Species differences in circulation, and inflammatory responses in children with common respiratory adenovirus infections

Haruna Nakamura,[*] Takao Fujisawa,[*] Shigeru Suga,[*] Kiyosu Taniguchi,[*] Mizuho Nagao,[*] Masahiro Ito[†], Hitoshi Ochiai[‡], Masami Konagaya[§] ,Nozomu Hanaoka[§] ,and Tsuguto Fujimoto[§]

[*]Department of Pediatrics, Mie National Hospital, Tsu, Mie, Japan

[†]Biwako Gakuen Yasu Medical and Welfare Center, Yasu, Shiga, Japan

[‡]Ochiai Pediatric Clinic, Kameyama, