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Viral genomic analysis of human adenovirus F (HAdV-F) and putative novel human adenovirus C (HAdV-C) recombinant through next-generation sequencing and bioinformatics

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FOLHA DE APROVAÇÃO

Dedication

I dedicate this work to my family and to all children that have been affected by gastroenteritis diseases around the world.

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I want to thank everyone that has contributed to this unforgettable journey of deep curiosity, creativity, learning, many flaws, and thrilling successes that basically is graduate school and science in general. I am extremely grateful to all of you for your support and dedication!

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One thing I have learned in a long life: that all our science, measured against reality, is primitive and childlike -- and yet it is the most precious thing we have.

Albert Einstein (1879-1955)

LIST OF ILLUSTRATIONS

LIST OF TABLES

LIST OF ABBREVIATIONS

AdVs	Adenoviruses
RVAs	Rotaviruses
NoVs	Noroviruses
HAstVs	Astroviruses
ICTV	International Committee on Taxonomy of Viruses
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
pTP	Precursor Terminal Protein
pol	DNA polymerase
RNA	Ribonucleic Acid
SAdV	Simian Adenovirus
HAdV	Human Adenovirus
CAR	The Coxsackievirus and Adenovirus Receptor
DNA	Deoxyribonucleic Acid
mRNA	Messenger RNA
TP	Terminal Protein
PeVs	Parechoviruses
AGE	Acute Gastroenteritis
dsRNA	Double-stranded RNA Viruses
ssRNA	Single-stranded RNA Viruses
NGS	Next-Generation Sequencing
PKR	RNase L-dependent Protein Kinase
VA-RNA	Viral Associated RNA
INFs	Interferons
PRB	Retinoblastoma Protein
TNF-α	Tumor Necrosis Factor
DBP	DNA Binding Protein
RID	Receptor Internalization Degradation
ADP	Adenovirus Death Protein
TRAIL	Tumor Necrosis Factor – Related Apoptosis-Inducing Ligand
MLP	Major Late Promotor
DNA-PK	DNA Dependent Protein Kinase
ARI	Acute Respiratory Infections
SDS	Sodium Dodecyl Sulfate
RGD	Tripeptide Arg-Gly-Asp
SUS	Sistema Único de Saúde (Brazilian Unified Health System)

CTNBIO	Brazilian National Technical Biosafety Commission
ELISA	Enzyme-Linked Immunosorbent Assay

ABSTRACT

Acute gastroenteritis in children remains a principal cause of hospitalization and an important public health problem in Brazil. Usually, it is caused by viral agents including rotaviruses (RVA), human enteric adenoviruses (HAdV) and noroviruses (NoV). Serotypes 40 and 41 (HAdV-F40/41) are commonly associated with gastrointestinal infection and are the only members of F species. There are few available data about HAdV-F40/41 regarding its incidence and molecular characterization. Brazil is a country with continental dimensions where continuous multiregional surveillance is vital to establish a more complete picture of the epidemiology of HAdV-F. The aim of the current study was to investigate the genetic diversity and frequency of HAdV-F in rural and low-income urban areas in northern Brazil using sequencing techniques in order to obtain full-genome data. Phylogenetic analysis was also carried out with the aim to obtain more information on the genetic comparison between Brazilian and global HAdV-F strains. A total of 251 stool samples collected between 2010 and 2016 from patients with acute gastroenteritis were screened for HAdV-F using next-generation sequencing techniques. HAdV-F infection was detected in 57.8 % (145/251) of samples. A total of 137 positive samples belonged to HAdV-F41 and 7 to HAdV-F40. HAdV-F40/41 dual infection was found in one sample. Single HAdV-F infections were detected in 21.9 % (55/251) of samples and mixed infections in 37.4 % (94/251), with RVA/HAdV-F being the most frequent association (21.5 %; 54/251). Genetic analysis indicated that the HAdV-F strains circulating in Brazil were closely related to worldwide strains. Surveillance of enteric viruses by NGS proposed in this study also allowed the identification and genomic characterization of a new recombinant strain of HAdV-C (HAdV-C BR-211). Recombination analysis revealed that strain HAdV-C BR-211 is a chimera in which the variable regions of Hexon gene combined HAdV-C1 and HAdV-C89 sequences. Therefore, HAdV-C BR-211strain possesses a genomic backbone of type HAdV-C89 and a unique insertion of HAdV-C1 in the Hexon sequence. In addition to the recombinant strain HAdV-C BR-211, the present investigation also described the full-length genome of a HAdV-C strain type 1 (HAdV-C BR-245). This was the first large-scale HAdV-F study in Brazil in which whole-genome data and DNA sequence analyses were used to characterize HAdV-F strains. Expanding the viral genome database could improve overall genotyping success and assist the National Center for Biotechnology Information (NCBI)/GenBank in standardizing the HAdV genome records by providing a large set of annotated HAdV-F genomes.

Keywords: Bioengineering. Human Adenoviruses. HAdV-F. HAdV-C. Metagenomic. Next-generation Sequencing. Bioinformatics.

RESUMO

A gastroenterite aguda em crianças ainda é uma das causas mais comuns de hospitalização e importante problema de saúde pública no Brasil. Geralmente, é causada por agentes virais, incluindo rotavírus (RVA), adenovírus humano entérico (HAdV) e norovírus (NoV). Os sorotipos 40 e 41, únicos membros da espécie F (HAdV-F 40/41), são os mais comumente associados à infecção gastrointestinal. Existem poucos dados disponíveis do HAdV-F 40/41 em relação a sua incidência e caracterização molecular. Brasil é um país com dimensões continentais e, portanto, espera-se encontrar uma diversidade genética viral elevada no país. Uma vigilância multirregional contínua é vital para estabelecer um quadro epidemiológico-molecular mais completo sobre o HAdV-F40/41 no Brasil. O objetivo do presente estudo foi investigar a frequência e diversidade genética de cepas de HAdV-F 40/41 em áreas rurais e urbanas de baixa renda no norte do Brasil usando técnicas de sequenciamento de nova geração (NGS), com foco na aquisição de sequências genômicas completas. Também foi realizada análise filogenética visando obter mais informações sobre a relação genética entre cepas brasileiras e mundiais de HAdV-F 40/41. Um total de 251 amostras de fezes coletadas de 2010 a 2016 nos estados do Pará e Tocantins foram rastreadas para o HAdV-F usando metagenômica. A infecção pelo HAdV-F foi detectada em 57,8% (145/251) das amostras. Um total de 137 amostras positivas pertenciam ao HAdV-F41 e 7 amostras ao HAdV-F40. Foi encontrada uma infecção dupla de HAdV-F40/41 em uma amostra. Foram detectadas infecções únicas pelo HAdV-F em 21,9% (55/251) das amostras e infecções mistas em 37,4% (94/251), sendo o RVA/HAdV-F a associação mais frequente (21,5%; 54/251). A análise molecular indicou que as cepas brasileiras de HAdV-F são geneticamente relacionadas às demais cepas de HAdV-F circulando mundialmente. A vigilância de vírus entéricos por NGS proposta no presente estudo também permitiu a identificação e caracterização genômica de uma nova cepa recombinante de HAdV-C (HAdV-C BR-211). A análise de recombinação revelou que a cepa HAdV-C BR- 211 é uma quimera na qual as regiões variáveis do gene Hexon combinam sequências de HAdV-C1 e HAdV-C89. Portanto, a cepa HAdV-C BR-211 possui um esqueleto genômico do tipo HAdV-C89 e uma inserção única de HAdV-C1 na sequência do gene Hexon. Além da cepa recombinante HAdV-C BR-211, a presente investigação também descreveu o genoma completo de uma cepa de HAdV-C tipo 1 (HAdV-C BR-245). Este é o primeiro estudo de HAdV-F utilizando sequenciamento de larga escala realizado no Brasil, onde os dados de genoma completo e análises de sequências foram usados para caracterizar as cepas de HAdV-F 40/41. A expansão do banco de dados de genoma viral pode contribuir significativamente para o melhoramento do sucesso da genotipagem de HAdV-F. Ainda, o aumento no conjunto de genomas anotados de HAdV-F poderá auxiliar o NCBI/GenBank a padronizar os registros de genoma dos HAdV.

Palavras-chave: Bioengenharia. Adenovírus humano. HAdV-F. HAdV-C. Metagenômica. Sequenciamento de Nova Geração. Bioinformática.

Table of Contents

1. INTRODUCTION

Acute Gastroenteritis (AGE) is a major cause of morbidity and mortality in children, responsible for an estimated 1.8 million deaths in children younger than 5 years in developing countries in 2004 (Fischer Walker et al., 2012). These countries are affected due to lack of adequate sanitation, hygiene, nutrition and access to healthcare, related to social inequality and poverty (Ferreita & Latorre 2012). In Brazil, in 2017, diarrheal disease caused an estimated (Network, 2018). Diarrheal disease also poses an enormous economic burden to already under-stress healthcare systems in low-income settings that lack appropriate public health and educational infrastructure (Krishnan, 2014). With such high incidence, diarrheal disease should be a high priority target for the healthcare industry. The main issue with reducing the burden of diarrheal disease is that many cases are of unknown etiology (Fontoura et al., 2018).

From what we do know, viruses cause approximately 75% of cases of diarrheal disease (Sidoti et al., 2015). Although a variety of pathogens, including viruses, bacteria and parasites are associated with AGE, viruses are the major etiologic agent of diarrhea in children. Six different viruses are associated with nearly all cases of viral gastroenteritis: rotaviruses group A (RVAs), human adenoviruses (HAdVs), noroviruses (NoVs), human astroviruses (HAstVs), human sapoviruses (HSaVs) and, more recently, parechoviruses (PeVs). (Lima et al., 2019; Crom et al., 2016; Amaral et al., 2015). RVAs and NoVs are known to be the most prominent causes of viral diarrea. Rotavirus, a dsRNA virus of the *Reoviridae* family, is the most common virus responsible for diarrheal disease globally (Esona & Gautam, 2015). About 40% of cases of diarrheal disease in Brazil resulting in hospitalization are caused by Rotavirus alone (Fontoura et al., 2018). This is because Rotavirus infection is frequently severe, making it one of the most dangerous of the gastroenteritis viruses (Esona & Gautam, 2015). Two safe and effective RVA vaccines (Rotarix, GlaxoSmithKline Biologicals, Belgium; and RotaTeq, Merck, Inc., USA) were licensed in 2006 and a significant decrease in the RVA-induced diarrheal disease burden has been observed by offering protection against Rotavirus infection as a whole, but mostly offering protection against severe Rotavirus illness (Carmo et al., 2011; Carvalho-Costa et al., 2019). A national immunization campaign in 2006 eliminated a large amount of Rotavirus, and children still regularly get vaccinated (Gurgel et al., 2008). This has given other viruses responsible for gastroenteritis an

opportunity to become more prevalent. Consequently, it has been speculated that a global emergence of other enteric viruses associated with the introduction of the RVA vaccine, including human enteric adenovirus species F (HAdV-F), has occurred.

Noroviruses and Sapoviruses, whose type species are Norwalk and Sapporo Virus respectively, belong to the *Caliciviridae* family, and are non-enveloped positive-sense RNA viruses (Robilotti et al., 2015; Tomoichiro et al., 2015).

Norwalk Virus is a common contaminant in food like shellfish and unwashed vegetables, and is the third most common gastroenteritis virus, whereas Sapporo Virus is rarer. Both cause moderate diarrhea with vomiting but only rarely fever.

Parechovirus is a member of the *Picornaviridae* family, non-enveloped positive-sense ssRNA viruses (Crom et al., 2016). Human Parechovirus type 1 causes gastroenteritis, but other types are responsible for different diseases. Human Parechovirus infection is the rarest of the six viruses commonly associated with gastroenteritis.

Astrovirus, the last of the six viruses commonly associated with diarrheal disease, is often overlooked (Siqueira et al., 2017). Astroviruses, of the family *Astroviridae*, non-enveloped positive-sense ssRNA viruses, often don't infect adults, and if they do, the infection is usually asymptomatic. However, Astrovirus is considerably dangerous in children, and found quite commonly, more so than Sapporo Virus and Human Parechovirus. In immunocompromised children, they can cause severe disease. They are highly diverse, and there is now increasing evidence of a high zoonotic potential. Nevertheless, research in viral gastroenteritis mostly focuses on Rotavirus, Adenovirus and Norovirus, with research on Astrovirus is lacking.

HAdV-F (genotype 40/41), the main study object of this work, is the second most prevalent etiologic agent of community-acquired pediatric AGE after commuity-acquired paediatric AGE after rotavirus, resulting in 2.8 – 11.8% infant diarrheal cases worldwide (Banerjee et al., 2017; Chandra et al., 2021; Uhnoo et al., 1984). These non-enveloped viruses encapsulate linear double-stranded DNA genomes that range in size from 26 to 45 kbp. The HAdV-F virion is icosahedral in shape, made up of a 252-capsomer protein capsid, from which 240 hexon trimers represent fundamental building blocks of the capsid shell while 12 penton-base pentamers, which each connect to a

fiber protein, are the second-most abundant subunit among the capsid proteins (Benkö et al., 2005; Fields et al., 2007 ; Robinson et al., 2013).

HAdVs belong to the *Adenoviridae* family, which is divided into 6 genera: Aviadenovirus, Mastadenovirus, Atadenovirus, Sidadenovirus and Ichtadenovirus. Mastadenoviruses infect a broad range of mammalian hosts, including humans, non-human primates, bats, bovines, canines, deer, dolphins, equines, murines, ovines, swine, sea lions, skunks, squirrels and tree shrews (Borkenhagen et al., 2019). HAdVs have been classified within the genus Mastadenoviruses with over100 serologically distinct types isolated. These have been characterized further and subdivided into seven distinct human species or subgenera (human mastadenovirus A-G), 1 simian specie, A (SAdVA), and 5 candidate simian species (SadVB-G). Recombination in the capsid protein-coding region undergo the antigenic shift and potentially contributes to evolution of different genotypes around the globe (Dhingra et al., 2019; Guo-Hong Huang, 2013; Nkogue et al., 2016; Raboni et al., 2014; Walsh et al., 2010). HAdVs can cause severe disease and infects multiple human systems (respiratory, ocular, intestinal, neurological and urinary) thereby symptomatically extending much further than diarrhea, vomiting and fever. The specific system HAdV infects depends on the serotype or genotype of that virus (Lion, 2014; Lynch et al., 2011). Although adenoviruses have the potential to cause severe disease, the majority of infections are mild. Their broad tissue tropism and relative ease at inhibiting replication at a molecular level enables their usefulness as adenoviral vectors or adenovirus-based vaccines. Their use is studied and employed to prevent infection from COVID-19, Middle East Respiratory Syndrome, Ebola, AIDS, Lassa fever, and Zika Virus disease (Garofalo et al., 2020).

Rowe and colleagues first recognized adenoviruses (AdVs) in 1950s during the maintenance phase of primary cell culture isolated from children after surgical removal of their tonsils and adenoids as they noticed cellular degeneration in presence of an unknown virus (Rowe et al., 1953). In 1954, Hilleman and Werne, in a study involving American recruits with respiratory infections, isolated an agent capable of inducing cytopathic effects in human cell cultures. These viruses were later listed as agents of adenoid degeneration, respiratory infection and pharyngoconjunctival fever, or acute respiratory disease (Hllleman & Werner, 1954). These agents were named adenoviruses due to the source, in which they were discovered (Enders et al., 1956).

In Brazil, there is a particular lack of information on the epidemiology of gastroenteritis, despite such a high incidence, and a lack of information on the etiology of the disease (Fontoura et al., 2018). Cases of diarrheal disease are often short-lived, so many patients seldom seek medical attention. For cases that are more chronic, antibiotics are given without diagnosis of the etiological agent. Given that the majority of gastroenteritis infections are of viral origin, the inappropriate use of antibiotics contribute to the ever-increasing global problem of antimicrobial resistance. When diagnostics are performed, typically this will include microscopic evaluation of stool for ova, cysts and parasites. Coproscopy may help to identify helminth eggs coproculture to identify bacteria for example. Rotavirus infection, typically via a rapid diagnostic test, is also investigated (Esona & Gautam, 2015). Treatment is limited for viral gastroenteritis other than symptom relief and intravenous fluids if the patient becomes severely dehydrated. This means that clinics and hospitals seldom aim to identify the etiology of viral gastroenteritis, as it is unlikely to help improve patient outcome. With patients not attending clinics to receive treatment, and with viral gastroenteritis of unknown etiology, the true prevalence and incidence of diarrheal disease is difficult to estimate (Fontoura et al., 2018). This is especially the case in more remote parts of Brazil, like the north or center of Brazil, where jungle and other rural terrain impedes travel and where funding for healthcare is lesser than in other parts of the country.

HAdVs are transmitted by multiple mechanisms including droplets, fomites, the fecal–oral route and autoinoculation. The infected host can develop a number of pulmonary complications including upper respiratory impairment, bronchiolitis and pneumonia, without significant seasonal variation (Lion, 2014). The virus also causes extrapulmonary pathologies including conjunctivitis, gastroenteritis and meningitis (Filho et al., 2007; Pratte-Santos et al., 2019). HAdV tropism is dependent on the serotype or genotype of that virus (Lion, 2014).

Serotypes 40 and 41 are commonly associated with gastrointestinal infection and are the two sole members of HAdV-F to date (Lima et al., 2019; Morris et al., 1975). HAdV-F40 and HAdV-F41 were first identified in stool samples in patients with diarrhea in 1975 (Morris et al., 1975). HAdV-F40/41 are distributed globally, with a frequency of detection ranging from 1.1–14% in children, depending on the geographical area (Giordano et al., 2001; Raboni et al., 2014; Ramani & Kang, 2009; Tran et al., 2010).

In low-income countries, the prevalence of HAdV-F40/41 ranges from 4.3–32% (Arashkia et al., 2019; Banerjee et al., 2017; Cruz et al., 1990; Gelaw et al., 2019). Similarly, additional HAdV species, such as HAdV-A (types 12, 18 and 31), HAdV-C (types 1, 2 and 5) and HAdV-D (types 28, 29, 30, 32, 37 and 43–46) and G (type 52), have also been implicated, although less frequently, with diarrhea (Kim et al., 2017; Li et al., 2005; Moyo et al., 2014; Primo et al., 2018). At present, there is no vaccine candidate for enteric HAdVs, making their development evenmore crucial for prevention, with great market prospect.

Next-Generation Sequencing (NGS), is a sequencing technique capable of identifying all the viruses present in a small sample, thus providing a unique solution to fill the void of information in viral gastroenteritis etiology across Brazil, and beyond (Levy & Myers, 2016). Multiple samples can be collected at once, given individual tags unique to their sample, and then sequenced together in one batch. This makes the procedure of NGS easy and straightforward. NGS is very sensitive, with similar sensitivity as PCR, capable of identifying viruses at a subclinical level. However, NGS isn't targeted, not requiring any specific primers to start the reaction, and yet still provides sequence information. Almost 30 HAdV-F complete genome sequences are available in the GenBank database, and yet no HAdV-F full-genome sequences have been reported in Brazil. Full-genome sequences, very few of which have been reported for any Adenovirus in Brazil, would provide useful information for understanding molecular epidemiology and tracing the spatiotemporal movement of viruses (Primo et al., 2018).

More accurate knowledge of molecular epidemiology, prevalence and diversity of enteric viruses, such as HAdV-F in Brazil are important to be able to put precautionary and control strategies in place (Barrella et al., 2009). It is likely that we will see an increase in the frequency of HAdV-F infections in the coming years, after the introduction of Rotarix vaccine in the Brazilian National Immunization Program. It is therefore necessary for us to enhance the acuity of epidemiological and laboratory-based surveillance for this specific virus since current data are too limited to elucidate their epidemiological role in infectious diarrhea.

In this retrospective study, we describe the molecular epidemiology of HAdV-F in northern Brazil between 2010 and 2016. We investigated the frequency of HAdV-F

infections in patients with gastroenteritis and molecular typing of positive samples throughout this period. In addition, this study assessed the genetic diversity of HAdV-F infections using full-genome phylogenetic analysis.

2. OBJECTIVES

The main objective of this retrospective study was to investigate the genetic diversity and frequency of human adenovirus F (HAdV-F) infections in northern Brazil between 2010 and 2016 through Next-Generation Sequencing technique and bioinformatics examination.

Furthermore, this study aimed to obtain crucial epidemic information as seasonality, location and point of origin of HAdV-F contagions through phylogenetic analysis.

Finally, the project intended to obtain full-length genomic sequences of HAdVs, not yet before described in Brazil.

3. BACKGROUND AND SIGNIFICANCE

3.1 VIROLOGY OF ADENOVIRUS

3.1.1 Classification

AdVs are common pathogens that have been detected in almost all types of vertebrates capable of replication in target host cells (Harrach, et al., 2019). Advs belong to the Adenoviridae family, of which there are 87 different known adenovirus species, subdivided into six genera, approved by the International Committee for the Taxonomy of Viruses (ICTV) (https://talk.ictvonline.org/ictv-reports/ictv_9th_report/dsdna-viruses-2011/w/dsdna_viruses/93/adenoviridae, accessed on 23 February 2022): Mastadenovirus, which includes HAdVs together with other Advs infecting mammalian species; *Aviadenovirus*, consists of Advs of avian host origin (Harrach et al., 2019; Harrach & Benkő, 2021); *Siadenovirus*, includes viruses that infect birds, amphibians and fish; *Atadenovirus*, have been reported from a wide variety of animals, including ruminants, birds, marsupials and reptiles (Order *Squamata*) and *Ichtadenovirus*, the only noted adenovirus with a single member from a fish which infects white sturgeon (Harrach et al., 2019) and the latest addition is *Testadenovirus* which has been designated for Advs discovered in testudinoid turtles (Harrach et al., 2019; Lefkowitz et al., 2018).

Adenoviruses of each of these genera differ from each other by several morphological and genetic characteristics. *Aviadenoviruses*, for example, present a viral particle with DNA of molecular weight varying between 28 and 30 Md, percentage in guanine and cytosine bases (G+C) varying between 51 and 57% and two fibers per vertex, which gives this virus the ability to recognize two distinct receptors on host cells. Adenoviruses of the Mastadenovirus genus have only one fiber per vertex, whose length varies between 10 and 30 nm. DNA has a molecular weight of 20 to 25 Md, with a percentage of G+C between 44 and 61%. Unlike other Mastadenoviruses, human adenovirus serotypes 40 and 41 have two fibers of different lengths, encoded by two distinct genes, but distributed one per vertex (Kidd et al., 1990; Pieniazek et al., 1990). One of the two fibers, called the long fiber, was likely acquired by recombination with DNA from adenoviruses that infect monkeys (Kidd et al., 1990). Figure 1 shows the phylogenetic distance tree of members of the *Adenoviridae* family (Harrach, 2014).

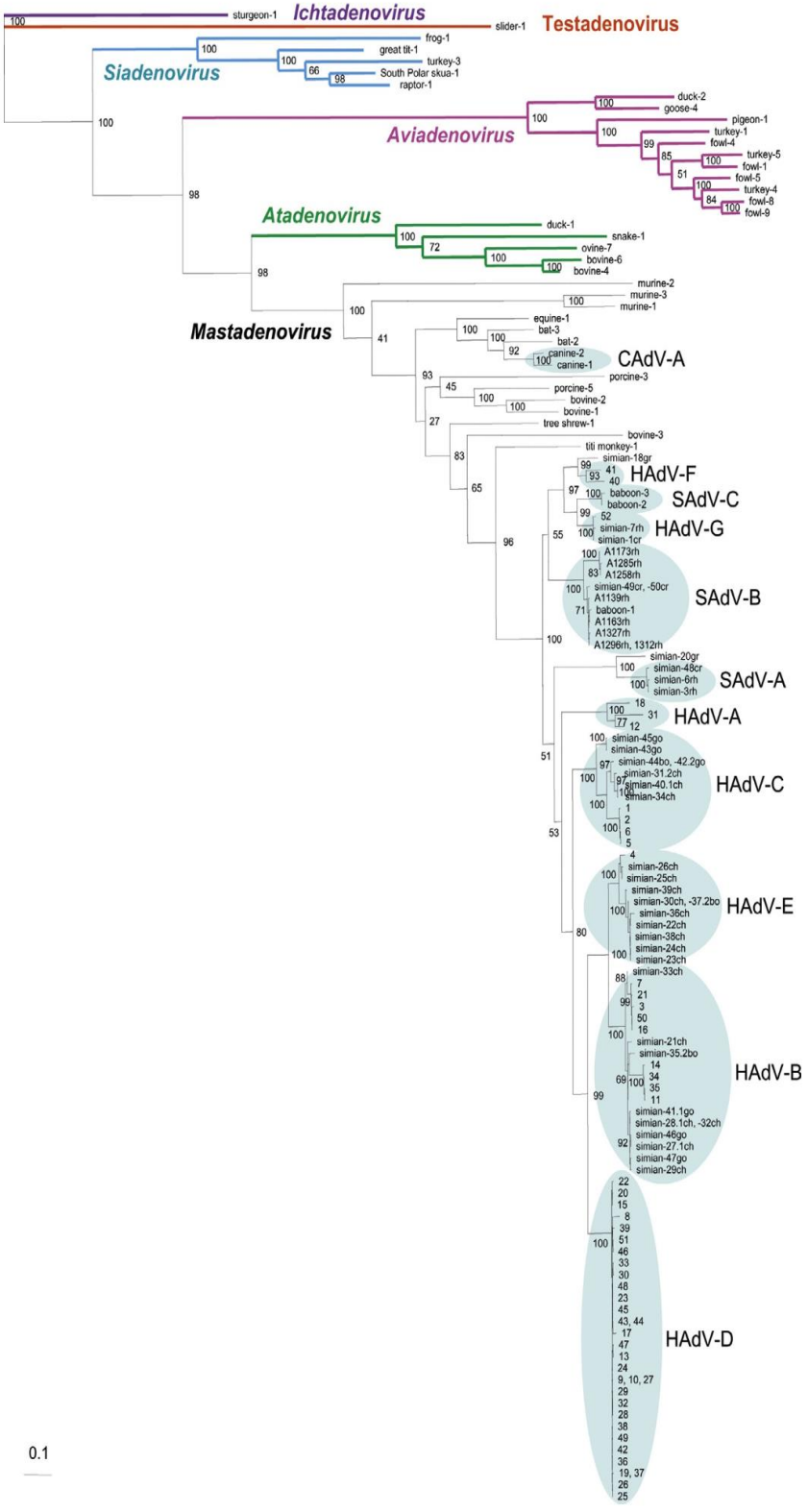


Figure 1: Phylogenetic tree reconstruction of adenoviruses obtained by maximum likelihood analysis using available DNA-dependent DNA polymerase amino acid sequences.
Font: Harrach (2014).

Human adenoviruses belong to the Mastadenovirus genus. These viruses are capable of infecting a wide range of tissues. As such, they are etiologic agents of several

pathologies, such as: respiratory syndromes, keratoconjunctivitis, enteric and renal infections. Advs classification is according to antigenic, morphological and molecular characteristics (Tiemessen & Kidd, 1995; Wadell, 1984).

The first classification, was based on the agglutination of erythrocytes from rats and from rhesus monkey (Rosen, 1960). Cross-reactions in the inhibition of hemagglutination were verified in some serotypes and intermediate lines (Adrian et al., 1985; Wigand, et al. 1987).

The distribution of adenoviruses into groups was suggested based on pathogenicity and oncogenicity in newborn hamsters. The current species A comprises the serotypes that have a high transforming capacity; species B comprises serotypes with moderate oncogenicity; the other species group together the adenoviruses considered non-oncogenic (Huebner et al., 1965).

The classification of adenoviruses based on the molecular weight of the structural polypeptides was proposed in 1979. A variation in the molecular weight of proteins II, III and IV was observed between each serotype (Wadell, 1979).

Taking into account the difference in the nucleotide sequence of the viral genome and knowing that the concentrations of G+C in the DNA of adenoviruses of different species is variable, in 1980 scientists analyzed the genome restriction patterns of several adenovirus serotypes after digestion with the enzyme Sma I. This enzyme has as its restriction site the sequence 5'CCC GGG. In this study, the authors observed that the number of fragments obtained was characteristic for each species and that members of the same species had several co-migrating fragments (Wadell et al., 1980).

Serotype distinction is made after carrying out tests to neutralize viral infection in permissive cell culture. Viruses that do not cross-react with other serotypes or that have neutralizing titers greater than 16 are considered to be distinct serotypes. In cases where cross-reaction occurs, hemagglutination and DNA restriction tests with restriction endonucleases are performed (Wigand & Adrian, 1986).

Table 1: Classification of HAdVs serotypes within species and their main characteristics.

	Species A	Species B	Species C	Species D	Species E	Species F
Serotypes	12, 18, 31	3,7,11,14,16, 21,3 4,35,50	1,2,5,6	8,10,13,15,17,19, 20,22-30,32,33,36-39,42-47,51	4	40-41
Similarity (%)[a]	48-69	89-94	99-100	94-99	4-23	62
G + C percentage	48	51	58	58	58	-
Constraint Profile with SmaI	4-5	8-10	10-12	14-18	16-19	9-12
Haemaggltinating pattern [b]	IV	I	III	II	III	IV
Oncogenicity	high	Weak	Negative	Negative	Negative	Negative
Fiber c receiver [c]	CAR	CD46, CD80 e CD86	CAR VCAM 1 Heparana Sulphate	CAR, sialic acid	CAR	CAR? C
VA-RNA gene Nº	1	2(B1) 1(B2)	2	2	2	2
ORF Nº in E3	6	9 (B1) 8 (B2)	7	8	9	5
Reasons recognized by penton-base [d]	RGD e LDV	RGD e LDV	RGD e LVD	RGD e LDV	RGD e LDV	LDV RGDA(40) IGDD (41)
Fiber Length (Repeat Patterns)	22	6 (B1) 6 (B2)	22	8	12	Length: 21-22 Short: 12
Tropism	Enteric	Renal Respiratory (B1) (B2)	Respiratory	Eyepiece	Respiratory eyecup	Enteric
Syndromes	Gastroenteritis	Acute Respiratory Persistent Kidney Infections	Acute Respiratory	Inapparent Keratoconjunctivitis	Acute Respiratory Conjunctivitis	Children's gastroenteritis

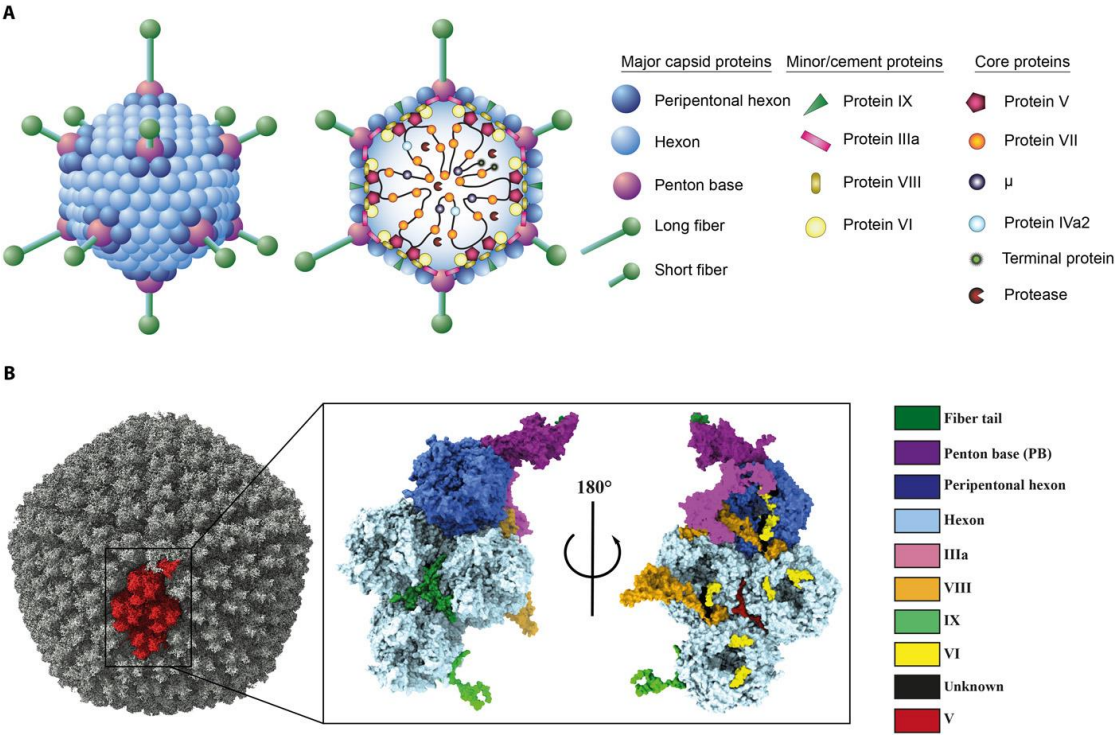
a: Homology percentage between species
b: Hemagglutination pattern: I- complete agglutination of monkey erythrocytes; II- complete agglutination of rat erythrocytes; III- partial agglutination of rat erythrocytes, IV- agglutination of rat erythrocytes after addition of heterotypic antiserum.
c: long fiber of species F binds to CAR, but short fiber has no known receptor.
d: reasons explained in penton-base through which recognition of secondary receptors, integrins, occurs.

Font: Modified from (Segerman et al., 2003)

3.1.2 Viral particle composition

Non-enveloped HAdVs with a linear double-stranded DNA are considered to have a complex known genome of ~35–kilo–base pairs packaged in a relatively large capsid of about 90 nm in diameter and molecular mass of ~150 megadaltons (Mangel &

Martín, 2014). The virion of HAdVs is composed of 13 different structural proteins. The outer capsid of icosahedral symmetry (Figure 2B) is made up of 3 major capsid proteins; 240 hexon trimers, 12 pentameric penton bases connected with a fiber situated at the capsid vertices and 4 minor capsid proteins responsible for stabilizing the hexon assemblies; IIIa, VI, VIII embedded in the inner surfaces of the capsid shell and IX in the outer surfaces (Figure 2A). The remaining 6 proteins account for the formation of inner core of nucleic acid, where double-stranded DNA bound with core proteins as shown in Figure 2. (Liu et al., 2010).



(A) Schematic representation of the capsid and core structure of HAdV-F41.
(B) Surface representation of the HAdV-F41 electron density with one asymmetric unit (ASU) highlighted in red (left) and a surface representation of the ASU of the HAdV-F41 atomic model viewed from the virion exterior (middle) and interior (right).

Figure 2: The overall structure of HAdV-F41.
Font: Rafie et al. (2021)

The IIIa, VI, VIII and IX proteins constitute the capsid and are associated with *hexon*, which is responsible for virion stabilization and flexibility in terms of its structure involving protein-protein interactions (Greber et al., 1998; Vellinga et al., 2005). The IIIa protein is located at the apex of the icosahedron, and together with the *hexon* form triangular faces, determining the shape of the virion necessary for viral

particle formation to occur. Viruses with mutations that affect IIIa are defective and form defective viral particles (Vellinga et al., 2005).

Proteins VI and VIII are associated with the inner surface of the viral capsid. Studies suggest that VI can interact directly with viral DNA, being the protein responsible for maintaining the contact between the *hexon* and the *core* (San Martin & Burnett, 2003; Stewart et al., 1993). During infection, protein VI can help the viral particle escape from the cellular endosome as it induces disruption of the endocytic membrane (Wiethoff et al., 2005). Another important function of VI is to facilitate the import of the hexon protein into the cell nucleus, where the viral particle is assembled (Matthews & Russell, 1995).

To date, protein VIII is the least known. Studies using defective mutants in this protein suggest that it may be related to the stability of the virion structure (Liu et al., 1985).

The protein of the adenovirus penton-base serotype 2 contains 471 amino acids and is complexed to fiber (582 amino acids). The adenovirus 2 fiber contains 582 aa and is divided into three domains: the tail (N-terminal portion) is attached to the base of the pentons; the stem, or central part, is connected to the globular region (C-terminal portion). Much of the fiber's amino acid sequence is not conserved, which causes antigenic variations between different adenovirus serotypes, however, the region near the N terminus is described as highly conserved between serotypes (Russel, 2009; Shenk, 2001; Tarassishin et al., 2000).

The viral core is composed of a double-stranded DNA molecule with 36Kb. Associated with the viral DNA are four proteins. TP (terminal protein), covalently linked to the 5′ end of each DNA strand, whose function is to initiate DNA replication (Shenk, 1996). The VII protein, the majority in the viral core, performs the histone function, being responsible for the compaction and organization of the genetic material inside the viral capsid (Chatterjee et al., 1986). The V protein anchors the viral DNA to the capsid apex, through interaction with the penton-base protein (San Martín & Burnett, 2003). The X protein is cleaved into the *mu* protein, which is present in mature viral particles, however, its function remains unknown (Shenk et al., 1996).

In table 2; the adenovirus proteins, the locations within the virion, as well as their respective functions, are reported.

Table 2: lists adenovirus proteins, virion locations, and their functions

Protein (polypeptide)	Virion location	Functions
II	Hexon monomer	Majority in capsid (structural)
III	Penton base	Cell Penetration
IIIa	Associated with penton base	Viral particle formation; stabilization
IV	Fiber	Primary Receiver Recognition
V	Core: associated with DNA and penton base; penton-associated peptide	Packing; Produces of stable progeny virions; Interacts with the viral DNA and other core proteins; Interacts with other viral capsid proteins; DNA encapsidation; virus assembly
VI	Hexon-associated peptide	Hexon contact with core, stabilization.
VII	Core	Cellular Histone; Nuclear transport; Prevents induction of innate immune response; Facilitates early gene transcription; Condenses AdV genomic DNA
VIII	Hexon-associated peptide	Stabilization
IX	Hexon-associated peptide	Stabilization
Mu/X	Core	Condenses AdV genome; Alters accumulation of E2 proteins, Is involved in increasing DNA transfection efficiency
TP	Genome	DNA Replication; Protects AdV DNA from nuclease activity
Protease	Core	Essential for virus maturation and production of infectious progeny virion; esential for the proper release of the incoming uncoated virion to the cytoplasm; Cleaves precursor adenoviral proteins IIIA,

		VI, VIII, Mu/X, TP and 52K/55K in virion; Cleaves 100K in the cytoplasm of transfected cells
IVa2	core	Activates AdV major late promoter; Packages adenovirus DNA; Acts as DNA packaging ATPase; involved in the insertion of the viral DNA in empty capsids

Font. Modified from (Kulanayake & Tikoo, 2021).

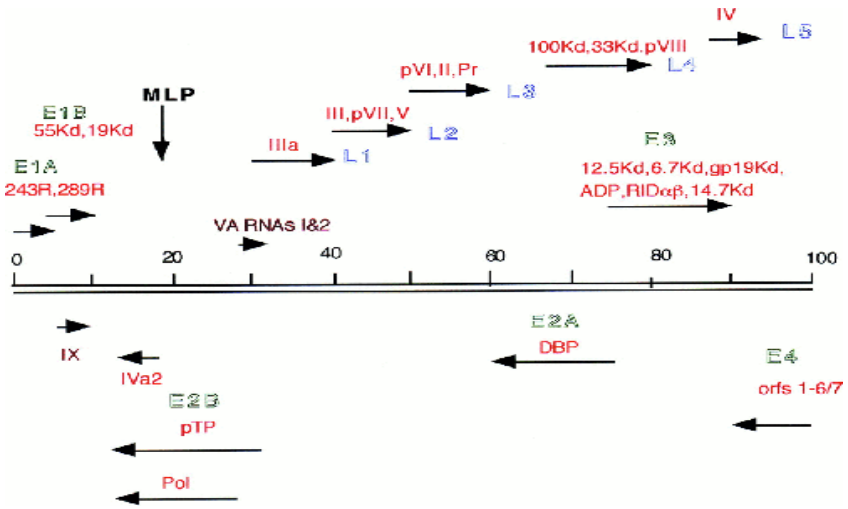
HAdV has 2 further proteins associated with mature viral particles; VIa2 and 23K protease, important in the processing of some structural proteins that enablethe process of viral particle entry into the host cell (Weber, 1976; Greber et al., 1996).

