 540, 537, 723, and 1004 bp fragments were amplified as shown in Fig. (3). The obtained results were assembled in one contig using a Seqman in a Lasergen software. Phylogenetic analyses of full genome of the two Egyptian human astroviruses revealed that they are closely related to HAstV type 4 and HAstV type 1 recovered from Italy and Russia; respectively, as clear in Fig. (4). The complete genome sequences of the two Egyptian human astroviruses share 95% and 98 % nucleotide identity with open reading frame 2 (ORF2) genes of classical Human astrovirus 1 strain ITA/2011/PA387 (accession number KY744141.1) and Human astrovirus 4 strain Rus-Nsc10-N358 (accession number KF039913.1), respectively.

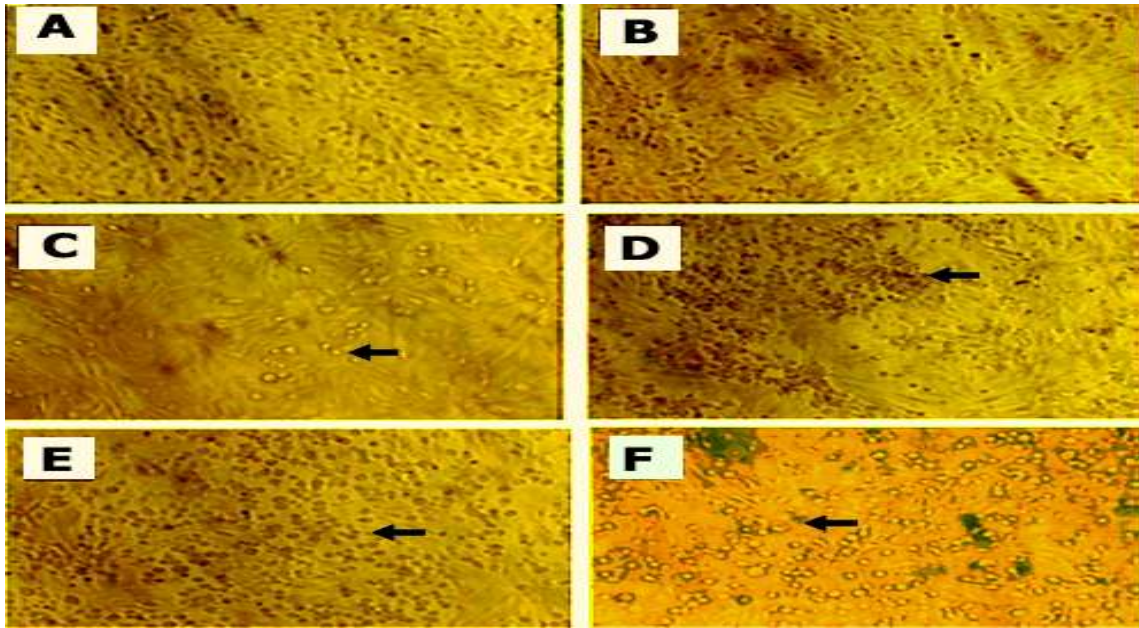


Fig. (1): Morphological changes in Vero cells due to propagation of human astroviruses recorded under an inverted light microscope. Signs of CPE started with rounding, darkness and clumping of cells, which may end up with complete destruction of the cell monolayer.

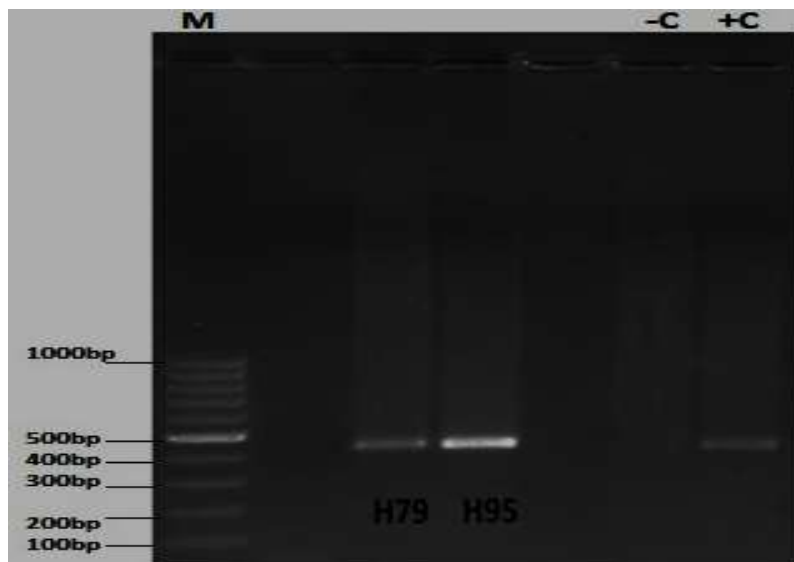


Fig. (2): Detection of HAstVs (H79, H95) from infected Vero cells at day 5 post infection using RT-PCR that targeting 442bp of RdRp region, as verified by including a 100bp molecular weight marker (M) on the same gels.

