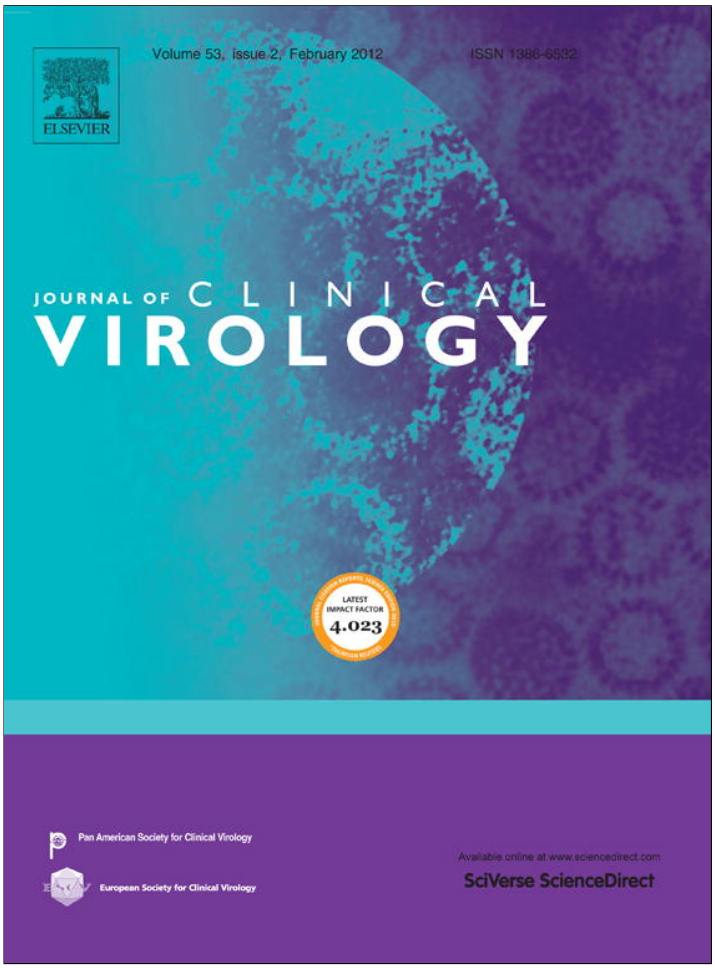

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Molecular typing of adenoviruses in pediatric respiratory infections in Buenos Aires, Argentina (1999–2010)

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ABSTRACT

Background: The human adenovirus (HAdV) types most commonly found in respiratory samples belong to HAdV species C (HAdV-C1, -C2, -C5, and -C6) and to HAdV species B (HAdV-B3 and -B7). Several studies in South America have shown the association between severe respiratory infections and subspecies B1.
Objectives: The aim of this study was to identify the adenovirus types associated with acute lower respiratory tract infections in children, found as single or coinfections, throughout a 12-year period.
Study design: All samples that tested positive for adenovirus by immunofluorescence assay from January 1999 to December 2010 were typed by evaluating a set of four viral genes (E1A, VA, hexon and fiber). Quantitative PCRs for HAdV-B and HAdV-C species were performed to compare the viral load found in single infections and coinfections.
Results: From a total of 743 HAdV, 654 (88%) were single infections and 89 (12%) coinfections. From the 654 single HAdV infections, members of four species were present: species B ($n = 492$, 75.23%), species C ($n = 138$, 21.1%), species E ($n = 19$, 2.91%), and species D ($n = 5$, 0.76%). Only members of species B ($n = 109$, 57.67%) and species C ($n = 80$, 42.33%) were detected in coinfections. HAdV-B7 and HAdV-B3 were the most prevalent types ($n = 308$, 36.54%; $n = 230$, 27.28% respectively) and HAdV-C1, -C2, -E4, -C5, -C6, -D8, -B11, -B14 and -B21 were also detected. Viral loads for species C viruses were higher in single infections than in coinfections ($p < 0.01$), whereas the opposite was observed for species B viruses ($p < 0.0001$).
Conclusions: This study provides a thorough description of adenovirus circulation and diversity in Buenos Aires in a 12-year period. The high proportion of coinfections found in this work shows that this phenomenom might be more common than expected.

