



Article

Evaluation of qPCR for the Selective Detection of Enteric Adenovirus Followed by Sequence-Based Genetic Characterization of F Strains Circulating in Brazil

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Abstract: Human adenovirus (HAdV) F40/41 is an important pathogen in pediatric acute gastroenteritis cases. However, the diversity of study designs and diagnostic methods often leads to misinterpretations of their impact. Our study explored the genetic diversity of HAdV-F40/41 in Brazil using a specific qPCR assay for HAdV species F, combined with a phylogenetic analysis of the partial hexon and fiber genes. Our results demonstrated that HAdV-F41 strains predominated and exhibited higher diversity than HAdV-F40 strains. Based on the hexon gene, Brazilian HAdV-F41 strains were grouped into two genome type clusters (GTC), further divided into subclusters, with most strains clustering to GTC2. The partial shaft region of the fiber gene exhibited higher conservation among HAdV-F41. The specific qPCR assay for HAdV species F identified HAdV-F in an additional 31.5% (34/108) of previously uncharacterized HAdV-positive samples detected using a non-specific HAdV qPCR assay. Both assays strongly correlated in detecting HAdV-F, and the specific qPCR assay for enteric types can enhance HAdV surveillance, especially when sequencing is not possible. Our study provides novel insights regarding the genetic diversity of HAdV-F species in Brazil.

Keywords: acute gastroenteritis; enteric HAdVs; genetic diversity; phylogenetic analysis; qPCR; Brazil



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1. Introduction

Acute gastroenteritis (AGE) is one of the leading causes of morbidity and mortality in children under the age of five, especially in low- and middle-income countries (LMICs) where proper access to health care, hygiene, and education is often limited [1,2]. Studies conducted in LMICs screening for multiple enteropathogens have detected rotavirus, norovirus, and human adenovirus (HAdV) F40/41 as the primary enteric pathogens detected in pediatric patients with AGE [3–6].

HAdV, members of the *Adenoviridae* family (genus *Mastadenovirus*), are non-enveloped, linear dsDNA viruses with a ~35 kbp genome [7]. Based on genomic and bioinformatics analyses, HAdV is currently divided into seven species with over 100 types characterized (hadvwg.gmu.edu/, accessed on 16 November 2023) [8]. Different types of HAdV exhibit different tissue tropisms, resulting in a diverse array of clinical symptoms. In immunocompetent individuals, symptoms are typically mild and lead to self-limited respiratory, gastrointestinal, and conjunctival infections. Severe disease usually impacts immunocompromised individuals, resulting in persistent and widespread infections that can also affect the genitourinary tract, liver, central nervous system, and cardiovascular system [9–11].

Species F comprises two well-established types (HAdV-40 and -41), which are known as enteric-HAdV due to their tropism for the gastrointestinal tract and association with AGE. The importance of HAdV in pediatric AGE was underestimated for many years.

However, the use of molecular detection techniques as a diagnostic method has revealed their substantial contribution to the global burden of pediatric AGE [12]. In a recent study in LMICs, the Global Pediatric Diarrhea Surveillance Network identified HAdV-F40/41 as the second most frequent pathogen detected in children under the age of five with AGE, behind rotavirus [3]. As different HAdV types can be detected in stool samples, regional studies worldwide have reported a wide range of HAdV positivity rates (enteric and non-enteric types), with rates ranging from 1.6% up to 39.1% [13–16]. The wide range in HAdV positivity rates is caused by multiple factors, including study design, scope, diagnostic methods, regional and temporal variations, and the introduction of rotavirus immunization [5,6].

Until early 2022, HAdV-F was a major pediatric pathogen in AGE cases, yet its genomic diversity remained poorly understood. In March of that year, multiple cases of severe hepatitis of unknown origin were detected in immunocompetent children. Further investigation revealed several cases dating back to October 2021 in over 40 countries [17,18]. The high detection rate of HAdV-F41 and the possibility of a clinical association of F41 with severe hepatitis of unknown origin increased the urgency for more comprehensive molecular phylogeny studies to better understand species F evolution, identify new emerging strains, and obtain more data for vaccine development [19].

Knowledge regarding HAdV-F40/41 genomic diversities and their actual contribution to AGE cases, particularly in Brazil, remains limited. To address this research gap, our study aimed to assess the efficacy of a species F-specific quantitative polymerase chain reaction (qPCR) protocol in determining the true impact of enteric HAdV in AGE cases. In addition, we conducted sequencing and molecular phylogenetic analysis of six hypervariable regions of the hexon gene, along with partial sequencing of the shaft region of the fiber gene from HAdV-F40/41 strains detected in AGE patients, to provide insights into the genetic diversity of species F in Brazil.

2. Materials and Methods

2.1. Stool Collection and Ethics Statement

This study included HAdV-positive stool samples collected between January 2018 and December 2020 from inpatients and outpatients (children and adults) with symptoms of AGE, as previously described [15]. AGE was defined as the sudden onset of diarrhea ($\geq$ three liquid or semi-liquid evacuations within 24 h) that may be accompanied by fever, nausea, vomiting, or abdominal pain. Stool samples were systematically sent to the Regional Rotavirus Reference Laboratory—Laboratory of Comparative and Environmental Virology (RRRL–LVCA) through sentinel sites at States Central Laboratories. The RRRL–LVCA is part of the national network for rotavirus surveillance, overseen by the General Coordination of Public Health Laboratories within the Brazilian Ministry of Health (MoH).

This study was approved by the Oswaldo Cruz Foundation (Fiocruz) Ethics Committee (approval number CAAE: 94144918.3.0000.5248). The surveillance was performed through a hierarchical network in which samples were provided by medical requests in hospitals and health centers, monitored by the Brazilian Unified Health System (SUS). This study was conducted within the scope of the RRRL/MoH as part of a federal public health policy for viral AGE surveillance in Brazil. Patient informed consent was waived by the Fiocruz Ethical Committee, and patients' data were maintained anonymously and securely.

2.2. Viral DNA Extraction

Nucleic acid extractions were performed from 140 μ L of clarified stool suspension (10% *w/v*) with the QIAamp Viral Mini kit (Qiagen, Valencia, CA, USA) on the automated QIAcube platform, following the manufacturer's instructions. The extracted viral nucleic acids were eluted in 60 μ L of the elution buffer AVE and immediately stored at -80°C until the molecular analysis. RNase/DNase-free water was used as a negative control in each extraction protocol.

2.3. HAdV-F Detection and Quantification

A specific qPCR assay for HAdV-F [20] was used to detect enteric types F40 and F41 in samples that had previously tested positive for HAdV using a degenerate set of primers and a probe targeting a conserved region of the first part of the adenovirus hexon gene [21]. Detailed information on initial HAdV detection and quantification methods was previously described [15].

The specific qPCR assay for enteric HAdV detection [20], targeting a conserved 118 bp sequence of the HAdV-F fiber gene, was carried out using the forward primer (5-AACTTTCTCTCTTAATAGACGCC-3); reverse primer (5-AGGGGGCTAGAAAACAAAA-3), and probe (5-CTGACACGGGCACTCT-3). Briefly, HAdV-F qPCR reactions were performed with 5 µL of the extracted DNA in a final volume of 20 µL, containing 10 µL of the 2x QuantiTect Probe PCR Kit (Qiagen, Valencia, CA, USA) and primers and probe with final concentrations of 0.5 µM and 0.25 µM, respectively. Reactions were conducted in the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following thermal cycling conditions: 2 min at 50 °C, 15 min at 95 °C, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. Samples that exhibited a characteristic sigmoid curve and crossed the threshold line with a cycle threshold (Ct) value < 40 were considered positive. All runs included negative and positive controls (stool sample), as well as a non-template control.

2.4. HAdV-F Genetic Diversity and Nucleotide Sequencing

To assess the genetic diversity of HAdV-F strains circulating in Brazil, positive samples initially characterized as types F40 and F41 [22] were resequenced. We targeted the six hypervariable regions (HVR1–HVR6) of the hexon gene using the primers S29 and S52 [23] and the partial shaft region of the long fiber gene using the primers AdF1 and AdF2 [24]. PCR reactions were performed using the Platinum Taq DNA Polymerase enzyme (Invitrogen, Carlsbad, CA, USA), with 5 µL of extracted DNA in a final reaction volume of 25 µL. The expected amplicons of 640 and 664 nt for the hexon gene and 508 and 530 nt for the fiber gene for types F40 and F41, respectively, were purified using the QIAquick Gel Extraction Kit (Qiagen), following the manufacturer's instructions. Sequencing reactions of the purified amplicons were performed using the Big Dye Terminator v. 3.1 Cycle Sequencing Ready Reaction Kit on an ABI Prism 3730 xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at the Fiocruz Institutional Genomic Platform for DNA sequencing (PDTIS).

2.5. Phylogenetic and Mutation Analysis of F40/41

Chromatogram analysis and consensus sequences were obtained using the Geneious Prime 2021.1.1 software (Biomatters Ltd., Auckland, New Zealand). The hexon and fiber nt sequences of types F40 and F41 were analyzed in terms of closest identity with sequences available in the GenBank database using the Basic Local Alignment Search Tool (BLAST) (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed on 5 May 2023). Sequences were aligned using ClustalW [25], and the maximum likelihood (ML) method was used for phylogenetic analysis using the randomized Accelerated Maximum Likelihood (RAxML) program [26] with the general time-reversible (GTR) model with a gamma-distributed heterogeneity rate across sites and 1000 bootstraps iterations for hexon and fiber genes, accessible on the CIPRES Science Gateway [27]. The output of RAxML runs was used in MEGA11 v11.0.13 [28] to visualize and construct the phylogenetic trees. Reference sequences were obtained from the National Center for Biotechnology Information (NCBI) GenBank database. In addition, to investigate the genetic diversity of the Brazilian HAdV-F strains, synonymous and non-synonymous mutations in the sequenced portions of the hexon and fiber genes were compared with prototype strains obtained from the GenBank database.

Nucleotide sequences obtained in this study were deposited in the GenBank database with the following accession numbers: OQ442226–OQ442299.

2.6. Statistical Analysis

Statistical analyses were performed using GraphPad Prism software version 9.0.0 (GraphPad Software, San Diego, CA, USA; www.graphpad.com, accessed on 15 October 2023). The Mann–Whitney U test was used to assess significant differences among qPCR protocols. Chi-square or Fisher’s exact tests were employed for analyzing categorical characteristics in contingency tables, and the correlation of Ct values between qPCR for HAdV (all types) and qPCR specific for HAdV-F was assessed using the Pearson correlation coefficient. For all analyses, a *p*-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Detection of HAdV-F40/41 Using a Specific qPCR Assay

In our previous study, conducted on samples collected between January 2018 and December 2020, we investigated the role of HAdV among patients with AGE in Brazil. Initially, we employed a degenerated qPCR primer set targeting a conserved region of the hexon gene (qPCR_HAdV) to test for the presence of HAdV in a total of 1235 stool samples. Positive samples with low Ct values were sequenced to further characterize HAdV species and types, with 39% (123/315) of HAdV-positive samples successfully sequenced [15].

In the present study, we used a qPCR protocol with HAdV-F40/41 specific primers (qPCR_HAdV-F) in 91% of previously sequenced samples (112/123). Using the qPCR_HAdV-F protocol, F species were detected in 93.5% (43/46) of sequenced samples previously identified as enteric types F40 and F41 and in 4.5% (3/66) of samples previously identified as non-enteric HAdVs. Three samples originally characterized as belonging to species C tested positive for species F, while three samples characterized as belonging to species F tested negative using qPCR_HAdV-F (Table 1).

Table 1. Comparison between HAdV degenerated and F-specific qPCR assays.

HAdV Classification	HAdV Degenerated qPCR ¹	HAdV-F qPCR		Positivity (%)
	N° of HAdV-Positive Tested Samples	Positive	Negative	
Enteric types	46	43	3	93.5
Non-enteric types	66	3	63	4.5
Non-sequenced HAdV	108	34	74	31.5
Total	220	80	140	36.4

¹ For qPCR protocol comparison, the table displays the original HAdV characterization results for the selected samples, as previously published [15].

In addition, we used the qPCR_HAdV-F assay for the group of HAdV-positive samples that could not be sequenced due to low viral detection (usually samples detected with Ct > 30). Among the non-sequenced HAdV-positive samples previously detected with the qPCR_HAdV, we detected HAdV-F in 31.5% of samples (34/108) with the new protocol (Table 1).

To evaluate the efficiency of the qPCR_HAdV-F assay, we compared the Ct values obtained using both assays for each sample in the HAdV-F positive group (*n* = 80). Scatter plots and regression analyses were conducted on each sample to determine the correlation between the Ct values for the two assays. A Pearson’s correlation coefficient of 0.96 (*p* < 0.0001) indicates a strong correlation (*r* value $\cong$ 1) between the estimated viral load obtained by the qPCR_HAdV-F assay and the qPCR_HAdV assay (Figure 1a). For each primer set, 66.3% of the samples tested (53/80) exhibited similar PCR amplification efficiencies (Ct 2.5 cycles) (Figure 1b). The estimated PCR efficiencies corresponded to an average of 1.75 Ct values between the Δ Cts of the samples, which ranged from 0.04 to 8.09. Only three samples showed a Δ Ct higher than 5.0 Ct. To evaluate the performance of the HAdV-F specific primer set, we compared the Ct values obtained by qPCR_HAdV-F and qPCR_HAdV assays on each HAdV-F positive sample. In 48.75% (39/80) of tested samples, the Ct values for the species F-specific primers were lower, suggesting that HAdV-F specific primers

can detect a higher viral load for the same sample compared to the degenerate primers (Figure 1c). When comparing the Ct values of the HAdV-positive samples previously detected on the qPCR_HAdV assay and non-sequenced, we observed that HAdV-F positive samples had significantly lower Ct values detected by qPCR_HAdV-F assay compared to non-F HAdV-positive samples detected by qPCR_HAdV using degenerated primers ($p = 0.0129$; median Ct values of 35.9 and 36.6, respectively). This indicates a higher viral load excretion in stool samples from patients detected with HAdV enteric types (Figure 1d).

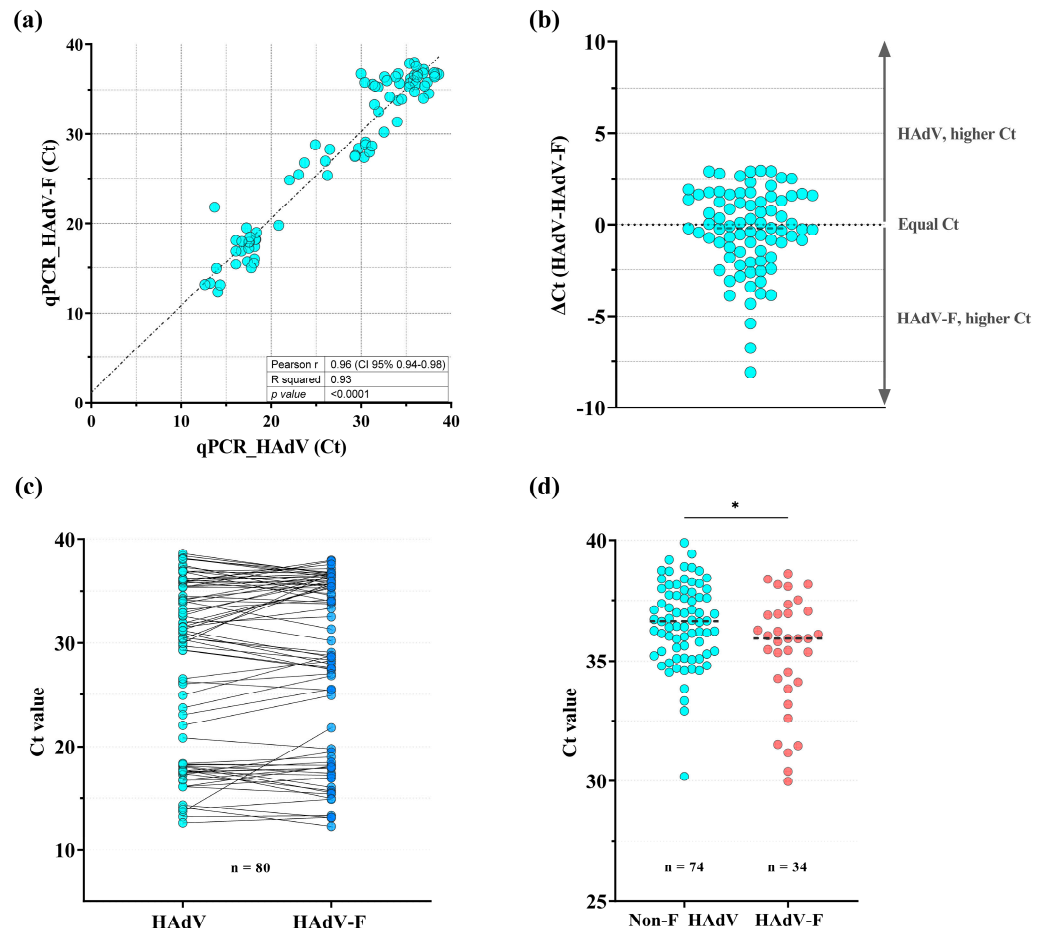


Figure 1. (a) Scatter plot of comparative Ct values showing the correlation between Ct values detected for the HAdV-F-positive samples ($n = 80$) using a HAdV degenerated primer set (qPCR_HAdV) and a HAdV-F specific primer set (qPCR_HAdV-F). Pearson's correlation coefficient indicates the strength of linear dependence between the different qPCR assays. (b) ΔCt difference between qPCR_HAdV-F and qPCR_HAdV assay Ct values for HAdV-F-positive samples with $Ct < 40$ ($n = 80$). Each blue dot represents a single sample. The dashed line indicates the point at which the difference in Ct values found in each qPCR assay equals zero ($\Delta Ct = 0$). (c) Paired comparison of qPCR Ct values obtained using qPCR_HAdV and qPCR_HAdV-F for each HAdV-F positive sample ($Ct < 40$), represented by light and dark blue dots, respectively. (d) Comparison of Ct values among HAdV-F-negative (blue dots) and HAdV-F-positive (red dots) on the unsequenced samples detected by the qPCR_HAdV and qPCR_HAdV-F, respectively. The horizontal dashed line indicates the median Ct values. * $p \leq 0.05$.

3.2. Phylogenetic Analysis of HAdV-F Hexon and Fiber Genes

Among the 43.9% (54/123) of samples previously characterized as HAdV-F, based on a conserved region of the hexon gene [15], we successfully sequenced 70% (7/10) of the F40 samples and 68.2% (30/44) of the F41 samples in this study for the HVR1-HVR6 of the hexon gene. In addition, we also amplified the partial shaft region of the long fiber gene in 100% (10/10) of the F40 samples and 61.4% (27/44) of the F41.

3.2.1. Hexon Gene

The HVR1-HVR6 phylogenetic analysis of the Brazilian strains revealed that the F40 strains were more conserved than the F41 strains. The seven F40 strains clustered together, sharing >99% nucleotide (nt) identity. Additionally, the HVR1-HVR6 regions of F40 strains showed >99% nt identity with the corresponding regions of the hexon gene of the prototype strain Dungan (AB330121), and to strains previously detected in Brazil (MT791000), India (KU884615, MT952448), Finland (KU162869), South Africa (MK955319), and China (MK883611) (Figure 2).