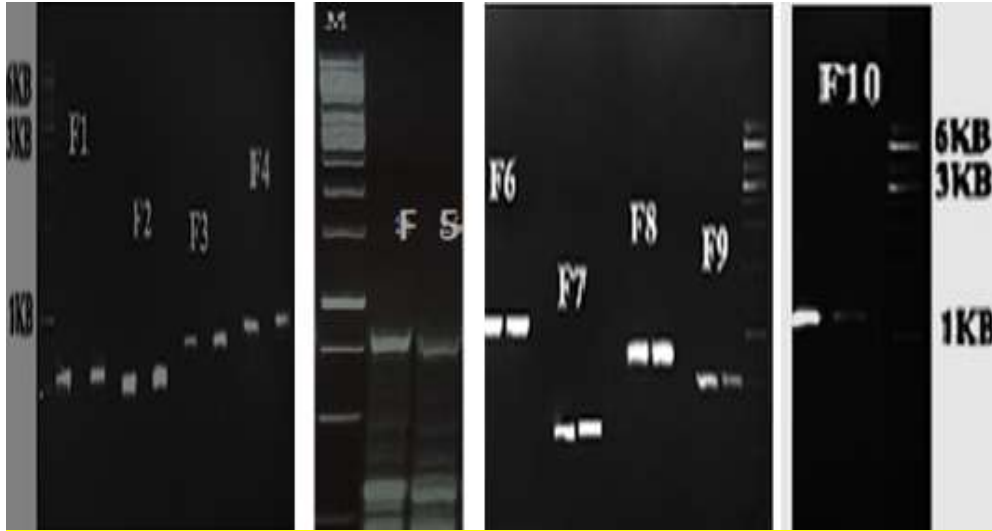


Fig. (3): Gel electrophoresis pattern of 10 amplicons from F1 to F10. The amplicons F1 to F10 molecular size are 838, 719, 822, 884, 835, 978, 540, 537, 723, and 1004 bp; respectively, separated by 1Kb molecular marker (M).

