Genetic heterogeneity and recombination in type-3 human astroviruses

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Abstract

Human astroviruses (HAstV) are important enteric pathogens and can be classified genetically and antigenically into eight types. During molecular surveillance for human astroviruses in Italy, sequence analysis of the diagnostic region C (about 400 nucleotide in length), located on the capsid (ORF2) gene, identified a novel type-3 strain. Upon sequencing of the full-length ORF2, the type-3 HAstV strain was characterized as a novel ORF2 genetic lineage, designated as 3c. By converse, in the ORF1b the virus was more similar to type-1 HAstVs, rather than to type-3 strains, suggesting a recombination nature, with the crossover site being mapped to the ORF1b/ORF2 junction region. Region C sequences of similar type-3 HAstV identified from European and extra-European countries were retrieved in the databases, suggesting the global distribution of this novel type-3 lineage.

Keywords

Astrovirus, genotyping, recombination, viral gastroenteritis, Italy

1. Introduction

Human astroviruses (HAstVs), genus *Mamastrovirus*, family *Astroviridae*, are important etiological agents of gastroenteritis in humans, mostly in young children, elderly people, and immunocompromised patients [Mendez and Arias, 2013]. HAstVs have a single stranded positive-sense RNA of about 6.8 kb in length that contains three overlapping open reading frames (ORFs). ORF1a, ORF1b, and ORF2 encode the serine protease, the RNA-dependent RNA polymerase, and the capsid protein precursor, respectively. The capsid protein precursor can be divided into a highly conserved N-terminal domain (amino acids [aa] 1 to 424), a hypervariable (HVR) domain (aa 425 to 688), and a highly acidic C-terminal domain [Wang et al., 2001]. The HVR domain of HAstV is believed to form the capsid spike and to control binding to cell receptors as neutralizing epitopes have been mapped inside this capsid portion [Dong et al., 2011].

Studies based on immune electron microscopy, immunofluorescence, ELISA and plaque neutralization assays have revealed that HAstVs are antigenically heterogeneous and that they can be classified into eight serotypes (HAstV-1 to HAstV-8). [Bosch et al., 2014; Mendez and Arias, 2013; Kurtz and Lee, 1984]. Sequence analysis of short fragments at either the 5' or 3' end of ORF2 (regions C and D) and RT-PCR genotyping protocols with type-specific primers have been used for genetic characterization of HAstV-1 to -8 [Belliot et al., 1997; Noel et al., 1995]. HAstV-1 appears to be the predominant circulating serotype worldwide followed by types 2–5 and occasionally by type-8, depending on the region. HAstV-6 and 7 are rarely detected [De Grazia et al., 2012; Gabbay et al., 2007; Guix et al., 2002; Liu et al., 2008; Medici et al., 2012; Mendez-Toss et al., 2004; Mustafa et al., 2000].

Also, sequence analysis of the small diagnostic regions located on ORF2 has revealed discrete sequence variation within some HAstV types, revealing intra-typic genetic lineages. Type-1 HAstV has been classified into four lineages (HAstV-1a to -1d), type-2 into four (HAstV-2a to -2d), type-3 into two (HAstV-3a and -3b) and type-4 into three (HAstV-4a to -4c) [De Grazia et al., 2012; Gabbay et al., 2007; Guix et al., 2002; Martella et al., 2013]. Cutoff values of 6% nucleotides (nt) identity in the full-length ORF2 and of 5% nt in small diagnostic regions have been calculated among the various lineages. Comparison of the full-length ORF2 has confirmed the classification of HAstVs into discrete lineages but also revealed several examples of intra-typic recombination within this genomic region [Martella et al., 2014]. Also, examples of recombination in, or, close to the ORF1b/ORF2 junction have been described, suggesting that this genomic region may be a preferential site for RNA cross-over [Babkin et al., 2014; De Grazia et al., 2013; Gabbay et al., 2007; Martella et al., 2013; Walter et al., 2001].

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Surveillance for HAstV in Parma, Northern Italy, has been carried out continuously since 2008 in children aged < 5 years hospitalized with acute gastroenteritis at the Maternal-Infantile Department of the University Hospital of Parma. All the HAstV strains identified between 2008 and 2013 were systematically genotyped in the diagnostic region C (nt 4571 to 4918 of accession no. L13745) [De Grazia et al., 2013; Martella et al., 2013; Medici et al., 2012]. In this 6-years period, the overall prevalence of HAstV infection was 1.34% (32 out of 2,383 children with gastroenteritis), ranging from 3.87% in 2008 to 0.49% in 2011. Five different genotypes were found to circulate in this time span, with type-1 HAstVs being predominant (23 cases, 71.88%), followed by type-2 (4 cases, 12.5%) and type-4 (3 cases, 9.38%), type-3 (1 case, 3.12%) and type-5 (1 case, 3.12%). The type-1 HAstVs were sub-typed as lineage 1a (16 cases, 69.56%), -1b (1 case, 4.35%) and HAstV-1d (6 cases, 26.09%). All the type-2 HAstVs were characterized as lineage 2d, and all the type-4 HAstVs as lineage 4c, while the type-5 HAstV strain was sub-typed as 5c. Based on the small sequence generated in ORF2 region C, the single HAstV-3 strain, PR1365/2012/ITA, detected in April 2012, segregated within a novel, yet unrecognized, type-3 lineage, along with similar strains detected globally. In order to investigate better the genetic signature of this strain, a 3.2 kb portion at the 3' end of the genome was sequenced and compared with HAstV-3 strains retrieved from the databases.