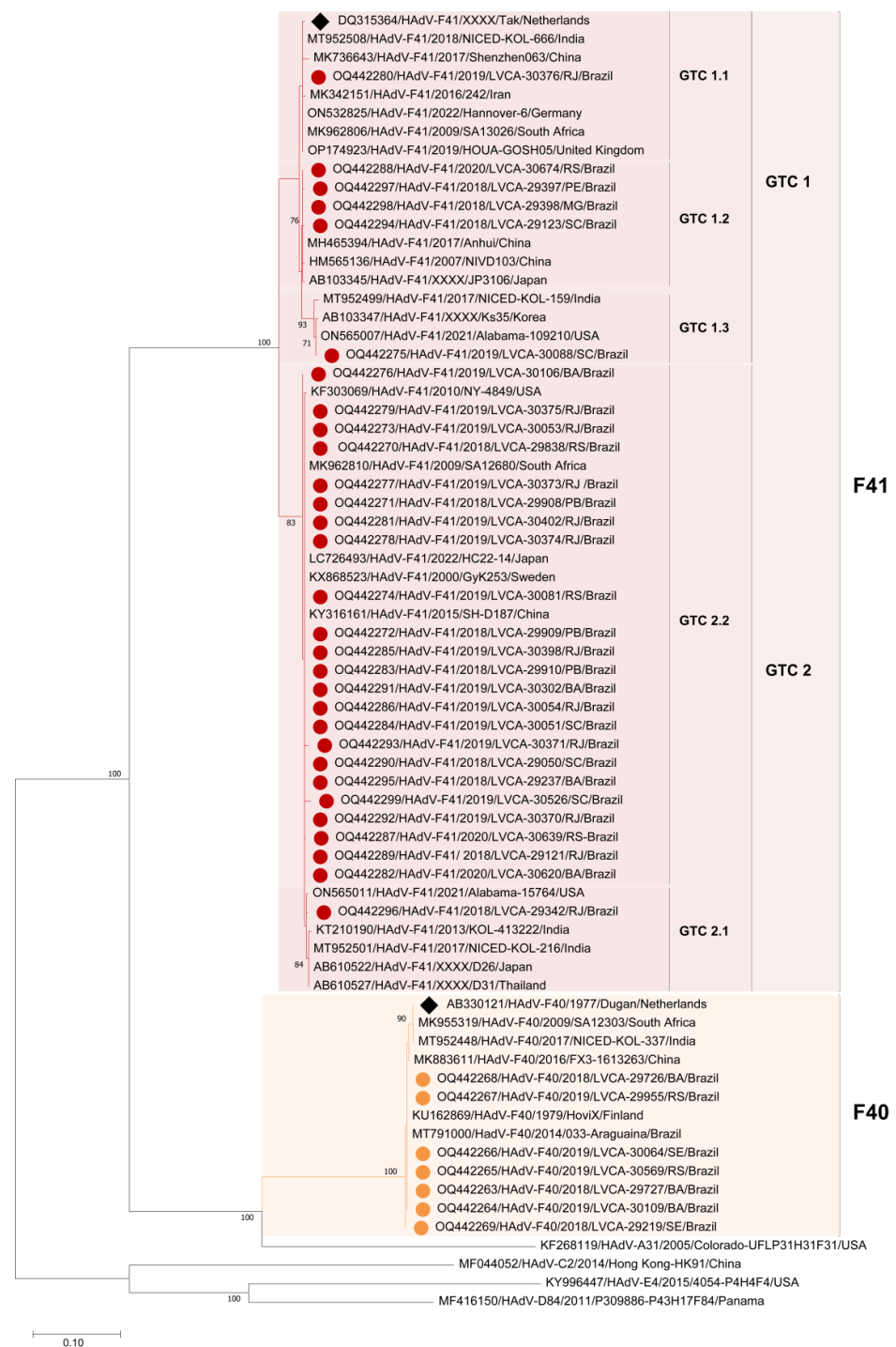


Figure 2. Phylogenetic tree based on the nucleotide sequence of the hypervariable regions (HVR1-HVR6) of the hexon gene. HAdV-F40 and -F41 strains isolated in this study are denoted with

an orange- and red-filled circle, respectively. Reference strains were downloaded from the GenBank repository and labeled with their accession number/HAdV-type/year of collection/isolate ID/country. Prototype strains for types F40 and F41 are marked with a black-filled diamond. The phylogenetic tree was constructed using the maximum likelihood method using the randomized Axelerated Maximum Likelihood (RAxML) program, with the general time-reversible (GTR) model with a gamma-distributed heterogeneity rate across sites and 1000 bootstraps iterations, and MEGA11 software v11.0.13 (Auckland, New Zealand) was used to construct and visualize the tree. The bootstrap percentage values of $\geq 70\%$ are shown at each branch point. Hexon genome type clusters (H-GTC) and subclusters are indicated for F41 strains.

The phylogenetic analysis of the HVR1-HVR6 in F41 strains showed that the Brazilian sequences clustered into two different lineages, H-GTC1 and H-GTC2 (H-GTCs: Genome type cluster based on the hexon gene). Among these, 20% (6/30) of the strains clustered into H-GTC1, while 80% (24/30) clustered into H-GTC2 (Figure 2). In terms of identity between the Brazilian isolates, H-GTC2 strains displayed a slightly wider variation range (95.9–100% nt identity) compared to H-GTC1 strains (96.2–100% nt identity). Regarding the prototype TAK (DQ315364), H-GTC2 (92.4–93.5% nt identity) exhibited a higher degree of divergence than H-GTC1 (96.5–99.8% nt identity).

Further analysis revealed that H-GTC1 was subdivided into three subclusters. H-GTC1.1 with one representative strain (LVCA_30376), which clustered with strains detected in Asia (MK342151 and KT210193), Europe (OP174923, ON532825, and MW567963), and South Africa (MK962806), sharing >99% nt identity. Four strains clustered into H-GTC1.2, and exhibited maximum nt identity (>99%) with strains from China (HM565136 and MH465394) and Japan (AB103345). H-GTC1.3 had a single representative strain (LVCA_30088), which clustered with strains from Asian countries (AB103347, MT952499, and DQ336391) and the United States (ON565007), displaying >99% nt identity.