Three viral capsid proteins are most exposed to the environment: hexon, penton-base, and fiber. Together, these proteins mediate the interaction of the adenovirus with the host cellular system during the initial steps of infection, being targets of the action of neutralizing antibodies and hemagglutination inhibitors.

3.1.3 Organization of the viral genome

The HAdV genome is a single linear molecule of double-stranded DNA, which can vary in length from 26,000 in the frog adenovirus (FrAdV-1, a *Siadenovirus*) to about 48,000 base pairs is the only AdV isolate from fish which has been found so far (WSAdV-1 *ichta adenovirus*) that encodes around 40 proteins (Davison et al., 2000; Doszpoly et al., 2019). The genome comprises inverted terminal repeats (ITRs) of 30-371 bp at both ends that are covalently linked to a virus-coded TP at the 5′ ends of each viral DNA strand (Davison et al., 2003). Currently, several adenovirus serotypes representing each species are already sequenced: HAdV-2 (Roberts, et al., 1985); HAdV-5 (Chroboczek et al., 1992); HAdV-40 (Davison et al., 1993); HAdV-41 (Toogood & Hay, 1988); HAdV-12 (Sprengel et al., 1994); HAdV-17 (Chillon et al., 1999); HAdV-35 (Gao et al., 2003; Vogels et al., 2003). Based on earlier studies of these genomes, it was observed that adenoviruses present a similar genetic organization, in which two origins of replication are identified, each one present at each end of the

genome and eight transcription units, dependent on RNA polymerase II, being five units of early transcription (E1A, E1B, E2, E3, E4), two intermediate transcription units (pIX, pIVa2) and the main late transcription unit (MLTU), which generates 5 families of mRNAs (L1 to L5) (Shenk, 1996) (Figure 3). The viral chromosome also has, depending on the species, one or two transcriptional regions of small double-stranded RNA (VA-RNA) transcribed by RNA polymerase III (Kidd et al., 1995; Shenk, 1996) (Figure 3).



OBS.: Initial transcripts are marked in gray (E1A, E1B, E2A, E2B, E3 and E4); and the late ones, in blue (L1-L5). Arrows indicate the direction of transcription. VA-RNAs are marked in brown. MLP: Major late promoter.

Figure 3: Transcription map of the HAdV genome.
Font: Russel (2000).

The two strands of DNA are transcribed: on the strand in which transcription occurs from right to left, transcripts E1A, E1B, IX, MLTU, VA-RNA and E3 result; and in the one where transcription occurs from left to right, transcripts E4, E2 and IVa2 result (Shenk, 1996).

The expression of the different units is synchronized, and can be divided into early, intermediate and late expression phases. The early phase is characterized by the expression of genes that modulate cellular functions, facilitating DNA replication and transcription of late genes. The estimated time for this process to take place is 6 to 8 hours in permissive cells, while the late phase lasts 4 to 6 hours (Russell, 2000).

3.1.4 HAdV Early Expression Regions

Five early transcriptional units are described in the literature, which are located on the two strands of viral genome DNA: E1A, E1B, E2, E3 and E4. The organization and temporal expression of these genes prevents the formation of complementary mRNAs, which could result in the formation of double-stranded RNAs (dsRNA). DsRNAs are recognized by dsRNA and RNase L-dependent protein kinase (PKR), which activate the IFN-induced defense system (Kitajewski et al., 1986; Katze et al., 1987).

Expression of early adenovirus genes begins with transcription of the E1A gene, the product of which stimulates the transcription of other early expression genes, such as the E1B gene and VA-RNA (Berk et al., 1979; Jones & Shenk, 1979).

More than 20 proteins are encoded by early regions, which perform various functions such as cell cycle regulation and viral transcription regulation, favoring an ideal environment for viral gene expression and viral progeny formation.

Next, the functions of early, intermediate and late adenovirus genes will be described.

3.1.5 The E1A gene

The early gene region 1A (E1A) of HAdVs is the first transcription unit to be expressed upon cell infection by tethering of viral genomes to host cell chromosomes. The E1A region encodes for two major proteins, E1A-289R and E1A-243R that expressed immediately after infection. The two of the E1A proteins play a central role in both the productive adenoviral infection and the cellular transformation (Russell, 2000; Shenk, 1996).

These proteins stimulate viral transcription (called transactivator) through TATA box, which are typically located 25-30 bp upstream of the transcription start site (Green et al., 1983). These products are capable of activating the promoters of some cellular genes, such as the genes expressing heat shock proteins 70 (Hsp70) and β-globulin (Kovesdi et al., 1987; Kirch et al., 1993).

The modulation of cellular metabolism induced by these proteins enhances cell susceptibility to viral replication and yet promoting cellular entrance into S phase of the cell cycle (Russell, 2000). For this reason, E1A proteins interact with the retinoblastoma protein (pRB) leads to the induction of transcription factor E2F, which in turn activates cellular genes initiating progression from G1 phase into S phase of the cell cycle to help establishing more favorable environment for viral DNA replication (Shenk, 1996)

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3.1.6 The E1B gene

The E1B gene region encodes polypeptides necessary for the lytic cycle of the virus, replication of viral DNA and that block apoptosis (Frisch & Mymryk, 2002; Hortwitz, 1996). Two proteins are encoded in this region: 19K and 55K. Both proteins have the ability to block cellular apoptosis, however, in a different way: The 55K protein inhibits p53-mediated apoptosis (Zhao & Liao, 2003). The p53 protein is a tumor suppressor that regulates the transcription of several genes involved in cell cycle and apoptosis. For this blockade, 55K, together with E4ORF6, form a complex that promotes p53 degradation in proteasomes (Harada et al., 2002).

The 19K protein is analogous to Bcl-2. This is an important inhibitor of apoptosis induced by various stimuli, such as Fas, tumor necrosis factor (TNF-α) and p53-dependent apoptosis (Chiou et al., 1994; Gooding et al., 1991; Russel, 2000). Both proteins are required to contain cellular apoptosis activated by proteins in the E1A and E4 regions (Russell, 2000).

3.1.7 The E2 gene

The gene products of the E2 region are subdivided into E2A [DNA binding protein (DBP)] and E2B [precursor terminal protein (pTP) and DNA polymerase (pol)]. These three proteins are required for viral DNA replication and subsequent transcription of late genes (Russell, 2000). Pol is one of the most conserved proteins among adenovirus serotypes (Ikeda et al., 1981). This protein belongs to the Pol family (DNA polymerases that have 3′5′ exonuclease activity) (Field et al., 1984).

DBP is an ATP-independent protein with high affinity for single-stranded DNA. This protein destabilizes the DNA helix during elongation and replication (Liu et al., 2003). The terminal protein, TP, covalently binds to the 5′ end of the viral DNA, acting as an origin of replication. TP forms a heterodimer with DNA polymerase necessary for the initiation of viral DNA replication. In addition, this protein has the function of protecting viral DNA from exonucleases and mediating its binding to the nuclear matrix (Roovers et al., 1993; Schaack et al., 1990; Webster et al., 1994).

3.1.8 The E3 gene

The E3 gene is not conserved, and the size and composition of units transcribed by the E3 region and varies considerably between adenoviruses of different species (Kajon et al., 2005). In adenoviruses of species F and D, there is an E3 region of approximately 3000 bp and 5200 bp respectively. Variations in this region are also observed with respect to different numbers of coding regions, for example, adenovirus species F has only 5 open reading frames (ORFs), adenovirus D has 8, subgroup B1 (serotypes 3 and 7) have 9 ORFs (Burgert & Blusch, 2000).

Some of the E3 genes are present in adenoviruses of all species (coding proteins 10.4K, 14.5K and 14.7K), others are present in most species (12.5k: species A-E; 19K: species B-E) and, some, appear to be species specific (29.4K and 30.7K of species A). Three of the proteins encoded by the E3 gene (10.4K, 14.5K, and 14.7K) are functionally conserved among adenoviruses. (Burgert & Blusch, 2000; Horwitz, 2001, 2004).

Figure 4 shows the genetic organization of the E3 region of the 6 human adenovirus species.

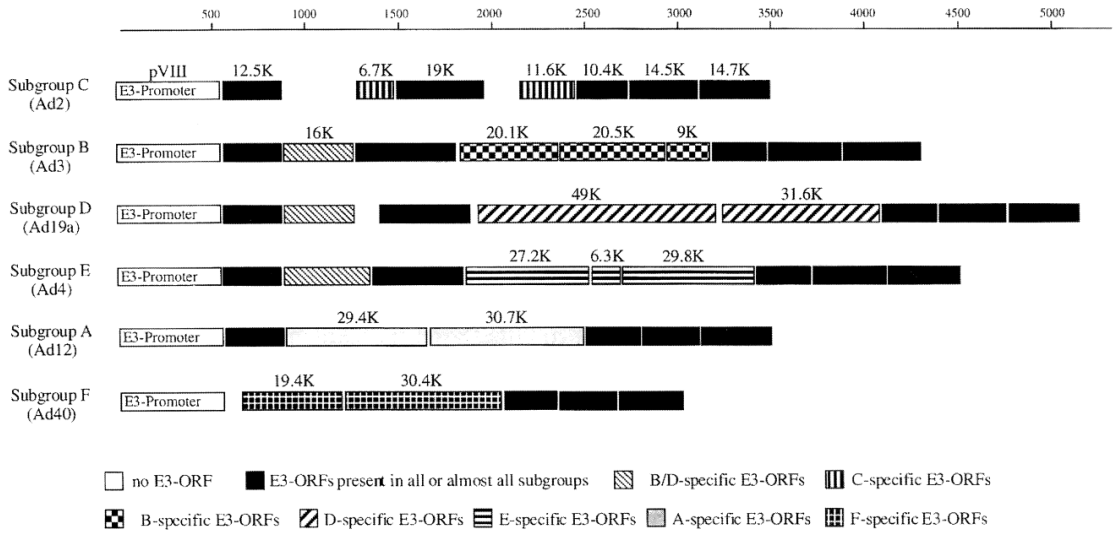


Figure 4. Genetic organization of the E3 region of adenoviruses of species A-F.

Font. (Burgert & Blusch, 2000)

The proteins encoded by the E3 region have an immunoregulatory function. They are not necessary for adenovirus replication in cell cultures, resulting in many adenoviral vectors devoid of these genes (Gonçalves & De Vries, 2006; Lichtenstein et al., 2004; Windhein et al., 2004). However, in vivo E3 gene products have the function of modulating the host's immune response (Fessler et al., 2004).

The 12.5K protein, encoded by all adenovirus species, except species F, is well conserved and its function is still unknown. Some studies have shown that deletion of the gene encoding this protein does not affect viral growth in cell cultures (Hawkins & Wold, 1992).

The 19K is the most abundant protein in the E3 region and is encoded by almost all human adenoviruses with the exception of species A and F. This protein has the property of binding to the Major Histocompatibility Complex (MHC-I), retaining it in the endoplasmic reticulum, thus preventing the exposure of viral peptides on the cell surface and the lysis of infected cells by cytotoxic T lymphocytes (Persson et al., 1980). *In vivo*, it was observed that the lungs of mice infected with wild-type adenovirus species C show a less severe immunopathology than animals infected with mutants whose 19K ORF was deleted (Ginsberg et al., 1989). The 16K protein, expressed by HAdV-B and E, is poorly studied. This protein has the same genomic location as the 6.7K protein (species C) and have homology in the C-terminal region; this suggests that

they may have a similar function during infection (Hawkins & Wold, 1995). The 6.7K protein appears to be involved in blocking cellular apoptosis (Lichtenstein et al., 2004). *In vitro* studies have observed that mutant adenoviruses with the ORF 16K deleted replicate the same as wild-type virus (Hawkins & Wold, 1995).

Proteins 20.1K and 20.5K are unique to adenovirus species B and have no established function. Studies show the synthesis of these proteins in cells infected by adenoviruses 3 and 7, which are found expressed in the membrane of infected cells and not in the cytoplasm (Hawkins & Wold, 1995).

The 7.7K protein is encoded by the B1 subspecies adenoviruses (serotypes 3, 7, 16, 21). This ORF shows a wide variation in its size and nucleotide sequence, being 7.7K in adenovirus 7p and 9.0K in adenovirus 3p, whilst absent in adenovirus subspecies B2 (Kajon et al., 2005).

Proteins 14.7K, 10.4K and 14.5K protect infected cells from TNF-α and Fas-mediated apoptosis (Krajcsi et al., 1996). The 10.4K together with 14.4K form a complex called RID (Receptor Internalization and Degradation) that induces the internalization and degradation of cell death receptors located in the cell membrane, such as: Fas, TRAIL-R1 and R2, preventing the interaction from these receptors to their ligands (Tollefson et al., 1990).

The 11.6K protein, also called ADP (adenovirus death protein) is one of the proteins of the E3 region that is expressed late, under the action of the MLP (major late promoter) (Tollefson et al., 1996). This nuclear transmembrane protein is expressed only by adenovirus species C and is related to cell lysis (Tollefson et al., 1996).

3.1.9 The E4 gene

The E4 region is highly conserved and has organizational similarity across all adenovirus serotypes. This region encodes around 6 polypeptides, named according to their ORF (from 1-6/7). Only the deletion of ORFs 3 and 6 produces defective viral multiplication in cell cultures and, consequently, are the most studied (Bridge & Ketner, 1989; Huang & Hearing, 1989). These ORFs form a complex with the 55K protein,

encoded by the E1B region, increasing the rate of viral replication and late protein synthesis (Leppard & Everett, 1999). These proteins also inhibit the activity of DNA-dependent protein kinase (DNA-PK), which is essential for a functional DNA repair system.

The role of ORFs 1-2 remains unknown. The ORF 4 appears to inhibit viral and cellular gene transduction and also induce p53-independent cellular apoptosis, mainly in transformed cells (Kleinberger, 2000; Kleinberger & Shenk, 1993; Mannervik et al., 1999).

3.1.10 Fiber protein and its role in infectivity

Adenovirus fiber protein is a homotrimer of protein IV, which is divided into three regions: the globular region (knob), the stem, and the tail, as can be seen in the Figure 5. Fiber protein in its trimeric form is resistant to SDS denaturation at 25-30 ° C, indicating that they are extremely stable proteins (Hong & Engler, 1996).

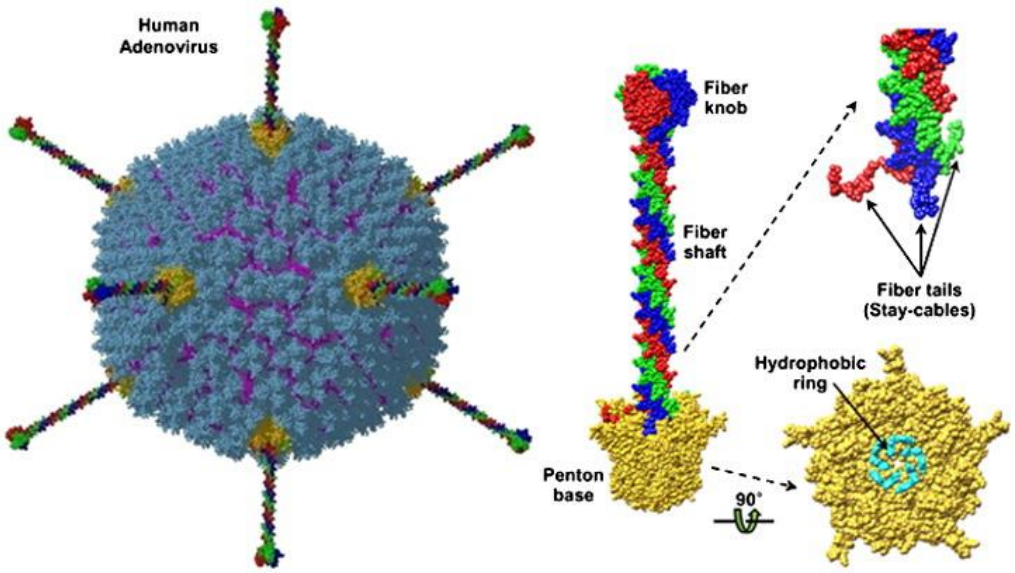


Figure 5: Model of the trimeric fiber and its interactions with the pentameric penton base of human adenovirus by cryo-electron microscopy.
Font: Liu et al. (2011)

The carboxy terminal (C-terminal) globular region of the fiber is responsible for recognizing the primary receptor by adsorbing the viral particle to the cell (Philipson

et al., 1968). This process can be easily inhibited by saturating the receptors with the purified fiber protein, indicating that the fiber and viral particle compete for the same receptor (Mei et al., 2002, Segerman e al., 2003). Several studies show that when the adenovirus fiber globular region is expressed in various expression systems (insect cells and bacteria) it naturally organizes into trimers, and only these are able to recognize the receptors, reinforcing that this recognition is dependent on three-dimensional fiber structure (Henry et al., 1994; Louis et al., 1994).

The stem region has repeats of approximately 15 amino acids, folded in a fibrous structure and extremely resistant to proteases (Stouten et al. 1992; Chrobockzek et al., 1995; van Raaij et al., 1999). The number of amino acid repeats varies by adenovirus species, yielding differently sized fibers (table 1)

The N-terminal tail region (MMKRARLEDDFNPVYPY) is conserved in all human adenovirus species and is responsible for interaction with the penton-base.

Changes in fiber, or even non-fiber particles, show a decline in infectivity in permissive cells, indicating that fiber protein is essential for efficient infection (Legrand et al., 1999; Von Seggern et al., 1999; Shayakhmetov et al., 2000; Rea et al., 2001; Havenga et al., 2002).

Due to the importance of fiber protein in viral tropism, this protein ends up under selective pressure, and fiber exchange is one of the most common natural events of recombination among adenoviruses, indicating a benefit in the emergence of new strains (Matumoto et al., 1958; Hatch e Siem, 1966; Jong et al., 1983; Flomemberg et al., 1987; Kajon et al., 1996).

A study suggested that chimeras consisting of adenovirus C capsid and adenovirus B fiber have intracellular traffic similar to adenovirus B. This data suggests that fiber is capable of influencing adenovirus intracellular traffic (Miyazawa et al., 1999).

The excess fiber produced during viral replication is released from the cell along with the penton base. In the extracellular environment, the fibers interact with proteins that make up the cell junctions, dismembering the epithelium, facilitating the

release of viruses and consequently their dispersion in the respiratory or enteric tract (Walters et al., 2002; Trotman et al., 2003).

3.2 EPIDEMIOLOGY OF HUMAN ADENOVIRUSES

Human adenoviruses (HAdV) cause a vast array of diseases, in adults and children, such as: respiratory, gastroenteric, urinary, ocular and central nervous system (Adhikary et al., 2004; Ebner et al., 2006; Erdman et al., 2002). These viruses have often been associated with generalized infections in immunocompromised patients, such as transplant recipients (Hierholzer, 1992; Ison, 2006). Adenoviruses are transmitted by direct person-to-person contact, via the respiratory route or fecal-oral route, through contaminated food and water. Some adenovirus serotypes have low pathogenicity and hence associated with asymptomatic infections.

Adenovirus species A, serotypes 12, 18 and 31 are associated with infrequent enteric infections in children under 1 year of age. Studies have shown that the serotypes of these species have a high transforming capacity in newborn hamster cells, however, there are no descriptions of association of these viruses with tumors in humans (Wadell, 1984).

Restriction analysis of the DNA allowed the division of adenovirus species B into two subspecies: B1 and B2. In subspecies B1 are serotypes 3, 7, 16, 21 and 50; frequently associated with cases of acute respiratory infection (ARI) and eye infections. Serotypes 7, followed by 3, are the most frequently isolated from ARI cases in Brazil and are associated with severe cases such as bronchiolitis, pneumonia and deaths. Infections caused by serotype 7 can range from mild upper respiratory tract illness and conjunctivitis to severe lower respiratory tract illness, spread to other organs, and death, particularly in children.

In subspecies B2, serotypes 11, 14, 34 and 35 are found. These serotypes are associated with kidney and urinary tract infections and are frequently isolated from patients undergoing kidney transplants from immunocompromised patients (Jong et al., 1999; Russell, 2009). Adenovirus serotype 14 has been linked to severe Acute

respiratory infections (ARI) outbreaks in the United States in recent years (Lewis et al., 2009; Tate et al., 2009).

Adenovirus species C, serotypes 1, 2, 5 and 6 are considered endemic, infecting adenoids and tonsils and presenting a high incidence in children under five years of age (Horwitz, 1996). Infections caused by these serotypes represent more than half of all adenoviruses isolated in the world (59%), with the main serotypes isolated being 1 (34.3%), 2 (42.8%) and 5 (18.6%) (Adhikary et al., 2004; Horwitz, 1996).

Species D has the greatest genetic variability including a large number of serotypes: 8-10, 13, 15, 17, 19, 20, 22-30, 32, 33, 36-39, 42-49 and 51. Most of these serotypes are rarely isolated, the exceptions being serotypes 8, 19a and 37, which are associated with outbreaks of epidemic keratoconjunctivitis (Russell, 2009). One study described that serotype 37 can be transmitted sexually and, in 1999, the isolation of serotype 51 in AIDS patients (Jong et al., 1981).

Species E has only one human serotype, 4. This serotype is responsible for outbreaks of respiratory infection in military recruits in the United States (Metzgar et al., 2005). A study presents the association of this serotype with conjunctivitis. Serotype 4 can be isolated from cases of conjunctivitis and respiratory infections (Wadell, 1984).

Serotypes of species F, 40 and 41 have enteric tropism and are considered to be of great importance in the etiology of infantile gastroenteritis (Tiemessen & Kidd, 1995). These viruses have biological, molecular and structural characteristics different from other adenoviruses, such as the presence of two fibers (Kidd et al., 1993).

A new adenovirus serotype, classified as 52, was isolated from patients with gastroenteritis in 2007. This serotype, although it has some morphological characteristics similar to adenovirus species F, such as the presence of two different fibers, and the tropism for the intestinal epithelium, has not yet been classified in any of the six known species. Phylogenetically, adenovirus 52 is similar to adenovirus isolated from monkeys, serotype 1 (SAdV-1 – *Simian Adenovirus serotype 1*) (Jones et al., 2007).

3.3 HUMAN ADENOVIRUS F (HADV-F)

One study used electron microscopy technique in the diagnosis of gastroenteritis describing a new adenovirus group present in large quantities in diarrheal stools (Flewett et al., 1974). A later study isolated an enteric adenovirus in HEK-293 or Grahan cell (Takiff et al., 1981), whilst a further Next, a study characterized enteric adenoviruses (serotypes 40 and 41) as members of species F (Jong et al., 1983). Currently, adenoviruses of serotypes 40 and 41 are represented by two strains “Dugan” and “Tak”, respectively, both isolated in the Netherlands from feces of children with diarrhea (Jong et al., 1983).

HAdV-F serotypes 40 and 41 (HAdV-40 and HAdV-41) of specific enteric tropism are considered of great importance in the etiology of acute childhood gastroenteritis (Tiemessen & Kidd, 1994, 1995). Enteric adenoviruses replicate very well in the intestines of children with diarrhea, releasing 10^{11} viral particles per gram of feces (Uhnoo et al., 1984). However, these viruses are fastidious in cell culture, suggesting a strong adaptation of viral replication in the intestinal epithelium (Tiemessen et al., 1993).

3.4 CHARACTERISTICS OF HAdVs-F

HAdVs-F (HAdV-40 and 41) have distinct molecular, biological and structural characteristics to other adenoviruses (Tiemessen & Kidd, 1995; Tiemessen et al., 1993).

HAdVs-F have modifications in the genes encoding early proteins (E1A; E1B E2, E3 and E4) and have two types of fibers, one long and one short, which are alternately distributed, one at each base (Pieniazek et al., 1990; Favier et al., 2002). This structural feature within the Mastadenovirus genus is unique to HAdV-40 and 41.

Another structural difference between HAdV-40 and HAdV-41 is the absence of the RGD (Arg-Gly-Asp) motif in the penton-base, which is important in the virus penetration stage in the cell. The HAdV-40 carries the RGAD (Arg-Gly-Ala-Asp) motif; and HAdV-41, the IGDD motif (Ile-Gly-Asp-Asp) (Albinsson & Kidd, 1999). Cell proteins that interact with the F-adenovirus penton-base are unknown.

3.5 THE FIBERS OF THE HADV-41

Unlike other human adenoviruses, the HAdV-F fiber (L5) gene has 2 ORFs (Kidd et al., 1993; Yeh et al., 1994). The first ORF (L5-1) encodes a protein called short fiber; and the second (L5-2) encodes a protein called long fiber. Interestingly, these proteins are evenly distributed in the viral particle, one in each base penton of the virus (Favier et al., 2002; Pieniazek et al., 1990).

3.5.1 Long fiber

The long fiber protein is a 562 amino acid polypeptide and has a molecular weight of 60.5kDa (Pieniazek et al., 1989; Kidd et al., 1990). The length of the long fiber (obtained from the C-terminal globular region to the N-terminal stem region) is 34nm (Chroboczek et al., 1995; Favier et al., 2002). The isoelectric point (p*I*) prediction analysis of the full long fiber indicates a p*I* of 7.51; whereas the p*I* of the globular region is 8.64 (Favier et al., 2004, 2002).

HAdV-F long fiber is known to recognize CAR, however, with lower affinity when compared to other human adenoviruses (Roelvink et al., 1998). This data suggests that the long fiber is used by HAdV-41 in the viral cell adsorption step to the host cell. The long fiber characteristics of the HAdV-41 can be seen in table 3.

3.5.2 Short fiber

The short fiber protein is a 387 amino acid polypeptide and has a molecular weight of 40.1kDa (Pieniazek et al., 1989; Kidd et al., 1990). The length of the short fiber (ie obtained from the C-terminal globular region to the N-terminal region of the stem) is 20nm (Chroboczek et al., 1995; Favier et al., 2002). The complete short fiber has a p*I* of 9.13, while the p*I* of the globular region is only 7.24 (Favier et al., 2004, 2002).

HAdV-41 short fiber is known not to recognize CAR (Roelvink et al., 1998). At the moment, the receiver used by the short fiber was not elucidated (table 3).

In a study it has been shown that purified HAdV-41 long and short fibers are resistant to both acid exposure (pH 2.0) and exposure to chymotrypsin. It was also observed that after treatment with acid or digestion, the long fiber had not changed in its CAR (extracellular domain) tropism. However, the same result was not obtained with HAdV-41 short fiber, that is, even after treatment with acid and chymotrypsin, there was no recognition of CAR by the fiber. Interestingly, the hypothesis that short fiber after exposure to gastric enzymes should recognize other receptors present in the cell should not be ignored (Favier et al., 2004).

Two researchers demonstrated that the C-terminal globular region of the HAdV-41 short fiber undergoes proteolysis following pepsin exposure. This result may indicate that HAdV-41 exposes epitopes after its exposure to enzymes present in the gastrointestinal tract, thus favoring their interaction with it.

Based on the published work, we believe that there are differences in HAdV-41 that must be elucidated in order to develop gene therapy vectors directed to the intestinal epithelium, as well as orally administered vaccine vectors (Seiradake & Cusack, 2005).

Table 3: Predicted pI values of adenovirus capsid proteins from different species.

Tropism	Serotype	Hexon	Penton-base	Fiber (knob)	Species	Receptor primary
Enteric	HAdV-41	5.49	5.75	7.51 (8.64) (long)	F	CAR
Enteric	HAdV-41			9.13 (7.24) (short)	F	
Nonenteric	HAdV-2	4.86	5.05	5.85 (6.35)	C	CAR
Nonenteric	HAdV-5	5.03	5.18	5.89 (5.91)	C	CAR
Nonenteric	HAdV-3	5.32	5.26	5.61	B	CD46 CD80 CD86

Font: Modified from (Favier et al., 2004).

3.6 ADENOVIRUS AND GENE THERAPY

Adenoviruses have been used in gene therapy as vectors, developed from types HAdV-2 and HAdV-5. These viruses have important characteristics for their use in gene

therapy, such as: growth in cell culture with high titers and space in the viral genome for the insertion of heterologous genes (Lemiale et al., 2007).

The intestinal epithelium has several characteristics for gene therapy, such as its easy access via the lumen, which would allow direct gene transfer *in vivo* by oral administration or endoscopy methods (Sandberg et al., 1994; Croyle et al., 1998). The intestinal epithelium has a large tissue area, good absorption, and the ability to secrete proteins into the bloodstream (Ledley et al., 1992).

Intestinal epithelium is the target of both oral immunization and treatment of genetic or metabolic disorders (such as cystic fibrosis, adenomatous polyps, phenylketonuria, and colon cancer) (Mestecky, 1987; Croyle et al., 1998).

Adenoviral vectors, derived from the HAdV-2 and HAdV-5 prototypes, when tested for their expression in the intestinal epithelium, have reduced efficacy (Croyle et al., 1998).

A study demonstrated that the adsorption and penetration capacity of HAdV-5 (species C) and HAdV-41 (species F) in enterocytes is distinct. In undifferentiated cells, the two serotypes behave similarly. However, in differentiated cells, HAdV-5 has its penetration and transduction affected, whereas HAdV-41 not only maintains its penetration capacity, but also aids in HAdV-5 transduction efficacy (Croyle et al., 1998).

Decreased β-integrin expression in the membrane of differentiated enterocytes is the likely reason for non-internalization of HAdV-5. In turn, given its structural characteristics, HAdV-41 should use other cellular proteins as receptors, some of which may be of significant expression in differentiated enterocytes (Croyle et al., 1998).

Recently, non-viral gene therapy vectors consisting of proteins or lipids have been developed. Some of these vectors are produced from recombinant viral proteins or synthetic peptides derived from viral proteins (Fender et al., 1997; Zhang et al., 1999).

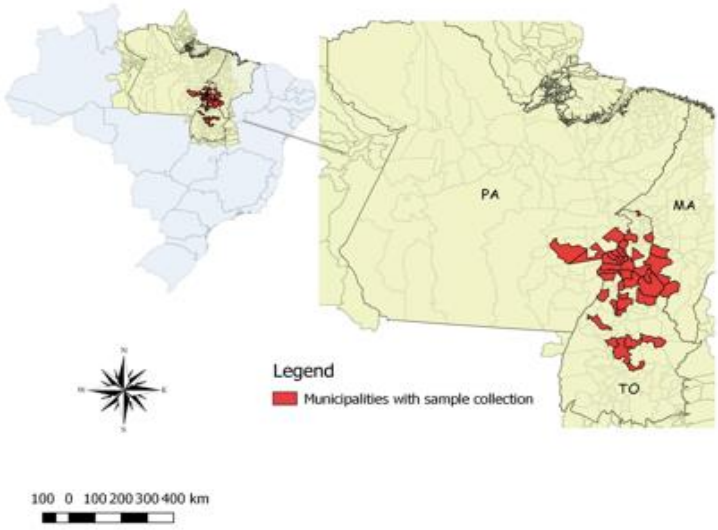
4. METHODS AND MATERIALS

4.1 SAMPLE SELECTION

From 2010 to 2016, the Laboratório Central de Saúde Pública do Tocantins (Lacen-TO) - surveillance laboratory in Tocantins - collected fecal samples from children under 5 years of age, with an episode of diarrhea, who came to see a local physician for diagnosis (total=251). These samples were then brought to the Virology Department of the Institute of Tropical Medicine in Sao Paulo for further processing.

The current cross-sectional surveillance study was carried out in the states of Tocantins and Pará, Northern and North regions of Brazil, respectively. This descriptive study was performed with surveillance specimens from patients presenting with the symptoms of AGE at Brazilian Unified Health System (SUS) units. The global aim of the study was to search human faeces for the presence of potential novel viral enteric pathogens in rural and low-income urban areas in northern Brazil using NGS techniques. Although screening for common enteric pathogens (e.g. HAdV-F) was not included in the original aims of the proposed NGS surveillance, metagenomic analyses offered an opportunity to obtain full-genome HAdV-F sequences in this rarely investigated Brazilian region.

Faecal samples were collected in 38 different localities. A total of 251 specimens were collected; 245 from the state of Tocantins and 3 from the state of Pará. Three samples were obtained from border municipalities (Estreito and Carolina) located between the state of Tocantins and the state of Maranhão (northeast region of Brazil) (Figure 6). A total of 237 stool specimens were obtained from children aged 1–5 years, 3 stool specimens were obtained from children aged 8–15 years, and 7 stool specimens were obtained from adults aged 20–78 years; all with symptoms of gastroenteritis. The age of the patient was missing for five stool samples. Gender data were available for 247 patients (98 females and 148 males; $\chi 2=6.85$, $P<0.05$).



OBS.: Up: Map of Brazil showing Brazilian Northern area. Down: Localities surveyed, including state of Tocantins (TO), Para (PA) and Maranhão (MA). Map was generated with QGIS software v2.14.9

Figure 6: Map of Northern Brazil highlighting municipalities (in red) from which samples were collected from patients with AGE, 2010-2016.
Font.: https://www.qgis.org/pt_BR/site/about/index.html.

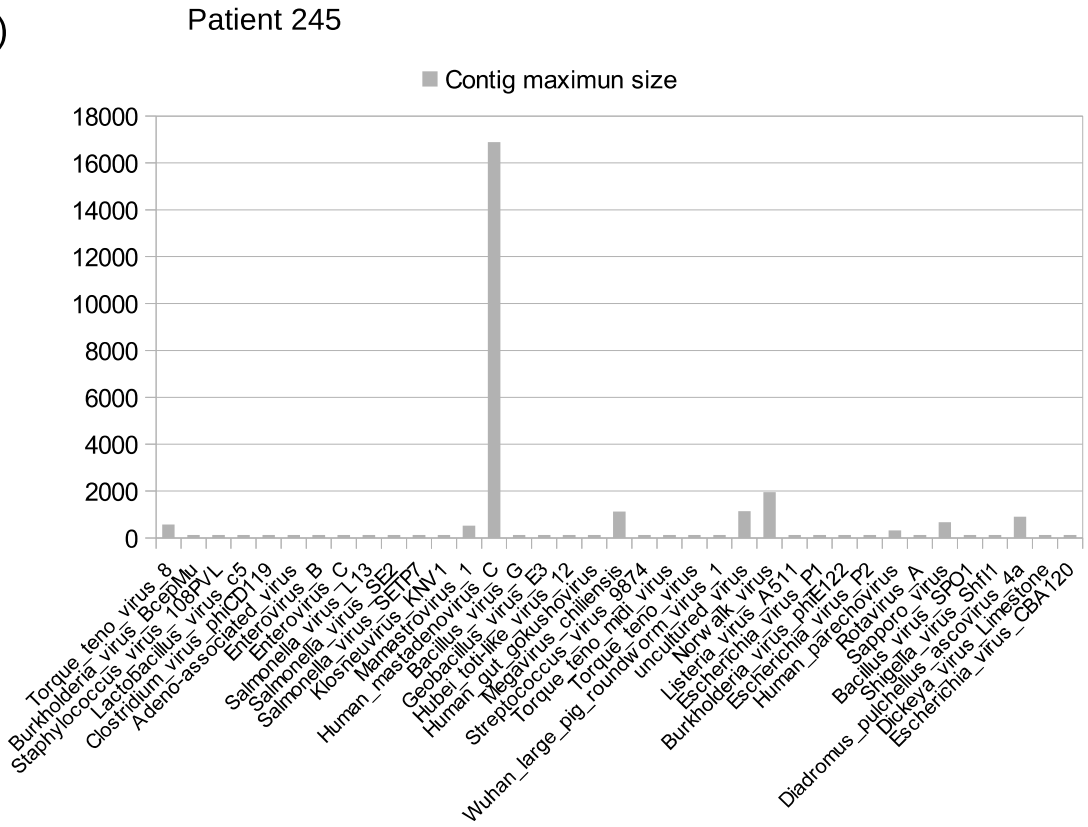
All specimens were sent to Tocantins Public Health Laboratory (LACEN-TO) together with relevant collection date, age and gender data where they were stored at −20 °C. Frozen faecal specimens were screened for bacteria (e.g., Escherichia coli and Salmonella sp.), protozoan (e.g., Giardia sp.) and helminths (e.g., Taenia Solum) using conventional parasitological and cultures techniques. Stored frozen fecal specimens were then forwarded to the São Paulo Institute of Tropical Medicine (IMT/USP) to identify enteric viruses, including RVAs, NoVs, HAstVs (Watanabe et al., 2018), and HSaV (Cilli et al., 2019), as well as rare (Ribeiro et al., 2019; Luchs et al., 2019; Rosa et al., 2019) or potentially novel viruses (Leal et al., 2019; Costa et al., 2018; Costa et al., 2018; Luchs et al., 2018; Tahmasebi et al., 2020; Costa et al., 2019), for NGS investigation.

This study was carried out retrospectively with surveillance specimens already available, without inclusion or exclusion criteria, and with no characterization of the participants. Therefore, epidemiological data (i.e. date of onset of diarrhoea, number of episodes of vomit or fever) were not available for all patients. In addition, the protocol

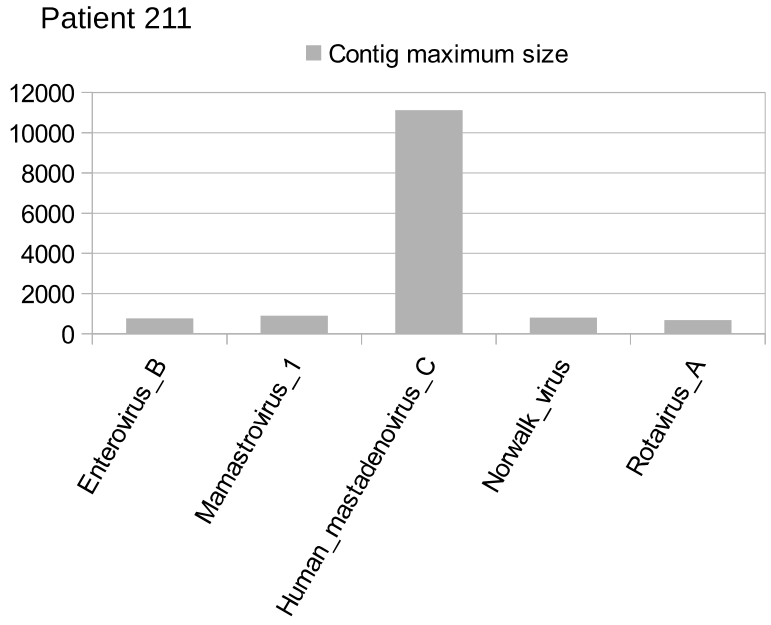
used failed to identify possible outbreaks. Descriptive statistics was used to describe changes in the distribution of HAdV-F-positive cases, and $\chi 2$ or Fisher's exact test for categorical variables. Temporal trends regarding the frequency of HAdV-F and other viruses identified during the study period were tested using logistic regression. Stata software version 13.0 with a two-tailed α error of 0.05 was used for statistical analysis, and a P value <0.05 was considered statistically significant.

Two HAdV-C strains were identified during the NGS investigation: HAdV-C BR-211 (potential novel HAdV-C recombinant) and HAdV-C BR-245 (HAdV-C1 strain), in which Norovirus was also detected (Figure 2). Both patients were experiencing AGE symptoms, such as diarrhea, vomiting and fever. In addition, the HAdV-C BR-211 patient presented coryzal symptoms. HAdV-C BR-211 (potential novel HAdV-C recombinant) was detected in a sample collected in 2015 in the city of Carolina, Maranhão from a 3-year-old female child, and the HAdV-C BR-245 (HAdV-C1) was collected in 2014 in the city of Araguaína, Tocantins from a 1-year-old female infant.

a)



b)



OBS.: Metagenomic analysis of viruses in children with gastroenteritis. Diagram showing all viruses identified in a certain biological sample.
X-axis represents viral species identified in each sample and
Y-axis represents the number of reads per species.
a) Viruses identified in the patient BR 211 and
b) Viruses identified in the patient BR 211

Figure 7: Viruses identified by metagenomic analysis.