1. Background

Human adenoviruses (HAdV) belong to the *Mastadenovirus* genus and are classified into six different species (A–F), with marked differences in tissue tropism and clinical manifestations.[1] The types most commonly found in respiratory samples belong to HAdV species C (HAdV-C1, -C2, -C5, and -C6) and to HAdV species B, subspecies B1 (HAdV-B3 and -B7) and B2 (HAdV-B14), which are endemic and epidemic respectively in pediatric populations.[2–4]

Rare cases of severe infection, outbreaks in closed populations, and even epidemic outbreaks have been associated with the emergence of new types or recombinant and intermediate strains.[4–7] Several studies in South America have shown the association, in particular, between severe respiratory infections and subspecies B1. Among types of subspecies B1, HAdV-B7h, which bears the hexon from HAdV-B7 and the fiber from HAdV-B3, has an increased lethality, which reaches 12–28% of the cases.[8–11] In some cases, lower respiratory infections caused by HAdV-B7 result in the development of chronic lung disease.[12–14]

Nowadays, molecular methodologies enable the identification of adenoviral types, molecular epidemiological analysis and allow detection of coinfections with other adenoviruses which may serve as a source for the emergence of new variants.[15,16] In Argentina, adenovirus infections of young children represent 4–9% of the viral etiology in acute lower respiratory tract infections (ALRI). However, no prevalence data are available for the individual types and only single cases have been reported.[17,18]

Abbreviations: HAdV, Human adenoviruses; IFA, Immunofluorescent Assay; qPCRs, Quantitative PCRs; ALRI, Acute lower respiratory tract infections; NPA, Nasopharyngeal aspirates.

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2. Objectives

The aim of this study was to identify the adenovirus types associated with ALRI in children, found as single or coinfections, by evaluating a set of four viral genes throughout a 12-year period.

3. Study design

3.1. Samples

Nasopharyngeal aspirates (NPA) from children hospitalized with clinical diagnosis of ALRI were received at the Virology Laboratory of "Dr. R. Gutiérrez" Children's Hospital (Buenos Aires, Argentina) from January 1999 to December 2010.

3.2. Viral studies

Rapid detection of adenovirus was performed by indirect immunofluorescence assay (IFA) with monoclonal antibodies (Light Diagnostics, Chemicon Int., Temecula, CA, USA).[19] This research included all samples that tested positive for adenovirus by IFA.

3.3. PCR typing

Molecular typing was performed according to the algorithm described in Fig. 1. For samples that presented more than one type or species, multiplex PCRs were split into monoplex separate assays, and the fiber-based species PCRs were also tested.[20] For samples that tested negative for any of the specific PCRs, other universal PCRs for hexon and penton were performed.[21–23] Samples that were positive only for universal PCRs were counted as non-typeables.

3.4. PCR controls

No template, extraction negative and positive template controls from a diluted isolate for all PCR sets were included in each run. Synthetic positive controls were obtained by cloning amplicons of HAdV-C2 and HAdV-B7 into pGemT® plasmids (Promega Inc, Madison, WI, USA). The sensitivity of the PCR protocols was determined by testing serial dilutions of purified plasmids to calculate the minimum number of copies detected. Specific PCRs were cross-validated by testing types from other species.

3.5. Cell culture

Samples were inoculated into Hep-2 or A-549 monolayers (ATCC CCL-23 and 185), checked daily until the cytopathic effect was evident, and frozen at −20 °C for further molecular characterization.

3.6. Viral load

Quantitative PCRs (qPCRs) for HAdV-B and HAdV-C species were perfomed and the number of copies of each was determined by absolute quantitation by means of a standard curve obtained from serial dilutions of the cloned plasmids.[24] qPCRs were performed in duplicate on an iQ5 real-time PCR detection system (BioRad, Hercules, CA, USA). Runs with efficiencies of $100 \pm 5\%$ and viral loads within the calibration curve ($30–3 \times 10^5$ copies) were used for the analysis. The viral load found in single infections was compared to that of coinfections and results obtained from NPA were compared to those from cell culture isolates. A reference gene (RNase P) was measured for all the samples to standardize the viral load per cell number.[25]

3.7. Statistical methods

Categorical variables were compared by Yates' corrected χ^2-test or Fisher's exact test. The viral load of single infections and that of coinfections were compared by the unpaired t-test (using GraphPad Prism version 5.04 for Windows, GraphPad Software, San Diego, CA, USA). Statistical significance was defined as $p < 0.05$.

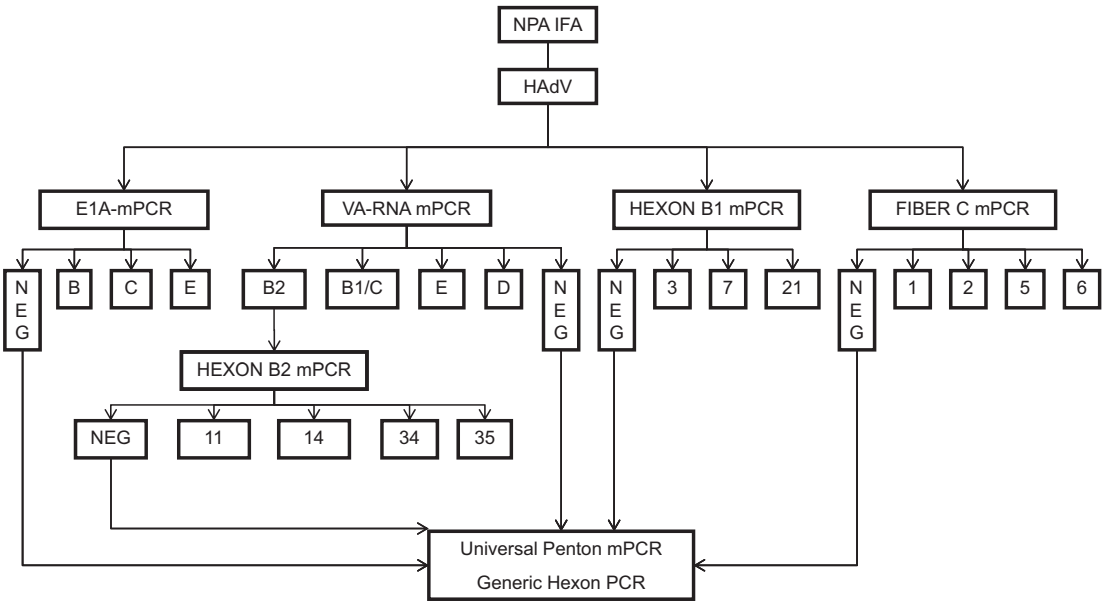

Fig. 1. Algorithm for molecular typing of Respiratory HAdVs. E1A multiplex PCR for HAdV species -B, -C and -E[23]; VA RNA-encoding regions PCR-RFLP to determine species, subspecies and groups of genomic types[31]; hexon gene multiplex PCR for HAdV-B3, -B7, and -B21 from subspecies B1[32]; and a fiber-based multiplex PCR for HAdV-C1, -C2, -C5 and -C6[33] were performed. An hexon gene multiplex to discriminate HAdV-B11, -B14, -B34 and -B35 among B2 subspecies was performed.[23] NPA, nasopharyngeal aspirates; m, multiplex; neg, negative.

Table 1
Summary of the findings. HAdV were included when positive for indirect immunofluorescence (IFA) and further characterized by molecular methods into species (C, D, E), subspecies (B1, B2) or coinfections (MIX).

Year	No. of samples	Positives by IFA	HAdV by IFA	HAdV molecular typing					
				B1	B2	C	D	E	MIX
1999	3684	1050 (28.50)	97 (9.24)	31	3	14	0	2	21
2000	3651	1542 (42.24)	74 (4.80)	28	3	11	0	0	9
2001	3237	1388 (42.88)	54 (3.89)	18	[a]	14	0	0	9
2002	3669	1416 (38.59)	137 (9.68)	89	2	11	0	0	14
2003	4018	1870 (46.54)	202 (10.80)	121	0	21	0	4	9
2004	3542	1682 (47.49)	59 (3.51)	18	1	17	0	7	3
2005	3975	1743 (43.85)	76 (4.36)	47	0	7	1	0	4
2006	4453	1834 (41.19)	47 (2.56)	28	0	10	0	0	5
2007	4800	2011 (41.90)	72 (3.58)	25	17	20	3	0	8
2008	4586	1849 (40.32)	49 (2.65)	33	3	5	0	0	7
2009	4394	1526 (34.73)	16 (1.05)	6	1	4	0	0	0
2010	4535	1441 (31.78)	33 (2.29)	17	0	4	1	6	0
Total	48,544	19352 (39.86)	916 (4.73)	461 (62.05)	31 (4.17)	138 (18.57)	5 (0.67)	19 (2.56)	89 (11.98)

[a] One additional sample was not typed as subspecies and was typed as species B. Number of samples and percentages are expressed for each year.