2. Material and Methods

A 3' RACE-PCR protocol was used to generate a 3.2 kb amplicon encompassing the 3' end of ORF1b, the full-length ORF2, the 3' untraslated region (UTR) through the poly-A tail [Wang et al., 2005]. Briefly, cDNA was synthesized by SuperScript III First-Strand cDNA synthesis kit (Invitrogen Ltd, Paisley, UK) with primer VN3T20 (5'-GAGTGACCGCGGCCGCT20-3'). PCR was performed with TaKaRa La Taq polymerase (TaKaRa Bio Europe SAS, Saint-Germain en-Laye, France) with forward primer panAstVFor1 (GARTTYGATTGGRCKCGKTAYGA) and the reverse primer VN3T20 [Chu et al., 2008]. The amplicon was purified and cloned using TOPO XL Cloning Kit (Invitrogen Ltd, Paisley, UK). A consensus sequence was generated on 3 clones. Additional primers were designed to determine the complete 3.2-kb sequence (corresponding to nt 3566–6787 of the Nsc08/336/2008/RUS/type3, GenBank accession no. GU732187) by an overlapping (primer-walking) strategy. Sequence editing, generation of multiple codon-based (translation) alignments and phylogenetic trees were performed with MEGA version 6.0 [Tamura et al., 2013]. Maximum composite likelihood (ML) algorithm and the neighbour-joining method were used for construction of the phylogenetic trees. The reliability of the phylogenetic trees was assessed by bootstrap re-sampling over 1000 replicates. SimPlot version 3.2 [Lole et al., 1999] was

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used to identify cross-over sites due to recombination. Additionally, recombination analysis was carried out with different algorithms implemented in the Recombination Detection Program v.4.43 (RDP4) (Martin et al., 2010), with default settings. The accession number of the strain PR1365/2012/ITA is KF668570. A total of 13 full-length ORF2 sequences of HAstV-3 and 29 partial (~350 bp) ORF2 sequences spanning the diagnostic region C were available in the NCBI databases and were included in the phylogenetic analyses.

3. Results and Discussion

The 42 HAstV sequences retrieved from the databases encompassed type-3 strains detected worldwide over a nearly 3-decade period. Three distinct lineages were clearly resolved by phylogenetic inference in either the full-length ORF2 or the region C of ORF2, and these clusters were statistically supported (Figure 1). Considering the whole data set, 30 (71.42%) sequences were characterized as lineage 3a, 3 (7.14%) as lineage 3b and 9 (21.42%) as the novel, yet unrecognized lineage, tentatively proposed as 3c. The vast majority of recent type-3 HAstVs could be sub-typed as lineage 3c, although 3c HAstVs were already circulating during 1998-99 in Germany and in the late 2000s in Tunisia, Egypt, India and Japan [Ahmed et al., 2011; Chan-it et al., 2010; Pativada et al., 2011]. However, no peculiar temporal or geographical pattern could be inferred from the data set. The Italian strain PR1365/2012/ITA in region C was grouped with other lineage 3c HAstVs and shared a nt identity of 96.3-99.1% with those strains and differed by 4.4 to 5.9% nt from HAstV-3a strains and by 6.9 to 7.2% nt from HAstV-3b strains.

In the full-length ORF2, nt identity between type-3 HAstVs of different lineages ranged between 90.4 and 93.7% while the nt identity within each lineage was higher than 95.3%. A nucleotide difference (cut-off value) of 6.3% was found among the various lineages on the basis of the full-length ORF2. These values are similar to the values calculated in the region C in other studies [Gabbay et al., 2007; Guix et al., 2002]. The aa variation within type-3 HAstVs reached 2.8% in the N-terminal domain (aa 1-424), 7.6% in the HVR hypervariable region (aa 425-688) and 8.5% in the highly acidic C-terminal domain. In the HVR, intra-lineage aa variation reached 4.9 and 1.2% for 3a and 3b strains, respectively, while inter-lineage aa variation ranged between 4.9 and 6.9%. The high inter-lineage variation observed in HVR, confirmed that this region is under stronger evolutionary pressure across HAstVs [Sanchez-Fauquier et al., 1994; Wang et al., 2001]. By converse in ORF1b-based analysis, strain PR1365/2012/ITA were genetically (93% nt identity) closer to the reference type-1 HAstV strain Beijing/293/2007/CHN (accession no. FJ755405), to a

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recombinant strain detected in Parma in 2009 with a type-1 ORF1b and type-2 ORF2 (accession no. JX087964) and to a recombinant strain detected in Palermo in 2003 (accession no. KC915035) with a type-1 ORF1 and a type-4 ORF2. In the ORF1b, the Italian strain PR1365/2012/ITA displayed 84% nt identity to the reference type-3 strain (Rus/Nsc08/326/2008/RUS, accession no. GU732187). By Simplot and RDP4 analysis, a putative recombination break-point event was mapped 30 nt upstream the ORF1b/ORF2 region, at nt 726 (corresponding to position 4295 of the strain Nsc08/336/2008/RUS/type-3, GenBank accession no. GU732187) (Figure 2), with a significant ($P < 0.05$) support with all the algorithms used.

Accordingly, the genotype-3 strain PR1365/2012/ITA was likely a recombinant strain with the ORF1b acquired from type-1 HAstVs. While the identification of recombination events in the ORF1b/ORF2 junction of HAstVs has been already documented [De Grazia et al., 2012; Martella et al., 2013], this is the first report of recombination between type-1 and type-3 HAstVs. The exchange of genome fragments via recombination is common in single-stranded RNA viruses and appears to occur at higher frequency in highly conserved genomic regions and between genetically related strains [Bull et al., 2005; Bull et al., 2007]. Virus recombination can affect phylogenetic groupings, increase the virulence/fitness of the agent, confuse molecular epidemiological studies, and have major implications in vaccine design [Bull et al., 2007]. As there is no data on the genomic make up of the other type-3c strains detected elsewhere, the question whether the type-1/type3c ORF1b/ORF2 signature is peculiar of strain PR1365/2012/ITA or it is a common feature of the 3c lineage remains open.

HAstV genome has undergone multiple breaking events, which had mainly occurred at, but were not restricted to, the ORF1b/ORF2 junction region [Babkin et al., 2012; Belliot et al., 1997; Martella et al., 2013; Walter et al., 2001; Wolfaardt et al., 2011]. Therefore, whole genome sequencing is optimal in order to characterize firmly these chimeric viruses and to understand better the frequency and the role of recombination in the evolution of HAstVs.

4. Conclusions

Although several sequences of the HAstV lineage 3c were already available in the databases, with the oldest strains dating back to the late 1990s, this different type-3 lineage had not been recognized thus far, and none of these type-3 viruses was characterized more in detail. Phylogenetic analysis based on the small sequences of the diagnostic region C of ORF2 was consistent with the analysis based on the full-length ORF2, confirming that region C is a good proxy for prediction of HAstV types and for distinction of intratypic genetic lineages. Conversely, partial genome sequencing or characterization based on a single genome region may not be fully suitable to say whether one

ha eliminato: . To strength statistical significance and to confirm the recombination finding, we used a set of six recombination detection methods implemented in RDP4 software package. The resulting data matched the results obtained with Simplot analysis. By RDP4 analysis the PR1365/2012/ITA strain was defined as recombinant as the crossover event at position nt 726 was found to be significant

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strain may be a good representative of a particular genotype. As most molecular epidemiological studies for HAstVs did not gather multi-target sequence data, it is possible that this phenomenon is largely underestimated and that we are at the very beginning to describe the role of recombination in HAstV.

The work of the present study was integrated into the activity of the Italian Study Group for Enteric Viruses (ISGEV) that monitors the epidemiology of enteric viruses in children through hospital based surveillance. The updated lineage classification of HAstVs is available on the ISGEV web site (http://isgev.net). Our findings reinforce the need for structured molecular epidemiological studies for HAstVs, in order to understand better the dynamics of HAstV circulation and the pathways followed by these viruses in their evolution.

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Figure legends.

Figure 1. The region C tree was constructed using an ~350-nt ORF2 fragment of the 42 sequences of type 3 HAstV strains available in the databases, of which 13 (highlighted in bold) were from the full-length ORF2 sequences. The full-length ORF2 tree was constructed with the 13 full length ORF2 (2385 nt) of type 3 HAstVs available in the databases. The ORF1b tree was constructed with the partial 3'end of ORF1b (836 nt) of the HAstV-3 strain PR1365/ITA/2012 and of reference strains retrieved from databases. Trees were built with the maximum-likelihood (ML) method, and bootstrapped with 1000 repetitions. Bootstrap values >80% are indicated The scale bar indicates the number of nucleotide substitutions per site. The genome organisation of HAstV and the locations of the various genetic targets used for phylogenetic analyses are also shown.

Figure 2. SimPlot analysis of the 3' end of ORF1b and full-length ORF2 sequences of PR1365/ITA/2012 HAstV-3 recombinant strain; window size, 200 bases; step, 20 bases. At each position of the window, the query sequence was compared to each of the reference strains (Nsc08/336/2008/RUS/type3, GenBank accession no. GU732187, 293/2007/CHN/type1, GenBank accession no. FJ755405). The X-axis indicates the nucleotide positions in the multiple alignments of the HAstV sequences (corresponding to nt 3566–6787 of the Nsc08/336/2008/RUS/type3, GenBank accession no. GU732187); the Y-axis indicates the percentage of similarity. The dashed line indicates the beginning of ORF2 (corresponding to nt 4321).

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