4.2 VIRAL METAGENOMICS

The protocol used to perform deep sequencing was a combination of several protocols applied to viral metagenomics and/or virus discovery and has been previously described by the study of the current author (Li et al., 2005; Costa et al., 2017). In summary, 50 mg of human fecal sample was diluted in 500 µL of Hanks' buffered salt solution (HBSS). This solution was then added to a 2 mL impact-resistant tube containing lysing matrix C (MP Biomedicals, Santa Ana, CA, USA) and homogenized in a FastPrep-24 5G Homogenizer (MP Biomedicals, Santa Clara, CA, USA). The homogenized sample was centrifuged at 12,000× g for 10 min, and approximately 300 µL of the supernatant was percolated through a 0.45 µm filter (Merck Millipore, Billerica, MA, USA) to remove eukaryotic- and bacterial-cell-sized particles. Approximately 100 µL, equivalent to one-fourth of the volume of the tube, of cold PEG-it Virus Precipitation Solution (System Biosciences, Palo Alto, CA, USA) was added to the filtrate, and the contents of the tube were gently mixed, then incubated at 4 ◦C for 24 h. After the incubation period, the mixture was centrifuged at 10,000× g for 30 min at 4 ◦C. Following centrifugation, the supernatant (~350 µL) was discarded. The pellet, rich in viral particles, was treated with a combination of nuclease enzymes (TURBO DNase and RNase Cocktail Enzyme Mix-Thermo Fischer Scientific, Waltham, MA, USA; Baseline-ZERO DNase-Epicentre, Madison, WI, USA; Benzonase-Darmstadt, Darmstadt, Germany; and RQ1 RNase-Free DNase and RNase A Solution-Promega, Madison, WI, USA) to digest unprotected nucleic acids. The resulting mixture was subsequently incubated at 37 ◦C for 2 h.

After incubation, viral nucleic acids were extracted using a ZR & ZR-96 Viral DNA/RNA Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. cDNA synthesis was conducted with AMV Reverse transcription (Promega, WI, USA) with the DNA Polymerase I Large (Klenow) Fragment (Promega, WI, USA) used for the second strand of cDNA synthesis. Subsequently, a Nextera XT Sample Preparation Kit (Illumina, San Diego, CA, USA) was used to construct a DNA library, which was identified using dual barcodes. The library was then purified using ProNex® Size-Selective Purification System (Promega, WI, USA). Following ProNex® purification, the quantity of each sample was normalized to ensure equal library representation in our pooled samples using the ProNex® NGS Library Quant Kit (Promega, WI, USA). For size range selection, Pippin Prep (Sage Science, Inc.) was used to select a 300 bp insert (range 200–400 bp), which excluded very short and long library fragments. Prior to cluster generation, libraries were quantified again by qPCR using the ProNex® NGS Library Quant Kit (Promega, WI, USA). The library was deep-

sequenced using a Hi-Seq 2500 Sequencer (Illumina, CA, USA) with 126 bp ends (Costa et al., 2017; Cilli et al., 2019; Costa et al., 2019). Bioinformatics analysis was performed according to the protocol previously described (Deng et al. 2015). The contigs, including sequences of rotaviruses as well as enteric viruses, humans, fungi, bacteria and others, sharing a percent nucleotide identity of 95% or less were assembled from the obtained sequence reads by de novo assembly. The resulting singlets and contigs were analyzed using BLASTx to search for similarity to viral proteins in GenBank. The contigs were compared to the GenBank non-redundant nucleotide and protein databases (BLASTn and BlastX). After identification of the viruses, reference template HAdV-F40/41 sequences were used to map the full-length genomes with Geneious R9 software (Biomatters Ltd L2, Auckland, New Zealand).

Based on the best hits of the BLASTx searches, HAdV-C genomes were chosen for further analyses. Sequences generated in this study have been deposited in GenBank: MN628614 (HAdV-C BR-245) and MN628615 (HAdV-C BR-211). All protocols and procedures were conducted within the enhanced laboratory biosafety level 2 (ABSL-2) facility of the Institute for Tropical Medicine, São Paulo University. The ABSL-2 facility consists of a laboratory in which all in vitro experimental work is carried out in class 3 biosafety cabinets, which are also negative pressurized (<−200 Pa). Although all experiments are conducted in closed-class 3 cabinets and isolators, special personal protective equipment, including laboratory suits, gloves and FFP3 face-masks is used. Air released from the class 3 units is filtered by High-Efficiency Particulate Air (HEPA) filters and then leaves via the facility ventilation system, again via HEPA filters. Only authorized personnel that have received the appropriate training can access the facility. The facility is secured by procedures recognized as appropriate by the institutional biosafety officers and facility management at São Paulo University and Brazilian National Technical Biosafety Commission (CTNBio).

4.3 ALIGNMENT AND PHYLOGENETIC ANALYSIS

Near full-length genomes of HAdV-F and HAdV-C were aligned using MAFFT software v.7 (https://mafft.cbrc.jp/alignment/software/) and clustal X version 2.0 software (Larkin et al., 2007). Subsequently, phylogenetic tree construction used the Maximum Likelihood approach, and branch support values were assessed with approximate likelihood-based measures of branch supports (approximate likelihood ratio test [aLRT] and a Shimodaira-Hasegawa [SH]-aLRT). Trees were then inferred using FastTree version 2.1 software (Price, Dehal, & Arkin, 2010). Evolutionary models

(general time reversible (GTR) model with gamma distribution) were selected based on the likelihood ratio test (LRT) implemented in the HiSeq Modeltest2 software (Posada, 2008). Estimates of genetic distances were calculated using the Maximum Composite Likelihood model plus gamma correction and equal nucleotide rates among sites. Distances were implemented in MEGA X version 10.0.5 (Kumar, Stecher, Li, Knyaz, & Tamura, 2018).

All positions containing gaps and missing data were eliminated (complete deletion option). The number of base substitutions per site from between sequences is shown; standard error estimate(s) are also shown and were obtained by a bootstrap procedure (200 replicates). There was a total of 33,317 positions in the final dataset.

4.4 DETECTION OF RECOMBINATION

The identification of potential parental sequences and the localization of possible recombination breakpoints were determined using the Recombination Detection Program (bootscan approach), RDP4 version 4.9.5. (Martin, Murrell, Golden, Khoosal, & Muhire, 2015), which was used with a window size of 50–350 bp and a step size of 50–100 pb, as well as Bonferroni correction with P values of 0.05 and 0.001 for the different detection methods, including RDP5, GENECONV, BootScan, MaxChi, Chimaera, SiScan and 3Seq (Martin et al., 2015).

4.5 HOMOLOGY MODELING OF HEXON STRUCTURE

The primary amino acid sequence of HAdV-C BR-211 hexon was submitted to the SWISS-MODEL workspace for homology modeling (Arnold et al., 2006). The SWISS-MODEL template library (https://swissmodel.expasy.org) was searched with BLASTp and HHBlits (Remmert et al., 2011) for evolutionary related structures that matched the target sequence (Cilli et al., 2019; Costa et al., 2018; Costa et al., 2019). The highest-quality templates were selected for model building. The models were built based on the target-template alignment using ProMod3. Coordinates that were conserved between the target and the template were copied from the template to the model. Insertions and deletions were remodeled by using a

fragment library. Side chains were then rebuilt. PyMOL v0.99 (https:pymol.org/) was used to generate the space-filling representation of the hexon structure.

4.6 SAMPLE PROCESSING AND NUCLEIC ACID EXTRACTION

The protocol for the processing of fecal samples and nucleic acid extraction was established in our lab using standard extraction protocols. The protocol is as follows: 50 mg of each fecal sample was diluted in 500 μL of Hanks' buffered salt solution (HBSS), added to a 2 mL impact-resistant tube containing lysing matrix C (MP Biomedicals, Santa Ana, CA, USA) and homogenized in a FastPrep-24 5G Homogenizer (MP biomedicals, USA). The homogenized sample was centrifuged at 12,000× g for 10 min, and approximately 300 μL of the supernatant was then percolated through a 0.45 μm filter (Merck Millipore, Billerica, MA, USA) in order to remove eukaryotic- and bacterial cell-sized particles. Approximately 100 μL, roughly equivalent to one fourth of the volume of the tube, of cold PEG-it Virus Precipitation Solution (System Biosciences, Palo Alto, CA, USA) was added to the obtained filtrate, and the contents of the tube was gently mixed, then incubated at 4 °C for 24 h. After the incubation period, the mixture was centrifuged at 10,000× g for 30 min at 4 °C. Following centrifugation, the supernatant (~350 μL) was discarded. The pellet, containing the viral particles, was treated with a combination of nuclease enzymes (TURBO DNase and RNase Cocktail Enzyme Mix-Thermo Fischer Scientific, Waltham, MA, USA; Baseline-ZERO DNase-Epicentre, Madison, WI, USA; Benzonase-Darmstadt, Darmstadt, Germany; and RQ1 RNase-Free DNase and RNase A Solution-Promega, Madison, WI, USA) in order to digest unprotected nucleic acids. The resulting mixture was subsequently incubated at 37 °C for 2 h. After incubation, viral nucleic acids were extracted using a ZR & ZR-96 Viral DNA/RNA Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol.

4.7 BIOINFORMATICS ANALYSIS

We performed bioinformatics analysis according to the protocol previously described in Deng et al. (2015) The sequence fragments obtained from deep-sequencing sharing a nucleotide identity of <95% were assembled by de novo assembly. The resulting singlets and contigs were analyzed using BLASTx to search for similarity to viral proteins in GenBank's

Virus RefSeq in order to sort them. Contigs include not only sequences of adenoviruses, but also sequences of other viruses. Only adenoviruses were studied, and fragments belonging to this virus family were compared to the GenBank nonredundant nucleotide database using the BLASTn tool to find a reference template sequence. The reference sequence was used to map the full-length genome using with Geneious R9 software (Biomatters Ltd L2, Auckland, New Zealand).

5. RESULTS

5.1 HADV-F DETECTION AND TYPING

HAdV-F was detected in 57.8% (145/251) of the samples, and most frequently detected in males (62.1%; 90/145) compared to females (37.2%; 54/145) ($\chi 2=6.05$, $P<0.05$). In one HAdV-F- positive sample (0.7%) the gender could not be determined. No trends or seasonal patterns were identified in HAdV-F identified over the study period (P=0.548): 54.5% (6/11) in 2010, 45.4% (5/11) in 2011, 75% (3/4) in 2012, 51.1% (23/45) in 2013, 56.5% (52/92) in 2014, 60.5% (52/86) in 2015 and 50% (1/2) in 2016 (Figure 8).

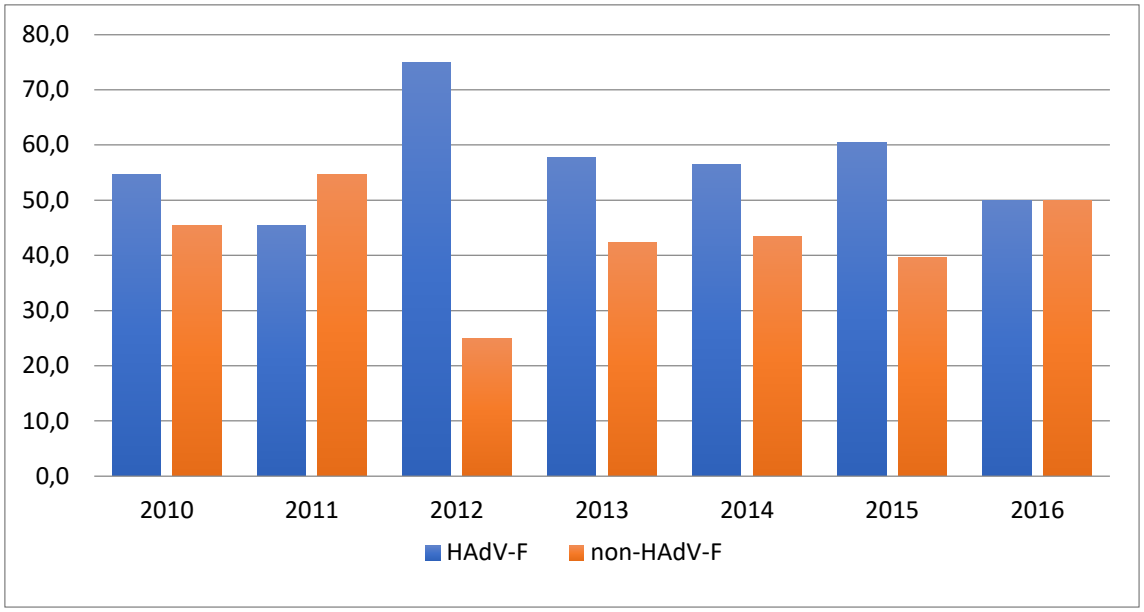


Figure 8: Detection rate (%) of HAdV-F-F41/41, and non-HAdV-F from patients with AGE in Northern Brazil by year, 2010–2016.

A total of 139 (95.8; 139/145) HAdV-F-positive samples were detected in the cohort of children ≤5 years. The patient's age was missing for one HAdV-F-positive sample. Two positive samples were from 50 year olds, one was from a 20-year-old, one was from a 7-year-old child and one was from a 6-year-old child. The ages of the subjects from whom HAdV-F-positive samples were obtained ranged from 1day to 50 years, with mean and median ages of 2.3 years and 1 year, respectively.

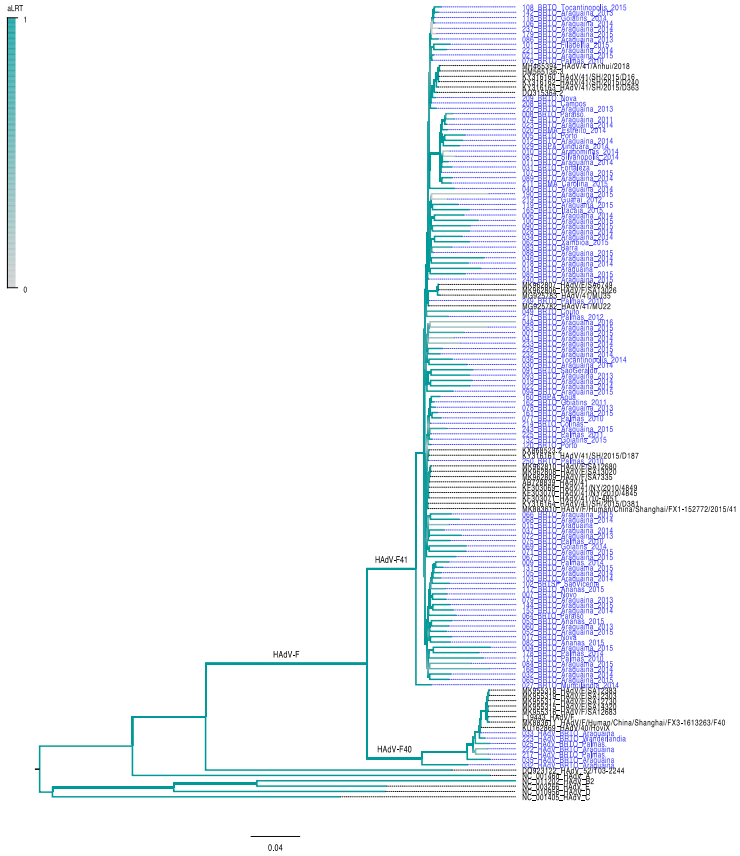
Single HAdV-F infections were detected in 55 (21.9%) of the 251 specimens, whereas mixed virus infections were detected in 94 (37.4%) of the same specimens. The

most frequent association of viruses was dual infection with RVA and HAdV-F (21.5%; 54/251), followed by triple infection with RVA/HAdV-F/NoV (10%; 25/251) and dual infection with NoV/HAdV-F (4.7%; 12/251). Dual infection with RVA and NoV was detected in 1.5% (4/251) of samples.

From the 251 samples that were subjected to Illumina MiSeq sequencing, near full-genome sequences (coverage >90%) were obtained from 82 samples, and partial genome sequences from 63 samples (Table 4). The type verification was performed using the blast web tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). HAdV-F41 was the most prevalent genotype (94.5%; 137/145), followed by HAdV-F40 (4.8%; 7/145). Dual infection HAdV-F40/41 was found in only one sample (0.7%).

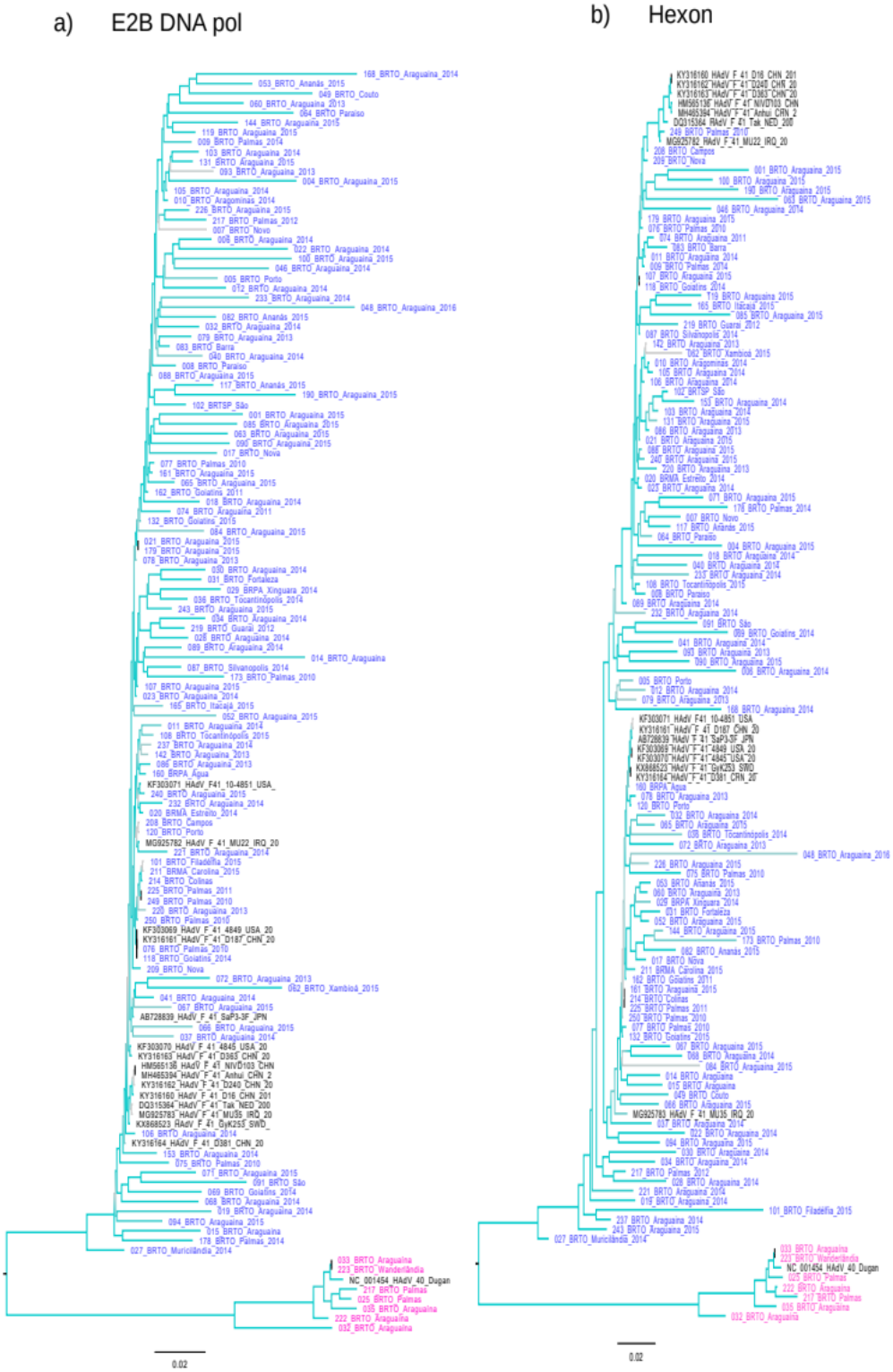
5.2 PHYLOGENETICS ANALYSIS AND GENOTYPING TREE

The near full-length genomes (approximately 35300 nucleotides) of Brazilian HAdV-F strains were sequenced and compared with previously described near full-length HAdV-F genome strains. Maximum-likelihood tree (Figure 9) showed that genotypes HAdV-F40 (reference strains: NC_001454-Dugan) and HAdV-F41 (reference strain: AB728839:SaP3-3F-JPN, DQ315364:Tak-NED, HM565136:NIVD103-CHN, KF303069:4849-USA, KF303070:4845-USA, KF303071:10–4851-USA, KX868523:GyK253-SWD, KY316160:D16-CHN, KY316161:D187-CHN, KY316162:D240-CHN, KY316163:D363-CHN, KY316164:D381-CHN, MG925782:MU22-IRQ, MG925783:MU35-IRQ, MG925783:MU35-IRQ) sequences clustered in distinct clades, both of them displaying high values of branch support. Brazilian strain 107 (highlighted in blue in Figure 9) is located within the HAdV-F41 clade. Another seven Brazilian HAdV-F strains (highlighted in magenta in Figure 9) are related to the HAdV-F40 genotype. The tree also demonstrated that reference sequences tend to cluster together, probably a bias of the oversampling from the Araguaina county area. For comparative purposes, trees using the putative E2B_DNApol, Hexon, Penton base and pTP proteins were also constructed (Figures 10 and 11). All partitioned trees presented high branch support and showed almost all references clustering together.



