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Molecular characterization of hospital-acquired adenovirus infantile respiratory infection in Chile using species-specific PCR assays

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Abstract

Background: Adenovirus serotypes 7, 2 and 1 are the second most common cause of viral acute lower respiratory tract infection (ALRI) requiring hospitalization in Chile. Nosocomial outbreaks have high secondary attack and lethality rates, and call for rapid and specific diagnosis.
Objective: We compared the results obtained on ALRI specimens by immunofluorescence (IFA) and virus isolation, plus restriction enzyme digestion (RFLP) typing, with universal, species-specific and 7h-specific PCR typing of adenovirus. A second objective was to determine the type of adenovirus implicated in nosocomial infection and nosocomial cross-infection rates.
Methods: Infants hospitalized for ALRI in the Roberto del Río Children's Hospital (Santiago, Chile) in 1995–1996 had nasopharyngeal aspirates obtained at admission and tested by IFA and virus isolation. Adenovirus isolates were identified by RFLP. When an index case was identified, samples were collected from contacts for 2 consecutive days and twice weekly thereafter for 2 weeks. Further typing of adenovirus isolates was undertaken with universal, species-specific and 7h-specific PCR performed in 2003 on the stored frozen samples.
Results: Fifteen index cases of adenovirus and their 65 contacts were identified. The nosocomial secondary attack rate using PCR was estimated as 46%. PCR had a higher sensitivity (98.7%) compared to virus isolation (90%) and IFA (50%) and facilitated identification of adenovirus strains more easily and accurately than RFLP (91.6% versus 55.8%). Fifty-three percent of the contacts had severe outcomes. The case fatality rate was 16.6% and was associated with adenovirus 7h.
Conclusions: Prompt, rapid and sensitive methods to identify adenovirus infection are necessary, especially for hospital-acquired adenovirus infections, because of their ease of spread and high fatality rate.
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Keywords: Adenovirus; Nosocomial infection; Infantile pneumonia

1. Introduction

Acute lower respiratory tract infections (ALRI) are a major worldwide health problem because of associated high morbidity and mortality rates. In Chile respiratory viruses are the leading cause of hospitalization in children less than 2 years of age (Avendaño, 1998; Avendaño et al., 1999; Kaempfer and Medina, 2000; Lagos et al., 1999; Young, 1996). Respiratory syncytial virus (RSV) and adenoviruses are detected at annual rates of 29% and 9.2%, respectively. Pneumonia and wheezing bronchitis are the most frequent clinical diagnoses among infants hospitalized for adenovirus infection. The severity of their symptoms is shown by prolonged hospital stay, frequent admission to the intensive care unit and high lethality rate. In Chile serotypes 7, 2 and 1 are the most frequent adenovirus strains associated with infant ALRI. Genome typing using restriction enzymes (RFLP) for strain characterization documented a high prevalence of adenovirus 7h, a genomic variant which emerged in the 1980s in the southern cone of South America and has been detected in Japan (Avendaño et al., 1991, 2003;

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Hashido et al., 1999; Kajón et al., 1994, 1996; Larrañaga et al., 2000; Palomino et al., 2004; Román et al., 1997; Wu et al., 1990). The laboratory techniques available – classic virus isolation in the 1950s, immunoassays (IFA) and polymerase chain reaction (PCR) in recent years – have facilitated the detection and characterization of respiratory adenoviruses (Adrian et al., 1986; Kidd et al., 1996; Larrañaga et al., 1990; Storch, 1999; Wadell et al., 1999).

Viral and host-related risk factors for severe adenovirus disease have not been clearly established. The occurrence of severe community epidemics and nosocomial outbreaks suggests the emergence of virulent adenovirus strains. Certain serotypes, especially 3 and 7, have been associated with severe adenoviral pneumonias in infants worldwide. Adenovirus genome typing has also shown a relationship between viral strains and severe clinical outcome (Carballal et al., 2002; Diaz et al., 1999; Echavarria et al., 1998; Ikeda et al., 2003; Kajón et al., 1993, 1994; Kajón and Wadell, 1994; Larrañaga et al., 2000; Mistchenko et al., 1998; Mitchell et al., 2000; Muñoz et al., 1998; Palomino et al., 2004; Simila et al., 1981). Adenovirus 7h nosocomial outbreaks with high secondary attack and lethality rates emerged in Chile during the 1990s (Hatherill et al., 2004; Palomino et al., 2000; Singh-Naz et al., 1993).

We studied adenovirus infection using IFA and virus isolation in cell culture for diagnosis (Larrañaga et al., 1990; Storch, 1999; Wadell et al., 1999). The adenovirus isolates were further studied by RFLP (Adrian et al., 1986; Wadell et al., 1999), which is a slow and cumbersome method. The PCR assay is better for adenovirus detection in clinical samples, since it is more rapid and sensitive than classic virus isolation or immunodiagnostic tests. The use of primers directed to the hexon gene allows the detection of all human adenoviruses, from species A to F (Echavarria et al., 1998, 2000; Elnifro et al., 2000; Kidd et al., 1996; Osiowy, 1998). Primers directed to the fiber genes permit identification of the adenovirus species. Primers have also been developed to recognize only adenovirus 7h among the species B strains. Finally, PCR has been used for classification of adenovirus according to species and serotype (Bruzzone et al., 2000, 2001; Xu et al., 2000).

During 1995 and 1996 we studied adenovirus nosocomial outbreaks using RFLP for adenovirus characterization (Palomino et al., 2000). We collected samples from 15 adenovirus infection index cases and from 65 of their hospital ward contacts. We found adenovirus infection in 36 ward contacts (55%). Four cases died (11.1%), three of them had underlying diseases. The aim of the current study was to compare the aforementioned results obtained by IFA, virus isolation and RFLP with results obtained using molecular assays, such as universal PCR for adenovirus detection, and species-specific and 7h-specific PCR assays for strain characterization of the original samples stored frozen for 8 years.

2. Patients and methods

2.1. Patients, ward description and contact follow-up

We studied community-acquired respiratory viruses in children <2 years of age admitted for ALRI to the Roberto del Rio Children's Hospital, in Santiago, Chile from 1988 to date, as described (Avendaño et al., 1991, 2003; Larrañaga et al., 2000). Between May 1995 and October 1996, infants hospitalized at two regular wards for community-acquired ALRI were evaluated for nosocomial adenovirus outbreaks using IFA, virus isolation and RFLP for adenovirus subgenus B and subgenus C (Adrian et al., 1986; Kajón et al., 1996; Palomino et al., 2000; Shinagawa et al., 1983).

2.2. Adenovirus genome typing using PCR techniques

In 2003, 181 nasopharyngeal aspirates (NPA), stored at −20 °C from 1995 to 1996, were processed again by PCR assay:

(i) *DNA extraction*. The 20 μl of NPA stored at −20 °C was added to a 1.5 ml tube containing 150 μl distilled H_2O, 60 μl of 5× TNE buffer (50 mM Tris–HCl, 5 mM EDTA, 50 mM NaCl (pH 8.0), 30 μl of 10% sodium dodecyl sulfate (SDS), and 10 μl of 10 mg of proteinase K per ml, and the tube was heated at 56 °C for 60 min. The DNA was extracted once with an equal volume of phenol, once with phenol-chloroform-isoamyl alcohol (25:24:1), and once with chloroform-isoamyl alcohol (24:1) (Xu et al., 2000).

(ii) *Oligonucleotide primers*. The oligonucleotide primers used for amplification of adenovirus strains were published by Echavarria et al. (2000) for universal PCR, Xu et al. (2000) for subgenera B and C, and Bruzzone et al. (2001) for adenovirus 7h.