4. Results

4.1. Viral detection

A total of 48,544 NPA were received at the Virology Laboratory of "Dr. R. Gutiérrez" Children's Hospital and tested for HAdV by IFA from January 1999 to December 2010. Overall, HAdVs were found in 4.7% of the samples, but in 1999, 2002 and 2003, these viruses were significantly more prevalent than in other years ($p < 0.000001$) (Table 1). Adenovirus circulation showed both an epidemic and an endemic pattern (Supplemmentary Fig. 1).

4.2. Molecular typing

PCR sensitivity and specificity were evaluated for each protocol by testing serial dilutions of purified plasmids and the lowest level of detection of PCRs was ten genome copies for each protocol. No cross-detection was recorded in any case.

A total of 841 out of the 916 HAdVs detected by IFA in NPA were adequate for molecular typing and 743 (81%) were typified according to the algorithm shown in Fig. 1. Five samples were only positive for the universal HAdV penton and hexon gene PCRs and were thus considered as non-typeables.

From the samples characterized, 654 (88%) were single infections and 89 (12%) coinfections. Therefore, a total of 843 HAdVs belonging to species B ($n = 601$, 71.29%), species C ($n = 218$, 25.86%), species E ($n = 19$, 2.25%), and species D ($n = 5$, 0.59%) were detected. Among HAdV species B, subspecies B1 was the most prevalent ($n = 461$, 93.7%) (Table 1). HAdV-B7 and HAdV-B3 were the most prevalent types ($n = 308$, 36.54%; $n = 230$, 27.28% respectively) (Table 2).

4.3. HAdV single infections

From the 654 single HAdV infections, members of four species were present: species B ($n = 492$, 75.23%), species C ($n = 138$, 21.1%), species E ($n = 19$, 2.91%), and species D ($n = 5$, 0.76%). From them, 619 samples (94.65%) were further classified into ten types (HAdV-C1, -C2, -B3, -E4, -C5, -C6, -B7, -D8, -B11 and -B21) and 35 (5.35%) remained untyped at different taxonomic levels (Table 2).

Among the most prevalent types, there was a switch in the prevalence from HAdV-B7 to HAdV-B3, the former more prevalent from 1999 to 2003 and the latter more prevalent from 2003 to 2008. This difference was significant in 1999, 2002, 2003, 2005, 2007 and 2008 ($p < 0.0001$) (Fig. 2a). The highest diversity of HAdVs, including the ten types detected in single infections, was found in the 7- to 18-month-old group (Fig. 2b).