H-GTC2 strains segregated into two subclusters, with the H-GTC2.2 harboring the majority of sequences. The shared nt identities ranged from 98.4% to 100% with strains from China (KY316161), Japan (LC726493), South Africa (MK962810), Sweden (KX868523), and the United States (KF303069). Finally, one sequence (LVCA_29342) clustered into H-GTC2.1, sharing a maximum nt identity (>98%) with strains from Asian countries (AB610522, AB610527, KT210190, and MT952501) and the United States (ON56501).

3.2.2. Fiber Gene

The partial shaft region of the fiber gene was more conserved than the hexon gene, among sequences of types F40 and F41. The ten F40 Brazilian strains clustered together, displaying nt similarity of >99%. The analyzed region of the fiber gene exhibited a maximum nt sequence identity of >98% with the reference strain Dungan (L19443) and with strains detected in Finland (KU162869), India (KU884631 and MT952560), and South Africa (MK955316) (Figure 3).

The F41 Brazilian strains shared >98% nt identity with the prototype TAK strain (DQ315364), and clustered into two genetic clusters (F-GTC: Genome type cluster based on the fiber gene). Within cluster F-GTC1, no strains clustered with subcluster F-GTC1.1. One Brazilian strain (LVCA_29630) grouped into subcluster F-GTC1.2, while three other strains clustered into subcluster F-GTC1.3, displaying maximum nt identities (>99%) with strains from China (KY316160, MT150355, and AB610544) and Japan (AB610540 and AB246870). All other F41 Brazilian strains ($n = 23$) grouped into F-GTC2 (subcluster F-GTC2.1) and shared nt identities ranging from 91.2% to 100% compared to the other clusters. The maximum nt identity (>99%) was observed with strains from South Africa (MK962809), the United Kingdom (OP174926), Iraq (MG925782), and Germany (ON532825 and KX868523) (Figure 3). Interestingly, no Brazilian strain grouped with subcluster F-GTC2.2.