(iii) *Adenovirus DNA amplification and detection*. Universal PCR was performed in 50 μl containing 40 μl of reaction mixture (final concentration of 10 mM Tris–HCl [pH 8.3], 50 mM KCl, 1.5 mM Mg_2Cl_2, 200 μl each deoxynucleoside triphosphate, 0.2 mM each primer (Hex 1, Hex 2), 2.5 U of *Taq* DNA polymerase [Invitrogen®]) and 10 μl of DNA extract (Xu et al., 2000). B and C specific PCR was performed in 50 μl containing 45 μl of reaction mixture (75 mM Tris–HCl (pH 9.0), 2.0 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$, 200 μM each deoxynucleoside triphosphate, 0.2 mM each primer (AdB1, AdB2, AdC1, AdC2), 5 U of *Taq* DNA polymerase [Biotools®]) and 5 μl of DNA extract (Xu et al., 2000). The 7h-specific PCR was performed in 50 μl volumes containing 45 μl of reaction mixture containing 0.2 mM each primer (REC1, REC2), 5 U of *Taq* DNA polymerase [Biotools®] and 5 μl of DNA extract (Bruzzone et al., 2001). The amplification reactions were carried out in a GeneAmp PCR System 2400 thermal cycler

(Applied Biosystems®) as described (Bruzzone et al., 2000, 2001; Xu et al., 2000). The products were separated by electrophoresis (0.5 h, 120 V) in a 2% (wt./vol.) agarose gels, stained with ethidium bromide, and photographed with UV trans-illumination and a digital camera Olympus C-3040. No other adenovirus species were analyzed.

2.3. Statistical analysis

Adenovirus infection was defined as a true positive if it was positive in more than one assay—either in two different samples or in two different tests (IFA, virus isolation or universal PCR) of the same specimen. A positive specimen by any assay was considered as a true positive sample for adenovirus infection if it was also positive by any other test. To distinguish true positive cases and samples in the final count from presumptively false positive results of the universal PCR, data from species-specific and 7h-specific PCR were considered.

The differences in proportion between IFA, virus isolation and universal PCR were assessed with the χ^2 or Fisher's exact test, where $p < 0.05$ was considered significant. The McNemar test for unknown gold standard was used; significant differences were defined by observed values higher than 3.841.

Since conventional cell culture is probably not sufficiently sensitive to be used as a gold standard assay for comparison in detecting adenovirus, we also considered an expanded gold standard method. The expanded gold standard defined a true positive specimen as one that was positive for adenovirus in more than one type of assay (IFA, virus isolation and universal PCR) (Pedneault et al., 1994; Rothbarth et al., 1991). The z-test of differences in proportion was used to compare the sensitivity, specificity and predictive value; z-values of less than 5% were considered significant.

Finally, the z-test of differences in proportion was used to compare virus isolation plus RFLP with species-specific PCR and 7h-specific PCR; z-values of less than 5% were considered significant.

3. Results

A follow-up of 15 adenovirus nosocomial outbreak cases was performed from May 1995 through October 1996. A total of 90 patients were enrolled, including 15 index cases and 75 contacts. Ten out of 75 contacts were excluded from the analysis because they were exposed less than 48 h. The mean exposure times with an index case was 2.7 days (range 2–16 days). Thirty-three of the 65 contacts (51%) were followed for 2 weeks and 32 for at least 1 week.

Adenovirus was detected in 45 of 65 contacts (69%). The results of cases and samples are shown in Table 1. IFA was positive in 32 infants (40%), virus isolation in 52 (65%), and universal PCR in 60 cases (75%). Fifty-four cases (67.5%) were positive by more than one assay. Six infants were positive only by universal PCR, but two of these were determined by 7h-specific PCR and one infant had adenovirus species C (by species-specific PCR). According to our adenovirus case definition, the remaining three cases could be considered false positives by universal PCR, or the strains belonged to an adenovirus species that would not be detected by the other assays. No negative PCR case was positive by any other method. Among the 15 index cases, IFA was positive in 14 cases (93%), virus isolation in 13 (86%) and PCR in all 15 patients (100%). Among the contact cases, IFA was positive in 18 cases (27.7%), virus isolation in 39 (60%) and PCR in 45 patients (69.2%) ($p < 0.01$). The IFA assay was more sensitive for adenovirus detection in index cases than in contacts ($p < 0.01$). No differences between index and contact samples were observed for PCR and virus isolation.

Since each enrolled case had more than one NPA sample available, a total of 181 specimens were tested. IFA was positive in 41 samples (22.7%), virus isolation in 77 (42.5%) and PCR in 104 (57.5%) ($p < 0.01$; Table 1). Eighty samples (44.2%) were positive by more than one assay; 31 samples (17.1%) were positive by only one method and 70 (38.7%) were negative by all techniques. PCR failed to detect adenovirus infection in 7 samples (3.7%) that were positive by other tests and were obtained from cases that had other positive samples.

Twenty-five samples that were initially positive only by universal PCR included 13 that were not positive by any assay in other specimens. Species-specific PCR and 7h-specific PCR gave the following results for these samples: (i) two samples were confirmed as species C and one as species B by species-specific PCR, (ii) five specimens were adenovirus 7h by 7h-specific PCR and (iii) five samples were negative for B and C species. According to our definition, the last five samples could be considered false positives by universal PCR, or they contained an adenovirus species not detectable by the other assays. The remaining 12 samples came from patients with other specimens positive by other tests. Overall, 10 specimens were positive for adenovirus 7h, 1 was species B and 1 was species C.

The sensitivity, specificity and both positive and negative predictive values were calculated for universal PCR using all specimens. Universal PCR was positive in 99 samples and significantly more sensitive than IFA (95.1%) or virus isolation (92.2%), while specificity and positive predictive value were lower. Also the use of the expanded gold standard confirmed the higher sensitivity of universal PCR (98.7%) (Table 2), and the specificity and positive predictive value of the universal PCR was substantially better than with other assays.

The universal PCR assay confirmed respiratory adenovirus infection in the 15 index cases previously reported and in 45 out of 65 contacts (69.2%) (Table 3), including 9 additional adenovirus-positive cases (45 cases versus 36 cases). All index cases were adenovirus 7h. Thirty-five (53.8%) contact cases were adenovirus species B; 30 of them

Table 1
Comparison of adenovirus results by universal PCR, IFA and culture isolation in 15 index cases and 65 contacts from adenovirus hospital-acquired respiratory infection follow-ups

Positive cases by					Positive samples by				
PCR[a]	VI[a]	IFA[a]	No.	%	PCR[a]	VI[a]	IFA[a]	No.	%
+	+	+	30	37.5	+	+	+	31	17.2
+	+	−	22	27.5	+	+	−	40	22.2
+	−	+	2	2.5	+	−	+	8	4.4
+	−	−	6	7.5	+	−	−	25	13.8
−	+	+	0	0	−	+	+	1	0.5
−	+	−	0	0	−	+	−	5	2.7
−	−	+	0	0	−	−	+	1	0.5
−	−	−	20	25.0	−	−	−	70	38.7
Total (%)									
60 (75.0)	52 (65.0)	32 (40.0)	80	100	104 (57.5)	77 (42.5)	41 (22.6)	181	100

Roberto del Rio Children's Hospital, May 1995 to October 1996. PCR, polymerase chain reaction; IFA, immunofluorescence assay; VI, virus isolation (CPE on cell culture plus confirmatory IFA). From 65 contact cases more than one sample was sequentially obtained. A total of 181 nasopharyngeal aspirates were studied by the three procedures.
[a] Assays.

were adenovirus 7h. Since all index cases were hospitalized because of adenovirus 7h ALRI, the secondary attack rate evaluated by molecular assays was estimated as 46.1%, considering that in five contacts in which the universal PCR was positive, we could not identify the species or adenovirus genome type with the PCR assays available. From 52 positive viral isolates evaluated by the RFLP assay we typed 29 isolates, all of which were adenovirus 7h (55.8%). Using universal PCR from frozen samples from 60 adenovirus-positive cases (15 index cases and 45 contacts) we were able to identify the adenovirus genome type or its species in 55 (91.6%) comparing with RFLP (55.8%) ($p < 0.001$).

Table 2
Comparison of universal PCR, virus isolation and IFA assay of 181 samples of nasopharingeal aspirates from 15 adenovirus hospital-acquired respiratory infection follow-ups

Assay results	Number of results		Statistical value		%			
			$p <$ value 0.01	Me Nemar > 3.84	Sensitivity	Specificity	Positive predictive value	Negative predictive value
PCR	Virus isolation		0.01	17.3	92.2	68.3	68.3	92.2
	Positive	Negative						
Positive	71	33						
Negative	6	71						
PCR	IFA		0.01	57.3	95.1	53.6	37.5	97.4
	Positive	Negative						
Positive	39	65						
Negative	2	75						
PCR	Expanded gold standard*		0.01	20.3	98.7[a]	75.2[b]	75.9[c]	98.7[d]
	Positive	Negative						
Positive	79	25						
Negative	1	76						
Virus isolation	Expanded gold standard*		0.01	0.69	90.0[a]	95.0[b]	93.5[c]	92.3[d]
	Positive	Negative						
Positive	72	5						
Negative	8	96						
IFA	Expanded gold standard*		0.01	39.02	50.0[a]	99.0[b]	97.5[c]	71.4[d]
	Positive	Negative						
Positive	40	1						
Negative	40	100						

PCR, polymerase chain reaction; IFA, immunofluorescence assay; VI, virus isolation.
* EGS, which was defined as a positive for more than one assay.
[a] $p < 0.01$ for sensitivities of PCR vs. virus isolation ($z = 3.36$) and PCR vs. IFA ($z = 10.5$).
[b] $p < 0.01$ for specificities of virus isolation vs. PCR ($z = 5.14$) and IFA vs. PCR ($z = 6.60$).
[c] $p < 0.01$ for the positive predictive values of virus isolation vs. PCR ($z = 4.51$) and IFA vs. PCR ($z = 5.90$).
[d] $p < 0.01$ for the negative predictive values of PCR vs. virus isolation ($z = 2.68$) and PCR vs. IFA ($z = 7.14$).

Table 3
Adenovirus strain genome types using restriction enzyme assays and PCR, and secondary attack rate per index case

Index cases	Index cases genome type by RFLP	Index cases genome type by PCR	No. of contacts	No. of positive contacts by IFA and VI	No. of positive contacts by PCR	Contact's genome type by RFLP	Contact's genome type by species-specific and specific 7h PCR	Secondary attack rate for B-7h (%)
1	ND	B-7h	6	4	5	7h (4)	B-7h (5)	83.3
2	B-7h	B-7h	4	4	4	7h (2)	B-7h (2) B-not 7h (2)	50.0
3	ND	B-7h	4	3	3	ND	B-7h (2) Undefined (1)	50.0
4	ND	B-7h	6	3	3	ND	B-7h (2) B-not 7h (1)	33.3
5	B-7h	B-7h	3		3	ND	B-7h (1) B-not 7h (2)	33.3
6	B-7h	B-7h	6	3	5	7h (2)	B-7h (5)	83.3
7	B-7h	B-7h	3	1	2	7h (1)	B-7h (2)	66.6
8	B-7h	B-7h	5	1	2	7h (1)	B-7h (2)	40.0
9	ND	B-7h	6	3	4	7h (3)	B-7h (3) C (1)	50.0
10	ND	B-7h	3	2	2	7h (1)	B-7h (1) Undefined (1)	33.3
11	B-7h	B-7h	4	2	3	7h (1)	B-7h (1) Undefined (2)	25.0
12	B-7h	B-7h	2	0	1	ND	Undefined (1)	0.0
13	B-7h	B-7h	2	2	2	7h (1)	B-7h (1) C (1)	50.0
14	B-7h	B-7h	5	1	2	ND	C (2)	0.0
15	B-7h	B-7h	6	4	4	7h (3)	B-7h (3) C (1)	50.0
Total	B-7h (10) ND (5)	B-7h (15)	65	36	45	7h (19)	B-7h (30) B-not 7h (5) C (5) Undefined (5)	46.1

Roberto del Río Children's Hospital. May 1995 to October 1996. PCR, polymerase chain reaction; IFA, immunofluorescence assay; VI, virus isolation; RFLP, restriction enzyme analysis; values within parenthesis are no. of cases.

The clinical outcome of the 45 adenovirus-positive contacts was: (i) 21 infants (46.6%) had mild symptoms or were asymptomatic (12 were adenovirus 7h, 3 were species B, 3 were species C, and 3 were not identified); (ii) 24 cases (53.3%) developed moderate or severe ALRI (13 were adenovirus 7h, 2 were species B, 2 were species C, and 2 were not identified). The remaining five infants died; four during hospitalization and one a few months after discharge due to chronic lung damage. All fatal cases were associated with adenovirus 7h; three of them had underlying diseases. The case fatality rate for secondary adenovirus 7h infection was 16.6% (5/30).

4. Discussion

Adenoviral infections produce a wide range of diseases depending on the serotype. Our previous studies in Chile demonstrated that serotypes 7, 2 and 1 are the most frequently detected in infants hospitalized for ALRI. During the 1990s, adenovirus 7h was the most prevalent, accounting for ∼50% of the cases. In South America adenovirus 7h has been more frequently associated with severe pneumonia, longer hospital stay, and a higher mortality than other genome types (Kajón et al., 1993, 1994, 1996; Kajón and Wadell, 1994; Larrañaga et al., 2000; Palomino et al., 2000).

In developing countries young children are hospitalized in multiple crib wards, because isolation is very difficult to accomplish. Furthermore, hospitalized children with underlying diseases, who have prolonged hospitalization, are at high risk of severe adenovirus infection and represent a source for nosocomial transmission. In this series, 53% of the adenovirus-positive contacts had a severe outcome and the lethality rate among adenovirus 7h positive contacts was 16.6%. Similar findings were reported for Cape Town, South Africa (Hatherill et al., 2004). In order to diminish the high risk of adenovirus nosocomial infection, rapid and sensitive tests to identify adenovirus carriers are necessary.

In the previous study, using IFA and virus isolation plus RFLP, we reported an adenovirus secondary attack rate of 36/65 (55%), without considering the viral genome type. At that time we could not calculate the secondary attack rate for adenovirus 7h, because only 52 isolates were available for typing (Palomino et al., 2000).

The implementation of adenovirus PCR has reanalysis of our stored samples and better characterization of outbreaks. Universal PCR assays demonstrated adenovirus infection in 45 out of 65 contact infants (69.2%), 13.8% more than we previously reported. Considering that 30 out of 65 contact cases were identified as adenovirus 7h by molecular assays, the adenovirus 7h secondary attack rate was 46.1%. Species-specific PCR and 7h-specific PCR were better for adenovirus characterization than virus isolation plus RFLP methods; they confirmed all 29 cases previously typed by RFLP and 26 additional frozen isolates, including index and contact cases.

Universal PCR was more sensitive for identifying adenovirus infection than IFA and virus isolation in 60 out of 80 cases and 104 out of 181 samples. Of these, these cases and five samples might have been false positives for the universal PCR. According to these results virus isolation provided the best balance of sensitivity, specificity, positive and negative predictive values. This imbalance is expected when using a method as sensitive as the universal PCR if all the precautions that the technique requires have been taken. In this study almost all the positive cases and samples were confirmed with specific PCR. Using the expanded gold standard, the specificity, positive and negative predictive values of universal PCR improve, because the number of false positives samples diminished.

During the years 2000–2005, there was a clear decrease in hospitalized community-acquired ALRI caused by adenovirus; from 9% in the 1990s to less than 4% currently. In this period, of 1928 infants hospitalized for ALRI we detected 74 new cases of adenovirus infection with the same surveillance system at the same hospital. Of these cases, 42 (56.7%) patients were characterized by species-specific PCR and 7h-specific PCR. Twenty-nine (69%) belonged to species B and 13 (31%) to species C. Only two cases were adenovirus 7h confirmed by 7h-specific PCR. None of these adenovirus cases died (data not shown).

The knowledge acquired by the medical staff as the result of the prior experience with nosocomial adenovirus infections (Palomino et al., 2000) led to the hospital assigning additional isolation rooms to diminish the risk of cross-infection by adenovirus. Furthermore infants with a clinical diagnosis of ALRI are now tested for respiratory viruses by rapid immunodiagnosis in the emergency room. If an adenovirus infection is diagnosed, the patient is admitted to an individual room and maintained there throughout hospitalization. At present community-acquired adenovirus infection is detected at low frequencies, and therefore nosocomial monitoring has not been warranted. However, the precautions mentioned above are routine in the hospitals and should help to control future outbreaks.

In conclusion, rapid and sensitive methods to identify adenovirus infection are needed because of their rapid spread and high fatality rate in the hospital. In developing countries where patient isolation and viral diagnosis is difficult, PCR for adenovirus looks promising as a tool for respiratory adenovirus management.

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