OBS.: Maximum-likelihood tree constructed with all available near full-length genomes of HAdV-F. The Brazilian strains are highlighted in blue and reference sequences of HAdV-A, HAdV-B, HAdV-C, HAdV-D, HAdV-E and HAdV-G (GenBank numbers: NC_001460, NC_011202, NC_001405, NC_010956, NC_003266 and DQ923122, respectively) are in black. The coloured scale in the upper-left area indicates the statistical support of each node calculated using the approximate likelihood ratio test (aLRT). Four HAdV-F Brazilian strains (BRTO025, BRTO032, BRTO033, BRTO035, BRTO217, BRTO222 and BRTO223) grouped within the genotype 40 clade; all other Brazilian HAdV-F strains (n=107) grouped into the genotype 41 phylogroup. Phylogenetic groups corresponding to HAdV-F genotypes 40 and 41 are indicated in the tree and their GenBank IDs are: MK955318, MK955319, MK955317, MK955316, MK955315, L19443, MK883611 and KU162869 for genotype 40; and MK962809, MK962810, KF303070, KF303069, MK962808, KF303071, KY316164, AB728839, MK883610, KX868523, KY316161, MH465394, HM565136, KY316160, KY316162, DQ315364, KY316163, MK962806, MK962807, MG925783 and MG925782 for genotype 41. The scale bar under the tree represents the nucleotide substitutions per site.

Figure 7: Phylogenetic tree of positive Brazilian HAdV-F Tocantins strains, northern Brazil, 2010–2016.



OBS.: (A) Maximum likelihood tree constructed with E2B DNApol gene (3660 nucleotides).
(B) Maximum likelihood tree constructed with the hexon gene (3880 nucleotides). The Brazilian strains are in blue while reference sequences are in black color in the tree. A colored scale is shown in the left upper area to indicate the statistical support of each node, each of which was calculated using the approximate likelihood ratio test (aLRT). Four HAdV-F Brazilian strains (BR030A, BR032, BR033 and BR034A) grouped within the genotype 40 clade, all others Brazilian HAdV-F strains (n=107) were grouped in the genotype 41 phylogroup. Phylogenetic groups corresponding to HAdV-F genotype 40 and 41 are indicated in the tree and their Genbank IDs are: NC_1454 and KU162869 for genotype 40; and MG925783, MG925782, KX868523, AB728839, KF303071, KY316164, KF303069, KF303070, DQ315364, KY316163, HM565136, MH465394, KY316060, KY316162 and KY316161 for genotype 41. The scale bar under the tree represents the nucleotide substitutions per site.

Figure 10: Phylogenetic trees of positive Brazilian HAdV-F Tocantins strains, Northern Brazil, 2010-2016.

OBS.: (A) Maximum likelihood tree constructed with pTP orf (1920 nucleotides).
(B) Maximum likelihood tree constructed with the penton base orf (1500 nucleotides). The Brazilian strains are in blue while reference sequences are in black color in the tree. A colored scale is shown in the left upper area to indicate the statistical support of each node, each of which was calculated using the approximate likelihood ratio test (aLRT). Four HAdV-F Brazilian strains (BR030A, BR032, BR033 and BR034A) grouped within the genotype 40 clade, all others Brazilian HAdV-F strains (n=107) were grouped in the genotype 41 phylogroup. Phylogenetic groups corresponding to HAdV-F genotype 40 and 41 are indicated in the tree and their Genbank IDs are: NC_1454 and KU162869 for genotype 40; and MG925783, MG925782, KX868523, AB728839, KF303071, KY316164, KF303069, KF303070, DQ315364, KY316163, HM565136, MH465394, KY316060, KY316162 and KY316161 for genotype 41. The scale bar under the tree represents the nucleotide substitutions per site.

Figure 11: Phylogenetic trees of positive Brazilian HAdV-F Tocantins strains, Northern Brazil, 2010-2016.

5.3 RECOMBINATION ANALYSIS AND GENETIC DISTANCES

The genetic distances of HAdV-F41 and HAdV-F40 were calculated in order to determine the diversity of these strains. The mean genetic distances of each genome and E2B DNApol, pTP, hexon and pentose base ORF region of the Brazilian HAdV-F strains are shown in Table 1. The overall diversity observed in Brazilian HAdV-F41 (0.09±0.001) was higher than that for the reference HAdV-F41 strains (0.02±0.001). Likewise, the diversity observed in Brazilian HAdV-F40 strains was superior to that of the HAdV-F40 references (0.01±0.001). The HAdV-F diversity noted in Brazilian strains may indicate that HAdV-F is highly spread in the regions surveyed or an artefact of the oversampling surveyed area.

5.4 GENETIC DISTANCES OF BRAZILIAN HADV-C

Viral sequences were identified through sequence identity (using BLAST) to annotated viral genomes in GenBank. Near full-length genomes were used to estimate the evolutionary distances of the strains detected here to HAdV-C reference strains. Once the mean genetic distances within each type were less than 10%, one reference sequence per type was selected in order to calculate pairwise distances between Brazilian and reference strains. The highest distance to strain HAdV-C BR-211 was with type 5 (43%), and the lowest was with type 57 (less than 4%). Equally, the highest distance to strain HAdV-C BR-245 was with type 5 (68%) and the lowest with type 1 (29%). The distances between HAdV-C BR-211 and HAdV-C BR-245 were 65%. It is important to mention that the higher distances of HAdV-C BR-245 are partially due to the poor quality of these sequences, which have many ambiguities and multiple gaps. To provide more detailed estimates and avoid errors of genome regions with poor quality, we also estimated distances between penton base, hexon and fiber genes separately (Table 5). Based on the closest evolutionary distances (Boldface words in Table 5), HAdV-C BR-245 can be classified as P1H1F1 and BR-211 classified as P89H1F89. Overall, genetic distances are in consensus with Blast results. Phylogenetic analysis confirms that strain BR-245 can be classified as HAdV-C1 and that strain HAdV-C BR-211 is possibly a recombinant strain.

Table 4: Genetic divergence of HAdV-C BR-211 and HAdV-C BR-245 strains.

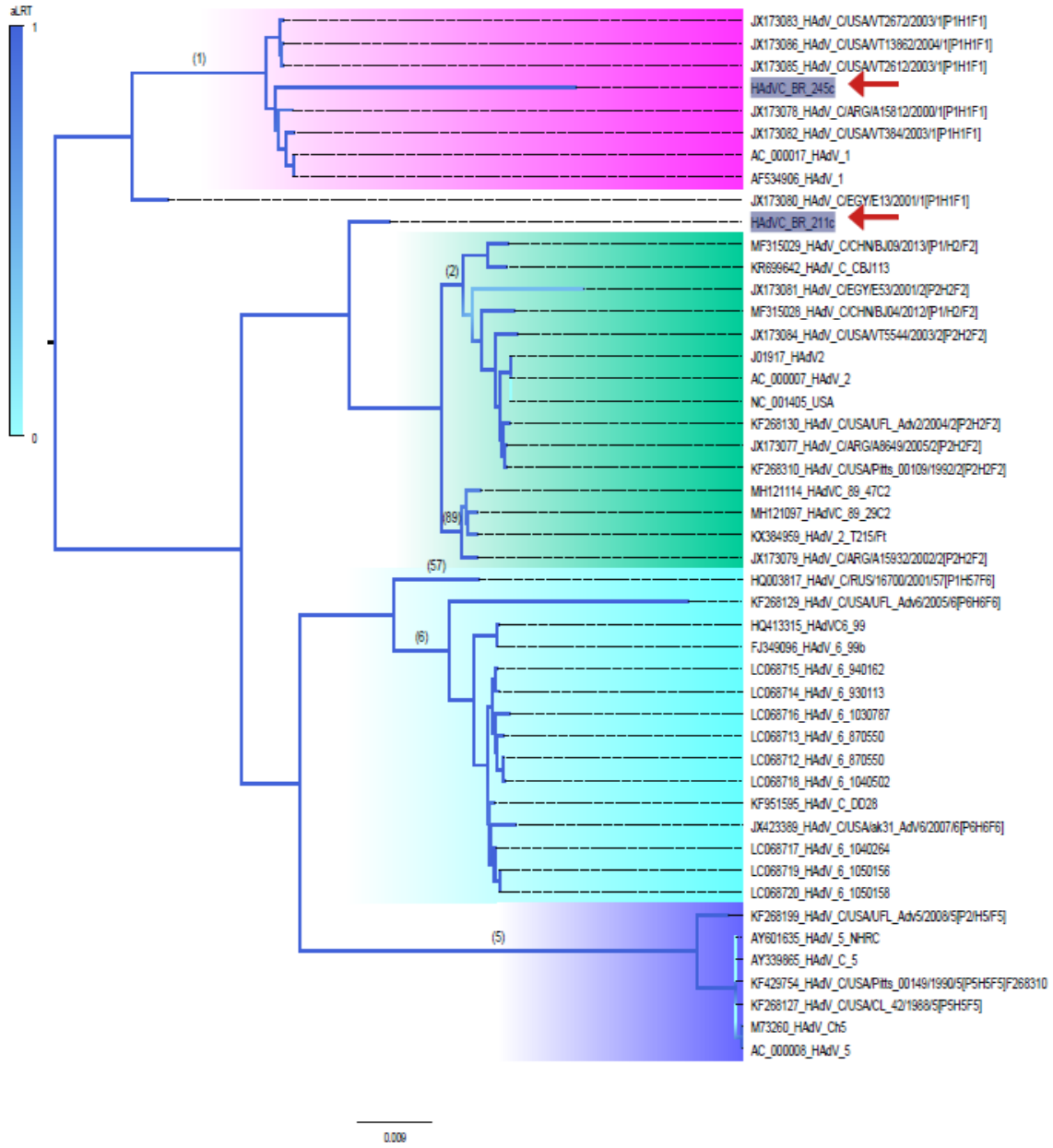
Gene region	Type 1	Type 2	Type 5	Type 6	Type 57	Type 89	Strain
Penton base (P)	0.00886	0.01065	0.01424	0.00886	0.00896	0.00510	BR-211
	0.00235	0.00886	0.01663	0.00471	0.00335	0.01543	BR-245
Hexon (H)	0.00242	0.16695	0.20007	0.19355	0.13400	0.17017	BR-211
	0.00446	0.017060	0.20023	0.19549	0.14343	0.17385	BR-245
Fiber (F)	0.777510	0.004093	0.722381	0.685276	0.692742	0.000994	BR-211
	0.025583	0.775442	0.691595	0.598394	0.605335	0.779578	BR-245

OBS.: Genetic distances were calculated using Maximum composite likelihood implemented in Mega X. Those based on the closest evolutionary distances are in boldface.

5.5 PHYLOGENETIC ANALYSIS OF NEAR FULL-LENGTH GENOMES OF HADV-C

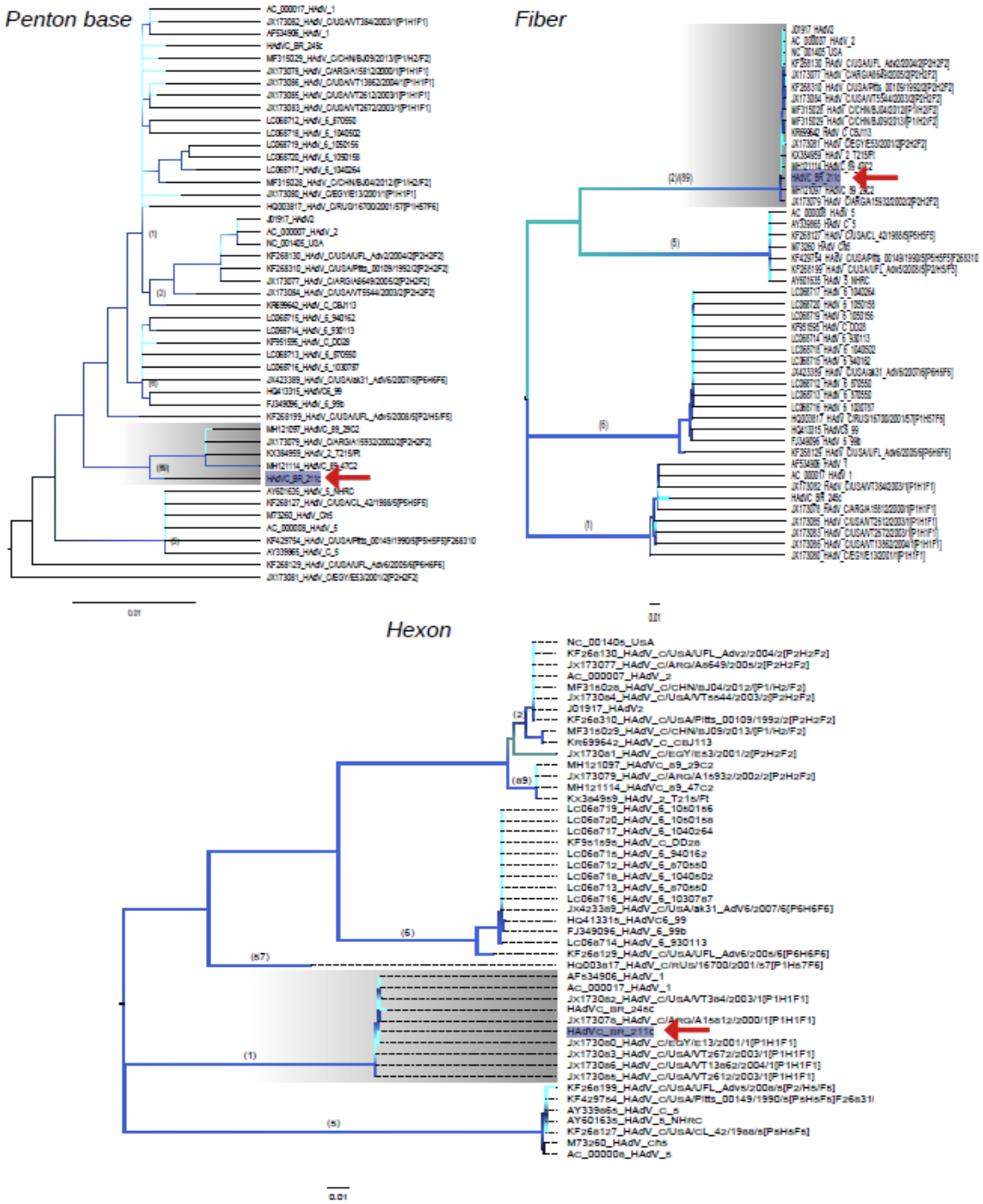
The Maximum Likelihood tree shown in Figure 12 was inferred using all near full-length genomes of HAdV-C available. Sequences formed well-supported monophyletic groups, represented by their corresponding prototype: types 1, 2, 5, 6, 57 and 89. Based on the phylogenetic tree, the HAdV-C BR-245 detected here grouped into the HAdV-C type 1 as expected. On the other hand, HAdV-C BR-211 strain was placed at the base of the clade formed by HAdV-C2 and HAdV-C89. It is important to mention that HAdV-C89 has been recently classified as a consequence of multiple recombination events (Rivailler et al., 2019; Yang et al., 2019; Mao et al., 2017).

In order to classify HAdV-C BR-211, the three coding regions (penton base, hexon and fiber) were analyzed separately. Each region was used to infer phylogenetic relationships between HAdV-C BR-211 and reference strains. All genetic trees displayed high bootstrap values based on approximate LRT. The phylogenetic analysis of the partitioned regions confirmed that strain HAdV-C BR-211 exhibited a close genetic relationship to HAdV-C-89 strains in its penton base and fiber regions, while the hexon gene grouped into the HadV-C type 1 (Figure 13).



OBS.: Red arrows point to the Brazilian strains described in the present study. Each node and corresponding branches are colored according to their statistical likelihood, calculated using aLRT. Numbers above branches of each phylogroup correspond to HAdV-C types, and the overarching clades they belong to are highlighted in different colors. The scale bar under the tree represents nucleotide substitutions per site. The Maximum Likelihood tree was inferred assuming GTR + G model and was constructed using the software FastTree (Price et al., 2010).

Figure 12: Maximum Likelihood tree constructed using near-full length genomes of HAdV-C.

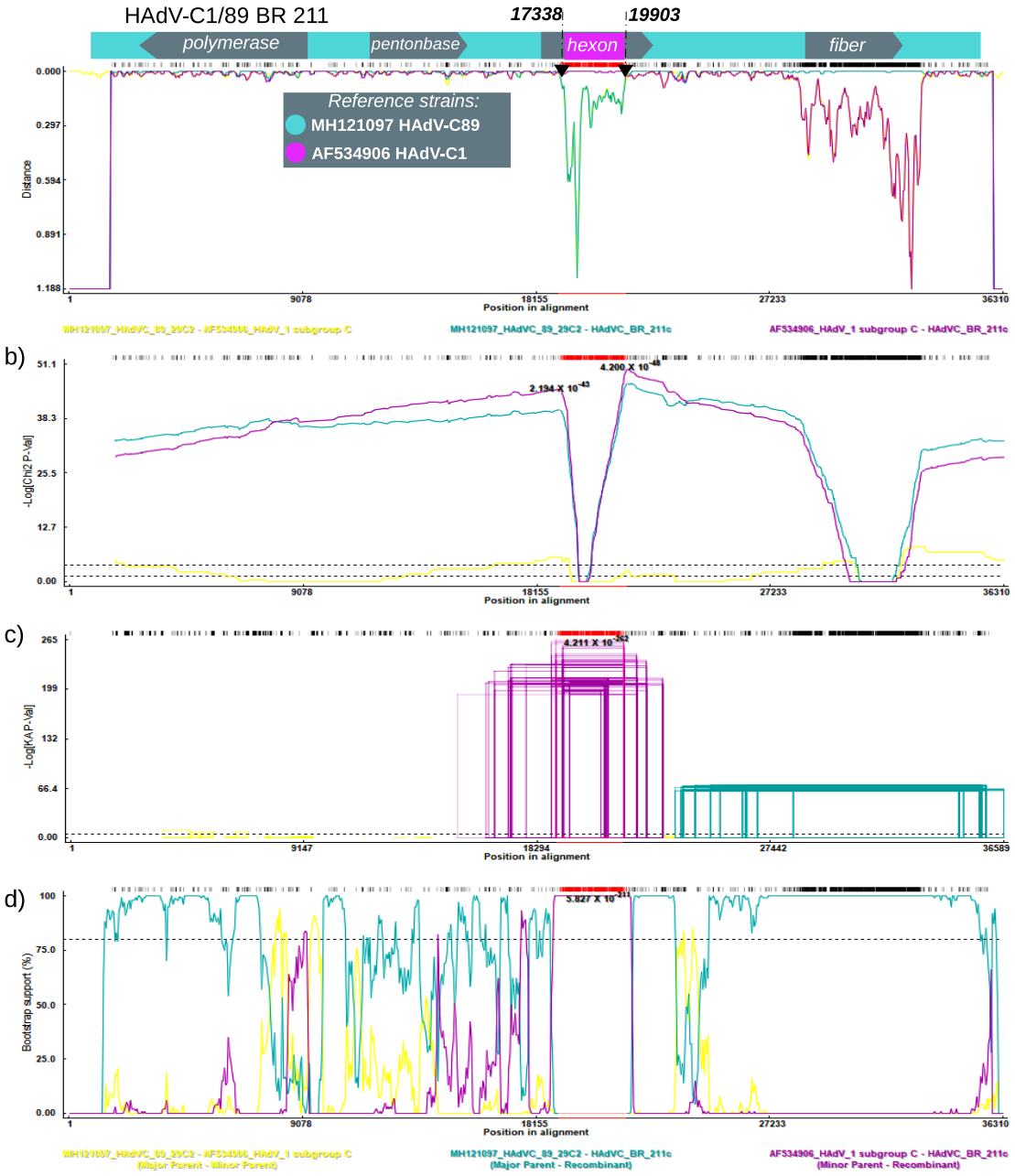


OBS.: A red arrow points to the Brazilian strain HAdV-C BR-211.Numbers above branches of each phylogroup correspond to HAdV-C types. Phyloclades in which the Brazilian strain HAdV-C BR-211 belongs are highlighted in grey. Each node and corresponding branches are colored according to their statistical likelihood, calculated using aLRT. The scale bar under the trees represents nucleotide substitutions per site. Maximum Likelihood trees were inferred using the most likelihood model according to the aLRT. All trees were constructed using the software FastTree (Price et al., 2010)**.**

Figure 13: Maximum Likelihood trees comparing penton base, hexon and fiber regions of sample and reference HAdV-C strains.