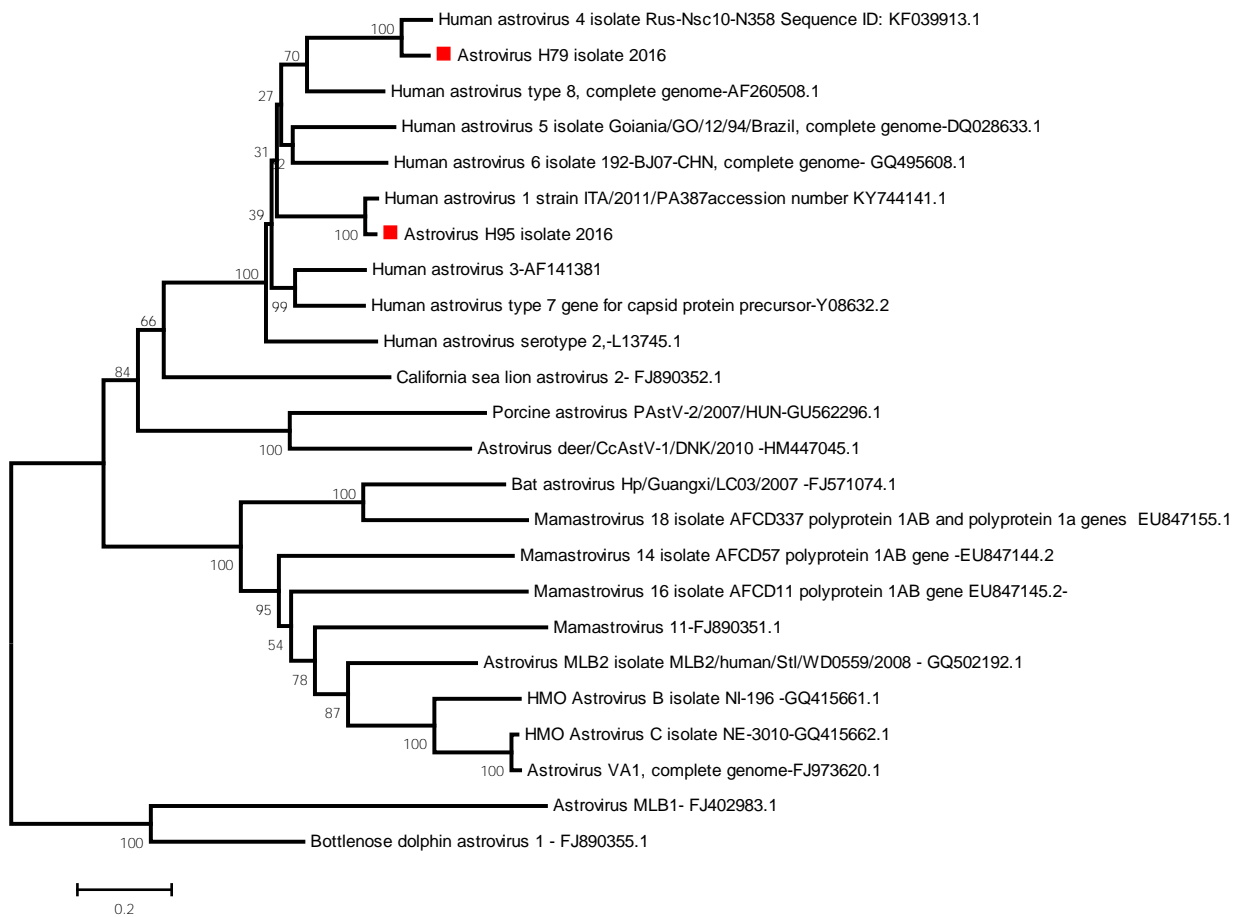


Fig. (4): Phylogenetic tree of two Egyptian human astrovirus isolates marked with red squares, compared with different viral genomic sequences obtained from Genbank of several genotypes.

4. Discussion

Astroviruses (AstV) are highly prevalent enteric viruses. Here we report, for the first time, the isolation and full genome sequencing of human AstVs detected in Egypt. In this study, Vero cells were found to be supportive for astrovirus growth. HAstV type 4 and HAstV type 1 successfully replicated in Vero cells after three successive passages. The propagated viruses were detected by RT-PCR. Previous study of Brinker *et al.*, (2000) showed that HAstVs were also successfully replicated well in Caco-2, Huh7.5, Huh7AI, and A549 cells.

Astroviruses are associated with diarrhea predominant in young children and immunocompromised individuals. Only two studies of Naficy *et al.*, (2000); Ahmed *et al.*, (2011), were conducted in Egypt to determine the distribution of astrovirus genotypes in children with diarrhea. Ahmed *et al.*, (2011) stated that HAstV-1 was the most circulating type, whereas previous study of Naficy *et al.*, (2000) reported also that HAstV-1 was the most frequent type, followed by HAstV-5, HAstV-8, HAstV-3, HAstV-6, HAstV-4 and HAstV-2. . Two isolates of human AstV genotype 1 and 4 were characterized in this study. Genomic structures were similar to the previously reported classical human astroviruses genotype 1 and genotype 4, detected in Italy and Russia, respectively. Glass *et al.*, (1996); Gaggero *et al.*, (1998) studies did not establish all the genotypes of the isolated astroviruses, however the most common astrovirus was reported as astrovirus genotype 1. In the current study, the primer sets constructed from the conserved regions of the reference strains of astroviruses could be useful in amplifying the polymorphic regions of genomes from several genotypes, and may be phylogenetically informative at both of the genus and genotype levels. Although we have not surveyed a large number of genotypes of human astroviruses, we believe that the primer sets will successfully amplify full genome of several strains.

Conclusions

Up till now, no data was available for the full genome of the circulating AstVs in Egypt. In this context, virus isolation and the full genomic sequencing of both isolates of human AstVs genotype 1 and 4 were conducted based on currently designed primers. The phylogenetic relationship of obtained sequences revealed that both isolates were closely related to HAstV 1 and HAstV4 isolated from Italy and Russia, respectively. Finally, the genetic diversity of the other HAstVs circulating in Egypt needs to be fully identified, as a first step in the control strategy of this neglected virus.

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Conflict of interest

The authors declare that there is no conflict of interests.

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