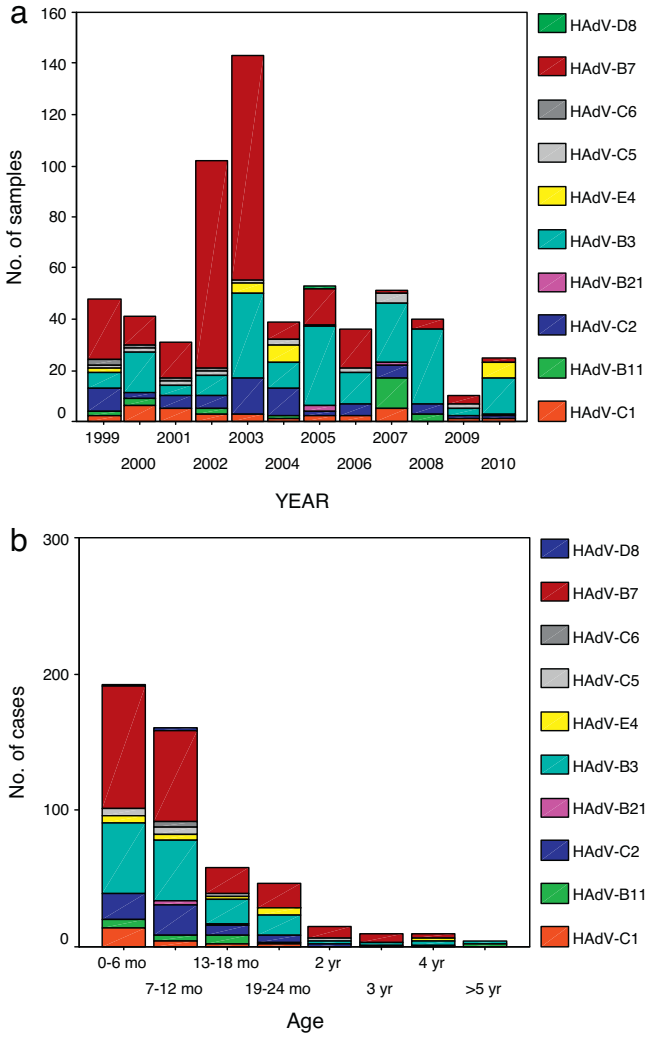

Fig. 2. Molecular typing of single HAdV infections in pediatric respiratory infections in Buenos Aires (1999–2010). (a) Prevalence of serotypes per year and (b) age distribution per serotype.

Children with single adenoviral infections presented bronchiolitis or pneumonia together with cough (HAdV-B3, HAdV-B7,

Table 2
Molecular typing of respiratory HAdV in children in Buenos Aires (1999–2010).

HAdV	Species B								Species C					Species D		Species E (HAdV-4)
	Subspecies B1				Subspecies B2			NT	1	2	5	6	NT	8	NT	
	3	7	21	NT	11	14	NT									
Species B																
Subspecies B1																
3	**189**	4	0	0	5	2	0	0	9	3	6	2	1	0	0	0
7	4	**264**	0	0	1	0	0	0	12	18	2	0	0	0	0	0
21	0	0	**4**	**0**	0	0	0	0	0	0	0	0	0	0	0	0
NT	0	0	**0**	**4**	0	0	0	0	0	2	0	0	2	0	0	0
Subspecies B2																
11	5	1	0	0	**23**	2	0	0	0	4	0	0	0	0	0	0
14	2	0	0	0	2	**0**	0	0	0	0	0	0	0	0	0	0
NT	0	0	0	0	0	0	**7**	0	1	0	0	0	0	0	0	0
NT	0	0	0	0	0	0	0	**1**	0	0	1	0	0	0	0	0
Species C																
1	9	12	0	0	0	0	1	0	**31**	1	0	0	0	0	0	0
2	3	18	0	2	4	0	0	0	1	**64**	1	0	0	0	0	0
5	6	2	0	0	0	0	0	1	0	1	**19**	0	0	0	0	0
6	2	0	0	0	0	0	0	0	0	0	0	**5**	0	0	0	0
NT	1	0	0	2	0	0	0	1	0	0	0	0	**19**	0	0	0
Species D																
8	0	0	0	0	0	0	0	0	0	0	0	0	0	**1**	0	0
NT	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**4**	0
Species E (HAdV-4)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	**19**

Single and double HAdV infections were counted. The number of single infections is marked in bold.

HAdV-C1, HAdV-C2, HAdV-C5, and HAdV-E4), conjunctivitis (HAdV-B3, HAdV-B7, HAdV-C2, and HAdV-E4) and pertussis-like syndrome (HAdV-B3, HAdV-B7, HAdV-C1 and HAdV-C5).

Almost all types presented as severe cases, with the exception of HAdV-C6 and HAdV species D. The mortality for each type varied from 1.1 to 5.3% for HAdV-B3 and HAdV-B7 respectively, but the latter was frequently detected in deceased patients (14/22).

4.4. HAdV coinfections

More than one HAdV type was detected in 89 samples. Some of these samples presented two (79, 88.6%), three (9, 10.11%) and four (1, 1.12%) types of the same or different species. No coinfections were detected in 2009 and 2010 (Fig. 3a).

From the 89 HAdV coinfections, only members of species B ($n = 109$, 57.67%) and species C ($n = 80$, 42.33%) were detected. Of them, 180 viruses (95.24%) were further classified into types and nine (4.76%) remained untyped at different taxonomic levels. Coinfection with subspecies B1 and species C was found in 64% of coinfections and HAdV-B14 was exclusively found in coinfection with HAdV-B11 or HAdV-B3. The highest diversity of HAdVs, including most of the mixtures of species and types detected in this study, was found in the 7- to 18-month-old group (Fig. 3b).

Children with coinfections of subspecies B1 and species C presented bronchiolitis (46%) or pneumonia (33%) combined with cough (7%) and conjunctivitis (2%).

Almost all coinfections showed moderate symptoms, with exception of mixtures between species B1 and C, which were severe. Mortality was similar to that of single HAdV-B7 infection in mixtures of HAdV-C2 and B7 (5.3%) but increased to 33.33% for mixtures of HAdV-C1 and B7 ($p < 0.005$).