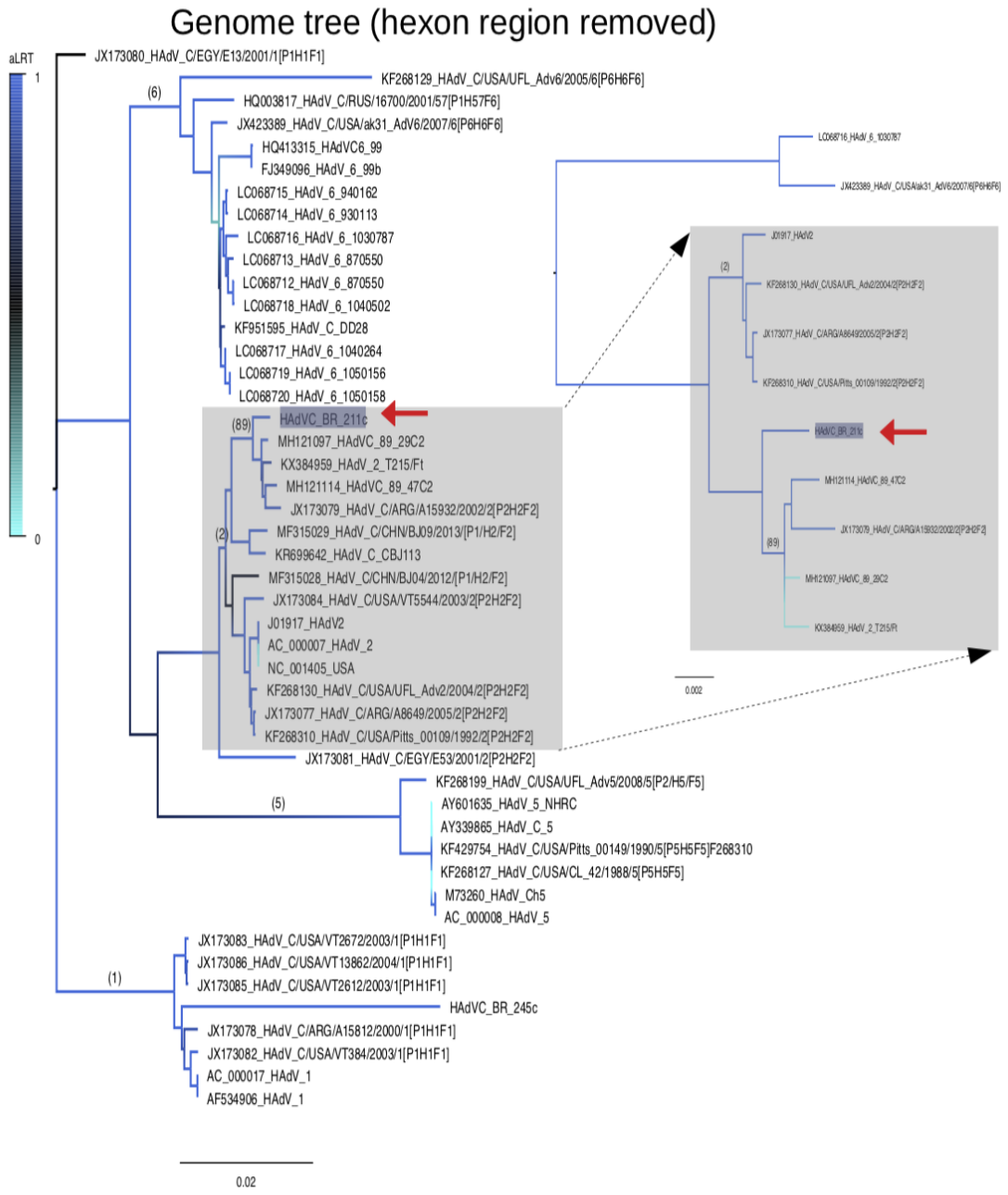
5.6 RECOMBINATION OF HAdV-C1 AND HAdV-C89

Recombination analysis of HAdV-C BR-211 strain (supposed P89H1F89 recombination) revealed a unique and unexpected feature: the presence of a potential chimeric hexon gene derived from HAdV-C1 and HAdV-C89. Two breakpoints located at positions 17,338 and 19,903 in the HAdV-C BR-211 genome were identified. The region delimited by the breakpoints is related to HAdV-C1 and was mapped within the hexon gene sequence, starting at position 17,186 and ending at position 20080. Hence, the HAdV-C BR-211 strain possesses a genomic backbone of type HAdV-C89 with a unique insertion of HAdV-C1 in the hexon sequence. The mosaic pattern of HAdV-C BR-211 is shown in Panel A in Figure 14, highlighting its composition of DNA from two distinct HAdV-C strains (represented by colored lines). To support our findings, the hexon gene sequence was partitioned in two: the first partition corresponds to nucleotides 1 to 17,000, and the second partition corresponds to the nucleotides 20,100 to 33,837, excluding the supposed HAdV-C1 insertion. Maximum Likelihood trees were constructed for each partition and confirmed that HAdV-C BR-211 belongs to type 89 in both regions (Panels B and C in Figure 14). We also constructed a genome tree without the hexon gene region (Figure 15) and characterized mosaic genome of BR-211 by other methods (Figure 16).



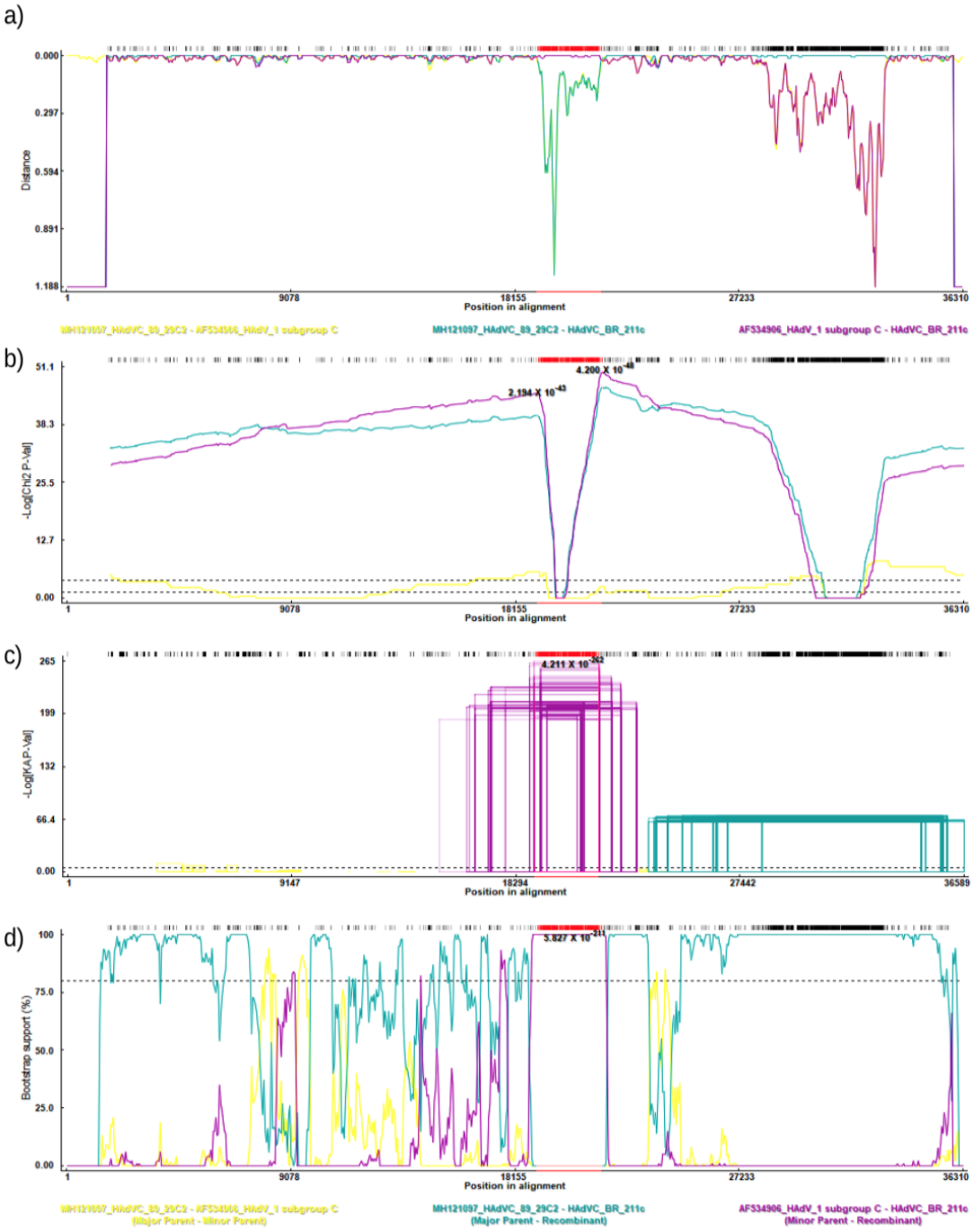
OBS.: (A) The Burt method was used to determine the parental types that compose the recombinant HAdV-C BR-211 strain. Colored lines represent the probability (given by the hidden Markov model) of genomic regions belonging to a certain parental HAdV-C type (greenish blue line – type 89 and magenta line – type 1). The x-axis represents the sequence length in base pairs (bp). The y-axis represents the probability at each base. Only results with a probability above 0.95 were considered. In the upper region of the figure a hatched horizontal line represents the informative genome site used to determine recombination, red area is the interval of breakpoints. Vertical gray lines indicate the confidence interval of breakpoints. All these analyses were performed using the RDP v4 software (Martin et al., 2015). Maximum Likelihood trees were constructed with an alignment corresponding to the genome positions 1 to 17000
(B) and with alignment corresponding to positions 20100 to 33837
(C). Red arrows indicate the Brazilian strain HAdV-C BR-211 and phyloclades formed by HAdV-C89 sequences are also indicated. The scale bar under the tree represents the nucleotide substitutions per site. Maximum Likelihood trees were inferred using the most likelihood model according to the aLRT. All trees were constructed using the software FastTree (Price et al., 2010).

Figure 14: Recombination pattern of chimera strain HAdV-C BR-211.



OBS.: Maximum likelihood tree constructed using near-full length genome of HadV-C in which the hexon gene region was excluded. The Brazilian strain BR211 described in the present study is indicated in the tree by red arrow. A colored scale indicating the statistical support of each node, calculated using aLRT, is shown in the tree. Phylogenetic groups corresponding to main genotypes are indicated by the numbers above branches of each phylogroup. The scale bar under the tree represents the nucleotide substitutions per site. A subtree constructed with few strains of types 2, 89 and 6 is also shown in the image. Maximum likelihood tree was inferred assuming GTR+gamma model and was constructed using the software FastTree v2.

Figure 15: Phylogenetic tree of HAdV-C genomes.

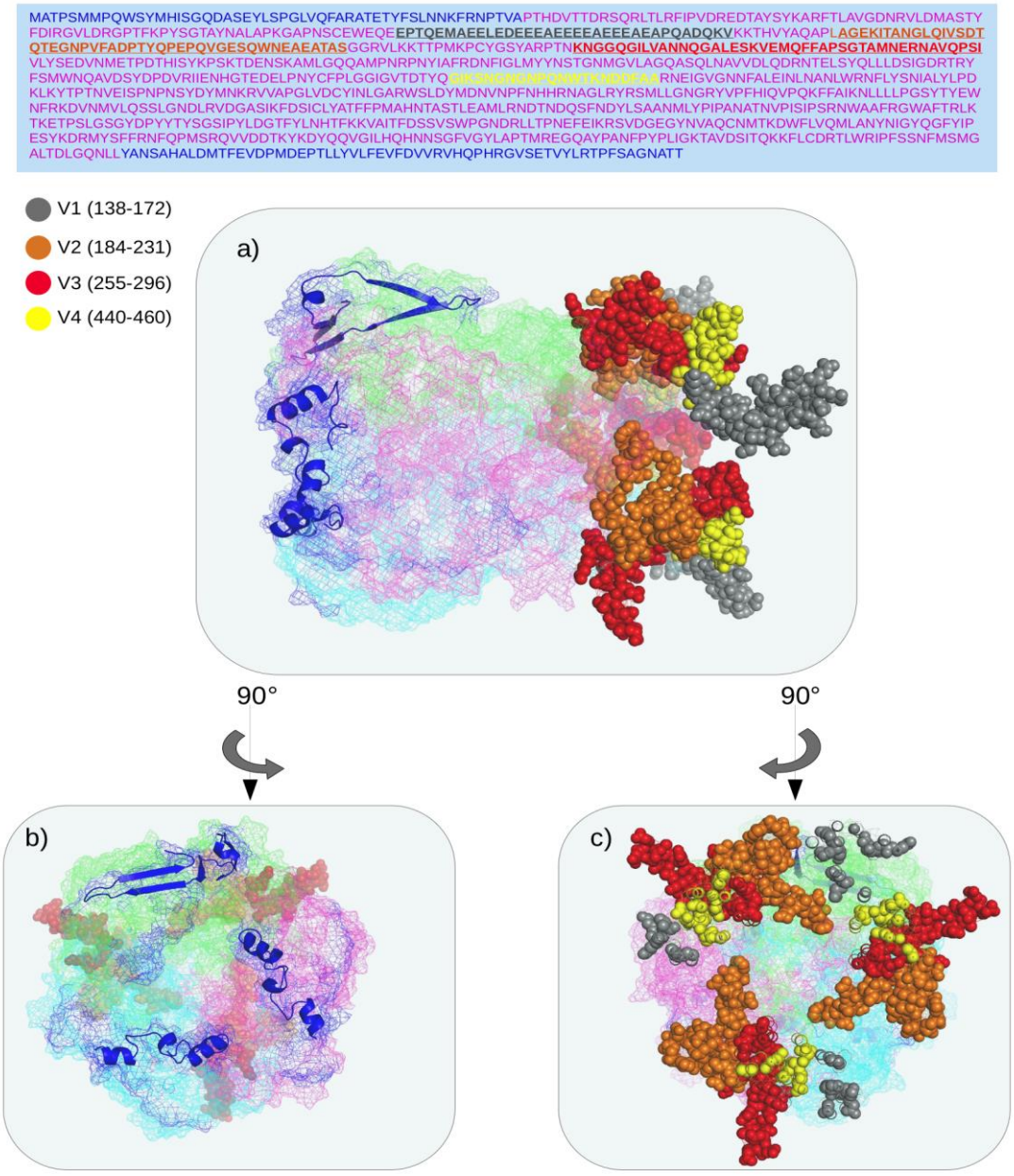


OBS.: Recombination pattern of chimera strains HAdV-C BR 211. The mosaic pattern of BR 211 is shown using distinct recombination methods that were described in material and methods section. **A)** The distance plot method. **B)** MaxChi method. **C)** Genconv method. **D)** Bootscanning method. Colored lines represent the probability (given in hidden Markov model approach) of genomic regions belonging to a certain parental HAdV-C type in this case type 89 (greenish blue line) and type 1 (magenta line). The x-axis represents the sequence length in base pairs (bp). The y-axis represents the statistical support in probability higher than 0.95. In the upper region of the figure a hatched horizontal line represent informative genome site used to determine recombination, red area is the interval of breakpoints. The confidence interval of breakpoints is indicated by vertical gray lines. All these analyses were performed using the RDP v4 software.

Figure 16: Genome mosaic pattern of HAdV-C 211.

5.7 STRUCTURE OF RECOMBINANT HEXON PROTEIN OF HADV-C

We were interested in understanding whether there were differences in the conformation of a chimeric hexon, so the structure of the hexon of the HAdV-C BR-211 chimera was investigated. Based on the high BLASTp similarity score between the amino acid sequences of the hexon proteins of HAdV-C BR-211 and HAdV-C2, the hexon protein structure 1P2Z from a HAdV-C2 strain was selected as a template to predict the structure of the HAdV-C BR-211 chimeric hexon. The HAdV-C BR-211 sequence was mapped onto the HAdV-C2 structure. Of note, the variable regions, present on the apical side of the hexon proteins, facing the outside of the virion, are derived from HAdV-C1, but remain unique (Figure 17). Variable regions of each monomer are interlaced to form the trimeric structure.



OBS.: The amino acid sequence of the hexon protein of the HadV-C BR-211 strain is shown in the upper panel of the image. Residues depicted in blue are those related to HAdV-C89 and residues in magenta are those related to HadV-C1. Variable regions, related to HAdV-C1, and present on the apical side of the protein, are indicated in colors: gray (V1: residues 138-172), orange (V2: residues 184-231), red (V3: residues 255-296) and yellow (V4: residues 440-460). (A) Side view of the predicted 3D model of the chimeric hexon protein of the HadV-C BR-211 strain based on the crystal structure of HAdV-C2 (PDB; 1P2Z). Mesh representation of each monomer of the hexon protein are distinguished by color: cyan, magenta and light green. Blue structures in the 3D model highlight regions of the HadV-C BR-211 hexon protein related to HAdV-C89. Variable regions (V1, V2, V3 and V4) of the hexon protein are shown as colored spheres in the 3D model. (B) and (C) show, respectively, left and right-sided views of the 3D hexon model. The homology model was based on highest BLASTp scores and was using Swiss-model tools (Arnold et al., 2006; Remmert et al., 2011).

Figure 17: Homotrimeric structure of chimeric hexon protein of HadV-C BR-211 strain.

5.8 CHARACTERISTICS OF PENTON BASE OF HADV-C BR-211

Trees constructed with the nucleotide or amino acid sequences of the penton base gene showed that the HAdV-C BR-211 strain forms a clade with HAdV-C89. Recombination analysis did not reveal any breakpoints in this region. Recently, HAdV-C89 was characterized as a new HAdV-C type, and this was achieved based on penton base residue analysis (Dhingra et al., 2019). After initial viral attachment, the penton base interacts with cellular integrins through an Arg-Gly-Asp (RGD) motif located in a hypervariable surface loop. This process results in virus internalization (Zubieta et al., 2006). It is with this hypervariable surface loop that penton base proteins can be distinguished. Key residues of the surface loop used to distinguish HAdV-C89 from HAdV-C2 (Yang et al., 2019) were used here to differentiate HAdV-C BR-211 from the other HAdV-C types. Predicted amino acid sequences of HAdV-C BR-211 penton base was compared to the amino acid sequences of reference strains. Table 6 summarizes these findings. Of the key residues on the hypervariable surface loop, HAdV-C BR-211 shares all the same residues as HAdV-C89 except for an amino acid substitution in strain HAdV-C BR-211 at position 157(K→N). In addition, motif ^{361}AAAP^{364}, present in HAdV-C BR-211 and other HAdV-C types like type 2, is absent in HAdV-C89. These features, which distinguish HAdV-C BR-211 from HAdV-C89, are in boldface in Table 6.

Table 5: Differences at the variable region of penton base of HAdV-C strains.