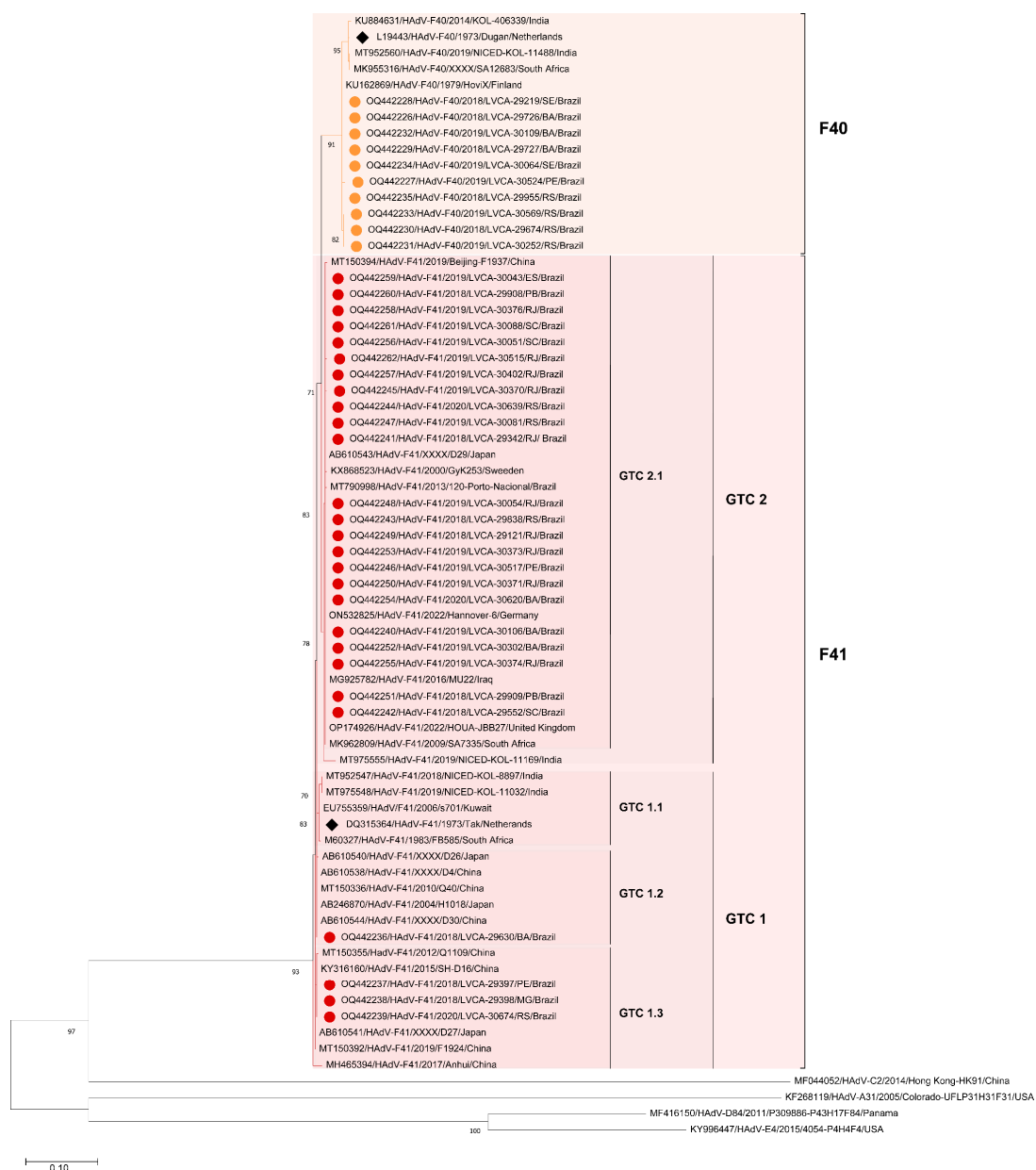


Figure 3. Phylogenetic tree based on the nucleotide sequence of the partial shaft region of the fiber gene. HAdV-F40 and -F41 strains isolated in this study are denoted with an orange- and red-filled circle, respectively. Reference strains were downloaded from the GenBank repository and labeled with their accession number /HAdV-type/year of collection/isolate ID/country. Prototype strains for types F40 and F41 are marked with a black-filled diamond. The phylogenetic tree was constructed using the maximum likelihood method using the Randomized Axelerated Maximum Likelihood (RAxML) program, with the general time-reversible (GTR) model with a gamma-distributed heterogeneity rate across sites and 1000 bootstraps iterations, and MEGA11 software v11.0.13 was used to construct and visualize the tree. The bootstrap percentage values of $\geq 70\%$ are shown at each branch point. Fiber genome type clusters (F-GTC) and subclusters are indicated for F41 strains.

3.3. Mutation Analysis of the Hexon and Fiber Protein of HAdV-F Strains Circulating in Brazil

3.3.1. Hexon Gene

Brazilian HAdV-F40 strains detected were mostly conserved among themselves within HVR1-HVR6. One sample (LVCA_29219) showed a transversion mutation (G850T), which resulted in an amino acid change from alanine (A) to serine (S). Compared to the Dungan

For the F40 Brazilian strains, the partial shaft region of the fiber gene accumulated more mutations compared to the HVR1-HVR6 region of the hexon gene. All F40 Brazilian samples clustered together, diverging from the reference Dungan strain with mutations resulting in amino acid changes in positions G476A [arginine (R) to glutamine (Q)], A914G

[lysine (K) to arginine (R)], and G946A [alanine (A) to threonine (T)]. Three of these F40 strains had an additional mutation at G502A [aspartic acid (D) to asparagine (N)].

In contrast, for the F41 Brazilian samples, the partial shaft region of the fiber gene was more conserved compared to the HVR1-HVR6 region of the hexon gene. Despite having fewer mutations, F41 strains were divided into two major F-GTCs. Compared to the reference TAK strain, the majority of the mutations resulted in synonymous substitutions. Only two non-synonymous mutations were detected. The first was found in all F41 Brazilian strains from both subclusters (G596A), which changed the amino acid from serine (S) to asparagine (N). The other non-synonymous mutation in the F41 fiber gene was detected only in samples from F-GTC2.2 (G748T), resulting in the amino acid change from valine (V) to phenyl-alanine (F).

4. Discussion

AGE caused by enteric HAdV imposes a significant and often underestimated burden on pediatric diarrheal disease in LMICs [3,12,29]. The lack of standardized surveillance methodology, including quantitative molecular detection methods specific to HAdV-F40/41, impairs a more accurate characterization of enteric HAdV and its impact on diarrheal diseases. Our study provides a comparative analysis of the analytical performance of two qPCR assays used for the detection of general and enteric-specific HAdV in stool samples and adds novel information regarding the genetic diversity of HAdV-F40/41 strains detected from AGE cases in Brazil.

By employing a qPCR assay specifically targeting HAdV-F, we confirmed its sensitivity through the detection of previously sequenced HAdV-F stool samples. Moreover, the new assay identified three samples previously classified as species C. This suggests a potential co-infection of distinct HAdV species within the same patient. All three samples belonged to patients under two years of age, with one patient additionally showing concurrent detection of rotavirus.

In our previous study, we also observed co-detections among different enteric viruses. Specifically, HAdV was identified in 30% of samples previously confirmed positive for either norovirus or rotavirus [15]. Our research team also reported detection rates of 32.1% for norovirus [30] and 12% for rotavirus [31] in studies conducted within the same regions of Brazil. These findings highlight the substantial circulation of these major enteric viruses in the country, particularly among children under the age of two. As expected, the likelihood of co-detections increases with the number of pathogens screened. Along the same line, a reanalysis of the Global Enteric Multicenter Study (GEMS) revealed the presence of two or more diarrhea-associated pathogens in 38.9% of samples [29].

Among HAdV non-enteric types, species A, B, C, and D are frequently detected in stool samples worldwide [13,14,32–34]. While non-enteric HAdV species are frequently detected, their precise role in cases of AGE remains uncertain [12]. Nevertheless, some studies have reported the detection of non-enteric HAdV as the sole agent in infants with AGE. For instance, in an AGE outbreak in Brazil, HAdV-A12 was the only enteric virus detected among several others screened, with high viral loads detected in five out of nine stool samples [35]. A case-control study conducted in China suggested a strong correlation between HAdV-B3 and diarrhea in children [36]. In Italy, a study involving hospitalized children with symptoms of AGE detected only non-enteric HAdV-C1, -C2, and -B3. Among the HAdV-positive samples, non-enteric types were detected in over 75% of patients as the sole enteric pathogen [37]. These studies emphasize the significance of the monitoring and characterization of non-enteric types, especially during AGE outbreaks.