4.5. Viral isolates

As an additional test, 234 NPA were cultured and molecularly typed. These NPA corresponded to 190 cases with single adenovirus infection and 44 cases with coinfection. Results from isolates and NPA were 100% identical.

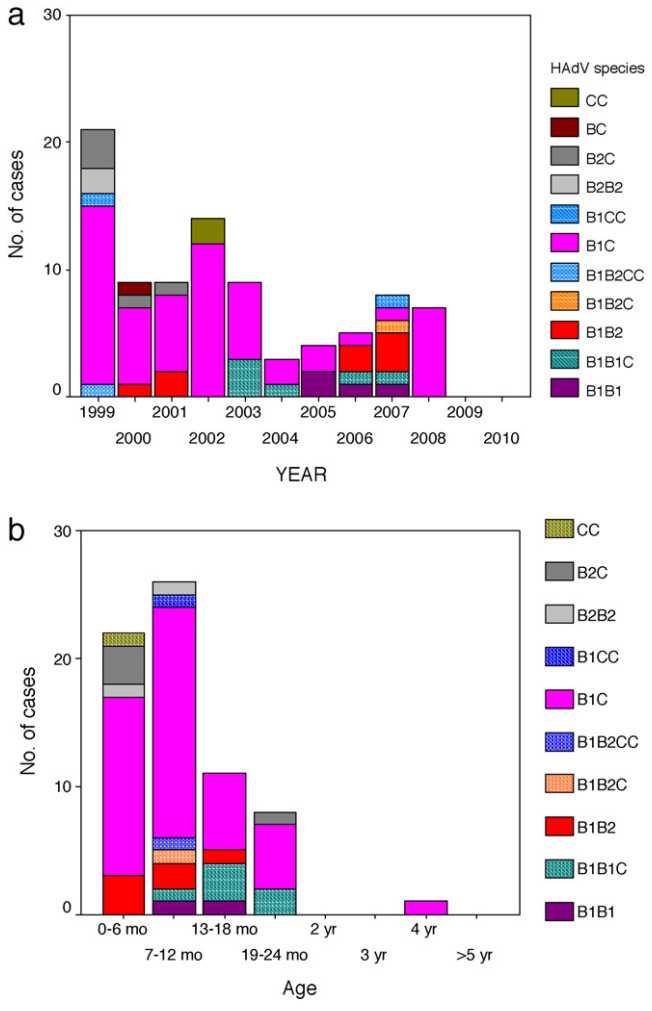

Fig. 3. Molecular typing of multiple HAdV infections. (a) Prevalence of multiple infection per year and (b) age distribution per species.

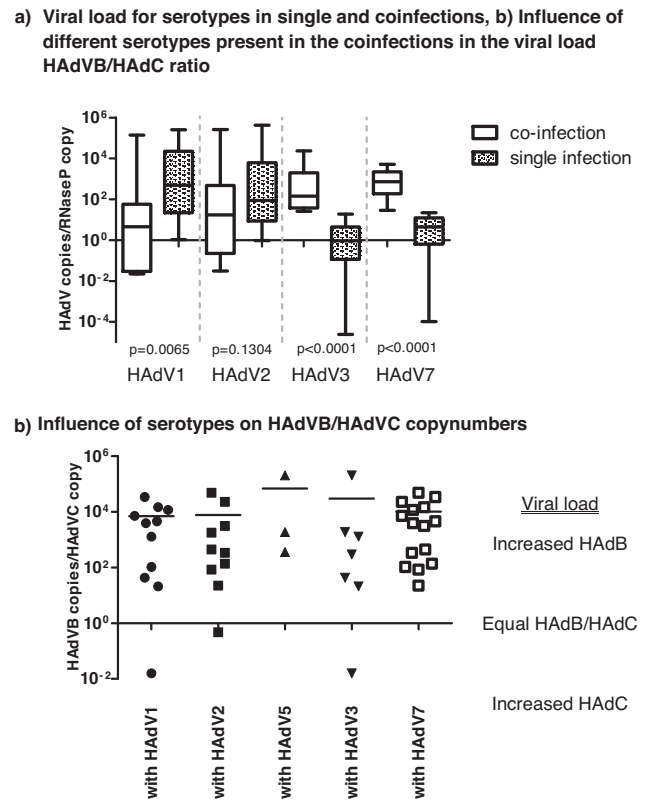

Fig. 4. Viral load in HAdV species B and C. (a) Viral load for serotypes in single and coinfections. (b) Influence of different serotypes present in the coinfections in the viral load HAdVB/HAdC ratio.