Penton base position	HAdV-C				
	BR-211	**Type 89**	**Type 2**	**Type 5**	**Type 1**
2	R	R	Q	R	R
153	Q	Q	L	P	L
157	**K**	**N**	K	N	K
312	S	S	N	S	N
458	R	R	S	R	R/S
361-364	**AAAP**	**Del.**	AAAP	AAAP	AAAP
367-369	EAA	EAA	Del.	Del.	EAA

IUPAC amino acid code. A = Alanine, C = Cysteine, D = Aspartic Acid, E = Glutamic Acid, F = Phenylalanine, G = Glycine, H = Histidine, I = Isoleucine, K = Lysine, L = Leucine, M = Methionine, N = Asparagine, P = Proline, Q = Glutamine, R = Arginine, S = Serine, T = Threonine, V = Valine, W = Tryptophan, Y = Tyrosine. Del. = Deletion. These features, which distinguish HAdV-C BR-211 from HAdV-C89, are in boldface.

6. DISCUSSION

In this study, NGS was used to assess enteric HAdV frequency and diversity in rural and low-income urban areas in northern Brazil. The value of NGS in HAdV detection and characterization has been described before (Zhang et al., 2018; Martínez-Puchol et al., 2020; Mohammad et al., 2020; Moore et al., 2015), including in Brazil (Duarte et al., 2019). The frequency of HAdV-F infections detected in this study (n=251; 57.8%) using NGS was higher than that observed in other studies carried out in Ethiopia (n=450; 32%) (Gelaw et al., 2019), Thailand (n=2312; 7.2%) (Kumthip et al., 2019), Kenya (n=278; 37.4%) (Magwalivha et al., 2010), Iraq (n=155; 34.2%) (Harb et al., 2019), Iran (n=2682; 5.2%) (Dashti et al., 2016), Bangladesh (n=871; 10.7%) (Afrad et al., 2018), Albania (n=142; 23.2%) (La Rosa et al., 2015) and PR China (n=2233; 10.9%) (Liu et al., 2014) using conventional PCR; Tanzania (n=1235; 3.5%) (Moyo et al., 2014) and the Republic of Congo (n=655; 5.5%) (Mayindou et al., 2016) using an enzyme-linked immunosorbent assay (ELISA); and Turkey (n=1154; 26.2%) (Ozsari et al., 2016) using an immunochromatographic test. The HAdV-F frequency obtained here was also greater than that for previous studies conducted in Brazil using distinct screening methodologies: in São Paulo (n=193; 3.1%) (1987–1988) (Timenetsky et al., 1993), Goiânia (n=557; 2.1%) (1987–1990) (Cardoso D. D. D. et al., 1992), Porto Velho (n-591; 2.0%) (2010–2012) (Amaral et al., 2015), Campo Grande (n=415; 3.6%) (2000–2004) (Andreasi et al., 2008), Belo Horizonte (n=392; 4.5%) (2005–2006) (Duarte, Mendes, Penna, Filho, & Magalhães, 2012), Juiz de Fora (n=377; .5%) (2007–2011) (Reis et al., 2016), Curitiba (n=255; 16.0%) (2010–2011) (Raboni et al., 2014), Belém do Pará (n=172; 43.0%) (1990–1992) (L. C. P. das N. Costa et al., 2017), Porto Velho (n=877; 6.4%) (2000–2002) (Magalhães et al., 2007) and the midwestern, south-eastern and southern regions (n=3003; 3.9%) in 2012–2017 (Primo et al., 2018).

Table 6: Pair-wise nucleotide distances of positive Brazilian HAdV-F Tocantins strains, Northern Brazil, 2010-2016.

HAdV-F	Genetic distances (standard error)*				
	Near-full genome	*E2B DNA pol gene*	*pTP gene*	*Hexon gene*	*Pentose Base gene*
Genotype 41 (n=107)	0.09±0.001	0.04±0.001	0.07±0.001	0.05±0.001	0.04±0.001
Genotype 40 (n=7)	0.07±0.001	0.08±0.01	0.04±0.01	0.03±0.01	0.05±0.02

- Calculation of the mean evolutionary distance was performed using Mega X software. Genetic distances were calculated using maximum composite likelihood and equal rates as implemented in the Mega software. All bootstrap analysis was performed using 1000 replications.

This discrepancy concerning HAdV prevalence should be considered carefully and with caution. The inconsistencies may be associated with differences in study design, settings, diagnostic methods, regional and temporal variation, sample storage conditions, the number of specimens tested and non-introduction of RVA immunization (Gray et al., 2007; Arowolo et al., 2019). Nevertheless, unexpectedly, similar differences in enteric HAdV prevalence were found in a multicentre study covering several countries in sub-Saharan Africa and Asia (Kotloff et al., 2012), employing a standardized detection protocol.

Gender has no recognizable role in HAdV infection, including in Brazil (Primo et al., 2018; Kumthip et al., 2019; Liu et al., 2014; Duarte et al., 2012). The suggested tendency of HAdV-F infections occurring more frequently in males, as found here, originates in sample collection bias itself, which has already shown a significant difference in relation to the gender of patients at the time of collection. A marked HAdV-F positivity rate was identified in children ≤5years, reinforcing the global observation that HAdV is an important pathogen in diarrhoeal disease in children (Primo et al., 2018; Afrad et al., 2018; Filho et al., 2007; Kumthip et al., 2019). Year-to-year fluctuations in HAdV-F positivity in the northern region of Brazil varied considerably, but no significant difference was observed. The divergences among the years seem to be related to the number of specimens tested from the convenient sampling.

The present data showed that there was a high percentage (37.4%) of co-infection in this study among HAdV-F-positive samples. The results obtained here were

consistent with those of previous studies, which have reported worldwide occurrence rates of viral co-infections ranging from 8.0–44.8% (Arowolo et al., 2019; Ferreira et al., 2012; Ouyang et al., 2012; Shigemoto et al., 2017; Akdag et al., 2020; Aktaş et al., 2019; Tatte & Gopalkrishna, 2019). The most common co-infection obtained was a dual infection with HAdV-F and RVA, with a detection rate of 21.5%, in contrast to reports from India (16.1%) (Tatte & Gopalkrishna, 2019), France (16%) (Tran et al., 2010), Nigeria (5.9%) (Arowolo et al., 2019) and previous studies conducted in Brazil (8.3%) (Raboni et al., 2014) with lower detection rates. This disparity among studies may be due to the number of viruses analysed, viral detection methods, season of sampling and sample size. Some studies have suggested that mixed infections may be responsible for more severe diarrhea (Román et al., 2003; Taylor et al., 1997), and it is assumed that once RVA infections cause enterocyte destruction from the top of the intestinal villus, the risk of opportunistic pathogen infection is increased (Chhabra et al., 2013).

A predominance of HAdV-F41 (94.5%) was noted throughout the investigation, corroborating with data obtained in studies conducted globally (Primo et al., 2018; Reis et al., 2016; Lennon et al., 2007). A downward tendency in HAdV-F40 detection has been observed worldwide, including in Brazil (Moyo et al., 2014; Primo et al., 2018; Reis et al., 2016; Filho et al., 2007; Soares et al., 2002). Studies suggest that an antigenic drift of HAdV-F41 led to its increasing prominence at the expense of HAdV-F40 (Moyo et al., 2014; Shimizu et al., 2007). HAdV-F genome sequences are largely conserved from isolate to isolate of the same type over time, as expected for dsDNA genomes (Seto et al., 2010; Mahadevan et al., 2010; Ismail et al., 2018). The phylogenetic analysis showed that the Brazilian HAdV-F sequences clustered together with sequences originating from distinct continents, suggesting that the HAdV-F strains circulating in Brazil were closely related to those strains circulating worldwide, and there is no evidence for the introduction of a particular HAdV-F variant in the country. The existence of some temporal order on genetic HAdV-F sequences was not observed. Although an increasing prevalence of HAdV- F41 was reported in AGE (Moyo et al., 2014; Primo et al., 2018; Li et al., 2017), only a few complete genomes of HAdV-F41 are currently available in the GenBank database. These are the first near full-length genomes sequences of HAdV-F40 and 41 acquired from Brazil. More complete genome sequences are needed to gain in-depth understanding of the molecular epidemiology and pathogenesis of HAdV-F species.

We characterized the near full-length genomes of two Brazilian HAdV-C strains. The detection of HAdV-C strains in stool samples may not necessarily be associated with diarrhea symptoms, as HAdV can exhibit a lingering shedding in feces after previous infection of other organs (Afrad et al., 2018). In fact, HAdV-C1, to which the HAdV-C BR-245 strain is identified here, is frequently associated with respiratory infections (Ghebremedhin, 2014). In addition, patient HAdV-C BR-245 tested positive for Norovirus infection. Patient HAdV-C BR-211 was experiencing respiratory symptoms in addition to gastroenteritis.

Previous studies have reported the detection of HAdV-C1 strains in Brazil associated with both respiratory and gastrointestinal symptoms (Ghebremedhin, 2014; Primo et al., 2018). Nevertheless, to the best of our knowledge, this is the first description of the full-length genome of a recombinant HAdV-C strain (BR-211) in the country. The HAdV-C1 BR-245 strain revealed to be related to Argentinean and North American HAdV-C1 strains according to our analysis, suggesting that this particular HAdV-C1 strain may have been circulating in the North and South American continents. However, the dataset used for this analysis presented an important limitation: the number of sequences analyzed was small, a restriction mainly due to the fact that we chose to look at only complete or near full-length genome sequences.

More in-depth analyses of HAdV-C1 strains detected worldwide involving full genomic characterization are needed in order to expand our understanding of HAdV-C1 genetic diversity.

NGS surveillance, described in the present investigation to study enteric viruses, has provided an opportunity to identify for the first time in Brazil a strain, BR-211, belonging to a new HAdV-C type derived from the recently described HAdV-C type 89. Because HAdV-C89 was only recently described as a novel HAdV-C strain (Dhingra et al., 2019), knowledge of the epidemiology and prevalence of HAdV-C89 is still very limited. This new HAdV-C type could only be determined based on the sequencing of its entire genome, since approximately 80% of the viral sequence shares high similarity to HAdV-C2 strains (Dhingra et al., 2019). Hence, without analyzing the whole genome of BR-211, it would have been virtually impossible to confirm its type and origins. Recombination events between the genes encoding major capsid proteins (hexon, fiber and penton base) are known to play an important role in the evolution of

HAdV-D types (Primo et al., 2018), but members of the HAdV-C species are considered to be more stable, undergoing fewer recombination events (Dhingra et al., 2019; Mohamed Ismail et al., 2019). HAdV-C types 57 and 89 are exceptions to this rule (Dhingra et al., 2019; Alonso-Padilla et al., 2016). The present study also identified, in HAdV-C BR-211, a chimeric hexon protein combining type 1 and type 89 sequences. Moreover, the variable regions in the hexon protein of HAdV-C BR-211 were found to be related to HAdV-C type 1 and not type 89. The variable regions of the HAdV hexon proteins represent the major antigenic portion of the protein, and various neutralizing antibodies have been detected that target them (Feng et al., 2018; Liu et al., 2018; Tian et al., 2018). It also has been shown that site-directed mutations in the variable regions of hexon proteins drastically reduce the replication of HAdV-C (Tian et al., 2018).

Characterizing recombination events allow one to indirectly identify HAdV-C strains that are circulating at a certain location and at a certain time. In other words, recombination means co-infection and therefore co-circulation of different strains (Mao et al., 2017). Our NGS data is not enough to determine the source and HAdV-C BR-211 infection. This strain may have emerged recently by the recombination of strains HAdV-C1 and HAdV-C89 that were co-infecting the patient BR-211. Another equally likely explanation is that patient 211 was infected by a HadV-C1/89 recombinant strain. For this reason, an increase in HAdV-C surveillance in this particular area of Brazil could provide valuable information about the presence and prevalence of strains HAdV-C1 and HadV-C89 and other recombinant HAdV-C strains.

In addition to the chimeric hexon with type 1 and type 89 sequences identified in HAdV-C BR-211, we also found unique signatures in the penton base (Table 6). The HAdV-C89 penton base sequence is highly diverse in the functional RGD loop compared to the HAdV-C types described in the past, which may result in differences in the binding properties of type 89 to secondary cellular receptors. However, whether such amino acid differences in penton base sequences might increase or not the virulence of circulating HAdV-C89 strains has yet to be assessed.

7. FINAL CONSIDERATIONS

The frequency and prevalence of infectious diseases is disproportionately high in developing countries, which lack adequate sanitary conditions as well as financial resources. Seemingly non-complex disease presentations, such as acute gastroenteritis, end up turning into a relevant concern for the public health system, due to the high costs of treatment and maintenance of patients in hospital units.

These observtions strengthen the objectives of this study, which were to investigate the genetic diversity of human adenovirus F infections in northern Brazil within a determined period through using genome sequencing techniques, such as Next-Generation Sequencing (NGS). Another aim was to perform the phylogenetic and bioinformatics analysis to get etiological and epidemiological data that allows a better understanding about the origins, evolution and current relations between different types and serotypes of the human adenovirus.

Diagnosis of adenovirus infection is usually performed by ELISA, for the detection of antigens, and by PCR or electron microscopy to check the viral presence in feces or secretions. Identification of the adenovirus typing and subtyping can only be performed in specialized laboratories. In Brazil, the absence of data on the molecular epidemiology of human adenoviruses makes it hard to understand the causal relationship between the virus and the disease, since the viral frequency and prevalence are not precisely known. The use of new advanced detection techniques, such as viral genomic sequencing, associated with phylogenetic and bioinformatic analysis, could provide trustworthy data, that would make it possible to establish more efficient epidemiological surveillance of human adenoviruses in the country.

Although acute gastroenteritis can also be caused by bacteria and parasites, in children viruses are the main etiological agents of the illness. Human adenoviruses F40/41 (HAdV-F) are the principal disease-causing of diarrhea in children under 5 years of age. We have seen in this study how prevalent HAdV-F (40/41) have been in children under 5 years of age in Tocantins between 2010-2016. Not only have many patients been infected with these viruses, but many have been infected with multiple different viruses at once. Despite a nation-wide campaign to vaccinate all Brazilians against Rotavirus, the virus persists, whilst other gastroenteritis viruses continue to

thrive. By understanding the distribution of these viruses across Tocantins, measures can be taken to control diarrheal disease, and reduce the mortality and threat to livelihood in children that accompanies it. In particular, better understanding the molecular epidemiology of HAdV-F in Tocantins provides information on a pathogen in an endemic region often overlooked.

Considering the importance of this concern, we performed the first large-scale HAdV-F study in Brazil in which whole-genome data and DNA sequence analyses were used to characterize HAdV-F strains. The results show that the sensitivity and efficiency of NGS, has huge of potential for characterizing the viruses found across the world.

One unanticipated finding was that we could identify a potential chimeric hexon gene originated from a genetic recombination between HAdV-C1 and HAdV-C89 strains, which means an alert about viral evolution of HAdVs in the coming years. In addition, further studies regarding this finding are needed. In this finding we described a near full-length genome of two HAdV-C strains from Brazil: the BR-245 strain, which is a common HAdV-C type 1 strain, and the HAdV-C BR-211 strain, a potential new recombinant HAdV-C strain with its own type, made up in part by a chimeric hexon gene with type 1 and type 89 DNA. Routine HAdV-C typing strategies may fail to detect new recombinants, likely resulting in incorrect typing of novel HAdV-C strains and missing the opportunity to discover new HAdV-C recombinants or even entirely new types. Expanding the viral genome database could improve overall typing success and help track the emergence of novel HAdV-C strains, thus helping to understand their evolution. In addition, future studies employing complete genomic sequencing on circulating HAdV-C strains in Brazil are needed to understand the clinical significance of the data presented in this study.

To further work and improve on the findings of this study, many parameters could be altered. A case control or cross-sectional study could be implemented instead of a retrospective study. Sample collection would be evenly distrusted in space and time, without skew in the average age or sex, and sample size would have to be higher to be able to draw more meaningful conclusions based on the results. New findings could be made on the symptoms associated with different viruses in Tocantins, and a true account of the outbreaks in Tocantins over last 7 years could be understood. Generation of full-genome sequences of the viruses presented in this study, and this

information could prove valuable when trying to understand the epidemiology of diarrheal disease in Tocantins.

This investigation has also shown that expanding the viral genome database could improve overall genotyping success and assist the National Center for Biotechnology Information (NCBI)/GenBank in standardizing the HAdV genome records by providing a large set of annotated HAdV-F genomes.

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APPENDIX

Table 7. Sequence raw data of positive Brazilian HAdV-F Tocantins strains, Northern Brazil, 2010-2016.

Sample_ID	GenBank_ID	Length of Consensus Sequence	Coverage*
TO-004	MT777454	33860	>10000
TO-005	MT791001	33491	>10000
TO-006	MT777455	33831	>10000
TO-007	MT826884	33420	>10000
TO-008	MT826888	33398	>13000
TO-009	MT826887	33417	>10000
TO-010	MT826885	33520	>28000
TO-011	MT826886	33518	>10000
TO-012	MT826889	33491	>24000
TO-014	MT777453	33420	>10000
TO-015	MT797127	33861	>10000
TO-017	MT826891	33862	>10000
TO-018	MT797099	33469	>10000
TO-019	MT797100	34263	>10000
TO-020	MT815556	33398	>10000
TO-021	MT815558	33519	>10000
TO-022	MT797101	34259	>10000
TO-023	MT815559	33939	>10000
TO-028	MT797103	33409	>10000
TO-029	MT815557	33396	>10000
TO-030	MT797102	33519	>10000
TO-031	MT797128	33421	>10000
TO-032	MT797104	33677	>10000
TO-033	MT791000	33879	>10000
TO-034	MT815560	33938	>10000
TO-035	MT797105	33897	>10000
TO-036	MT797106	33396	>10000
TO-037	MT797108	33396	>10000
TO-046	MT797107	34195	>10000
TO-052	MT815561	33415	>10000
TO-053	MT815563	33420	>10000
TO-060	MT815562	33414	>10000
TO-062	MT797131	34278	>10000

TO-063	MT777457	33859	>10000
TO-064	MT815564	33420	>10000
TO-065	MT777456	33859	>10000
TO-066	MT797109	34223	>10000
TO-067	MT797113	33998	>10000
TO-068	MT797110	34212	>10000
TO-069	MT797112	34371	>10000
TO-071	MT797111	34290	>10000
TO-072	MT797114	34211	>10000
TO-074	MT815565	33931	>10000
TO-075	MT797115	34204	>10000
TO-076	MT797118	34204	>10000
TO-078	MT815566	33419	>10000
TO-079	MT815567	33418	>10000
TO-082	MT815568	33420	>10000
TO-083	MT797116	34275	>10000
TO-084	MT797117	33412	>10000
TO-085	MT797120	34220	>10000
TO-086	MT797119	34228	>10000
TO-087	MT815569	33519	>10000
TO-088	MT797121	34228	>10000
TO-089	MT815570	33520	>10000
TO-090	MT815571	33854	>10000
TO-091	MT797122	34298	>10000
TO-093	MT797123	34259	>10000
TO-094	MT797125	34207	>10000
TO-100	MT797124	34209	>10000
TO-101	MT797126	33464	>10000
TO-102	MT815572	33420	>10000
TO-103	MT815573	33409	>10000
TO-105	MT815574	33417	>10000
TO-106	MT826890	33418	>10000
TO-107	MT826892	33879	>10000
TO-108	MT826893	33420	>10000
TO-117	MT826894	33416	>10000
TO-118	MT826895	33417	>10000
TO-119	MT797128	33407	>10000

TO-120	MT797127	34130	>10000
TO-131	MT826896	33418	>10000
TO-142	MT826897	33419	>10000
TO-144	MT826898	33419	>10000
TO-153	MT826899	33420	>10000
TO-160	MT797129	33410	>10000
TO-162	MT826900	33419	>10000
TO-165	MT797130	34155	>10000
TO-168	MT797132	33418	>10000
TO-173	MT797133	33421	>10000
TO-178	MT797134	33419	>10000
TO-211	MT826901	33852	>24000
TO-221	MT826902	33938	>24000

* Only sequences with average genome coverage greater than 10000x were considered. For genome assembly, only readings with quality were considered Q score of 30 (Q30)