Partial sequencing of the enteric HAdV capsid proteins might also be an effective method for phylogenetic analyses and monitoring new emerging lineages when whole genome sequencing is not available. Different species present different mechanisms of evolution. For instance, the emergence of new types in HAdV species B, C, and D is associated with homologous recombination within species types [38–40]. Differently, for species F there is no evidence of recombination between types F40 and F41, and its molecular

evolution appears to be driven by the accumulation of point mutations in various genome regions within each type. Recent studies analyzing the whole genome of HAdV-F41 have shown that the hexon and fiber (both short and long) genes, along with the E3 and E4 regions, are the primary hotspots for mutations [41–43]. Recent phylogenetic studies of type F41 identified three different lineages or genetic clusters [41]. To better elucidate the phylogeny and genetic diversity of the circulating enteric-HAdV in Brazil, we sequenced the HVR1-HVR6 of the hexon gene and the partial shaft region of the fiber gene from HAdV-F positive samples collected between 2018 and 2020. We also compared the F41 Brazilian strains with the F41 sequences obtained before and during the severe hepatitis cases.

In line with results obtained in eastern India [44], our analysis demonstrated that Brazilian HAdV-F40 strains were less predominant and more genetically conserved than HAdV-F41 strains. The F41 Brazilian strains were divided into two main lineages or clusters based on phylogenetic analysis of the partially sequenced hexon and long fiber genes, with the majority of strains circulating in the country clustering with GTC2. Other countries have reported co-circulation of various F41 strains, with a predominance of lineage 2 [41,44]. The higher detection rates of F41 compared to F40 may be related to its greater genetic diversity, a trend reported in studies across Brazil and several other countries [13,14,34,36] which can confer a genetic advantage. The phylogenetic analyses of the hexon and fiber genes from both F40 and F41 reveal that the Brazilian strains circulating in the country have high genome similarity to those circulating globally, with no evidence of a new emergent HAdV-F variant.

In late 2021 and early 2022, the emergence of acute hepatitis cases in children was reported in several countries worldwide. These cases were initially linked to high detection rates of HAdV-F41, with no classical acute hepatitis viruses detected [45,46]. At first, this observation suggested the circulation of a potentially virulent new strain of HAdV-F41. In our study, we detected a few mutations in the partially sequenced hexon and long fiber genes of type F41 strains circulating before the cases of severe hepatitis. Additionally, several F41 strains identified in Brazil over two years prior to the onset of severe hepatitis cases exhibit high nucleotide identity with recently sequenced F41 strains detected in samples from affected children. Our findings align with studies from the UK, Germany, and Kenya that analyzed the genomic diversity of circulating F41 strains before and during the emergence of severe hepatitis cases. The majority of the strains belonged to lineage 2, implying that there may be additional factors involved in the emergence of these cases [41,43]. More recently, a study conducted in the UK also reported that F41 lineages detected pre- and post-COVID were already circulating in the UK [42]. All these studies detected a third lineage, showing a significant divergence in the short fiber, suggesting a new recombinant that has yet to be detected in Brazil.

Recent findings have indicated that the co-infection of adeno-associated virus 2 (AAV2) with other pathogens, including HAdV-F41, may lead to liver disease [47,48]. A wastewater-based epidemiological study from Ireland also determined a correlation between the burden of severe hepatitis cases and daily viral load of HAdV and AAV2 in wastewater, indicating community circulation of these pathogens may be associated with the outbreak [49].

Our study has limitations. Firstly, we were unable to perform qPCR-F testing on all HAdV-positive samples, nor could we sequence all positive samples of types F40 and F41. This could lead to the misinterpretation of the main clusters circulating in the country and potentially miss key information on the diversity of HAdV-F. Additionally, our evolution analysis of the species F was based on partial segments of the hexon and long fiber genes rather than the complete genome. Other genes, such as the short fiber gene and the E3 and E4 regions, are known hotspots for mutations and their analysis could provide important new data regarding HAdV evolution [41–43].

5. Conclusions

In the current study, we explored the genetic diversity of HAdV-F40/41 strains by partial amplification of the hypervariable regions of the hexon and long fiber genes from

patients with AGE. The phylogenetic analysis revealed that the majority of Brazilian F41 strains clustered within the GCT2 lineage. Conversely, F40 strains were more conserved in both analyzed genes. Our study provides new insights into the genetic diversity of HAdV-F species in Brazil. Additionally, we demonstrated the usefulness of both enteric and non-enteric HAdV detection qPCR assays, which can significantly enhance HAdV surveillance, especially in cases where sequencing is not feasible. These assays should be considered for the implementation of optimized molecular diagnostic assays in viral AGE monitoring programs. Finally, our study offers comprehensive data on the genetic diversity and evolution of HAdV-F strains, thereby supporting future vaccine development efforts.

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Data Availability Statement: The datasets generated and analyzed during the current study are available in the GenBank repository under accession numbers OQ442226–OQ442299. This study is registered in the Brazilian National System for Genetic Heritage and Associated Traditional Knowledge Management (SisGen, No. A837EB6).

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