4.6. Viral load

In order to compare viral loads in single infections and coinfections, qPCRs were performed in NPA ($n = 179$) from patients presenting with the most prevalent coinfections, namely species B and C, and randomly selected samples of patients infected with only one member of these. The ratio between the viral load of species B and the viral load of species C (qB/qC) was calculated and compared to the viral load of single infections.

Viral loads for species C viruses (HAdV-C1 and HAdV-C2) were higher in single infections than in coinfections ($n = 17$ vs. $n = 13$, $p < 0.001$; $n = 25$ vs. $n = 13$, $p = 0.13$), whereas the opposite was observed for species B viruses (HAdV-B3 and HAdV-B7) ($n = 24$ vs. $n = 16$, $p < 0.0001$; $n = 26$ vs. $n = 25$, $p < 0.0001$) (Fig. 4a). Regardless of the types involved in the B1-C coinfection, we found that the HAdV-B viral load outnumbered the that of HAdV-C coinfection in 93.48% of the samples tested ($N = 46$) (Fig. 4b).

5. Discussion

In this study, 748 samples were analyzed and ten types (HAdV-C1, -C2, -B3, -E4, -C5, -C6, -B7, -D8, -B11 and -B21) of four species (HAdV-B, C, D, E) were found in single infections, whereas only members of species B and C were found in coinfections, including the first HAdV-B14 description in Argentina. This study provides a thorough description of adenovirus circulation and diversity in Buenos Aires in a 12-year period. Although prevalences presented in this work may represent only the cases that required hospitalization, the circulation of other types in the community cannot be ruled out.

The highest diversity and number of adenovirus infections were found in the 7- to 18-month-old group both in single and coinfections. Since children represent a group at risk for adenovirus infection that is different from adult patients, they deserve special attention. In this study, HAdV-B7, and HAdV-B3 were the most prevalent types whereas HAdV-C (1, 2, 5 and 6) were the most prevalent types in Israel.[26]

The implementation of molecular typing in adenovirus diagnosis complements the general use of IFA because it helps to detect a broader spectrum of both commonly found and newly emerged types. NPA proved to be a good source of virus and allowed us to perform molecular typing without previous amplification in cell culture.

The complexity of adenovirus infections led us to evaluate four viral genes to determine the type or the mixture present in each sample and to a continous and retrospective revision of the results with the most adequate protocol. Genes from early (E1A), intermediate (VA RNA) and late (hexon and fiber) blocks were targeted and protocols were updated through the years. The high number of PCRs performed allowed us to type them accurately. More than 99 samples were also confirmed by direct sequencing of the E1, hexon and fiber genes (data not shown).

By applying molecular typing methods, we were able to detect the presence of multiple adenovirus types in the same sample as coinfections in a high proportion of children (10.6%). Other authors have reported the presence of coinfections in low percentages in children (2.1–3.5%) bearing only two types.[15] Although the role of coinfections in the pathogenesis, evolution and molecular epidemiology of adenoviruses remains to be revealed, coinfections are the obligate source of recombination and emergence of new variants. The high proportion of coinfections found in this study shows that this phenomenon might be more common than expected.

In our study, there was a significant decrease in the prevalence of HAdV-B7 whereas HAdV-B3 increased from 2004. Although both were found in coinfections, there was a markedly decrease in the total number of cases since 2004, including the lack of severe and fatal cases and even of coinfections in the last 2 years (2009–2010) maybe due to viral interference as has been described for rhinovirus and the FluAH1N1 pandemic in 2009, or with other viruses.[27,28] Some authors have postulated that herd immunity may have diminished HAdV-B7 circulation.[29,30] The diversity and pathogenicity of HAdV-B3 and HAdV-B7 are currently being studied.

Although temporal and geographical restrictions have been shown for adenovirus circulation by detailed molecular typing studies, the diversity of adenovirus has not been elucidated yet. The presence of a high prevalence of coinfections, from which recombinant viruses with altered tropism and pathogenicity may arise, highlights the need for continued surveillance and molecular epidemiology of the *Adenoviridae*.

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Competing interests

None declared.

Ethical approval

Not required.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2011.11.001.

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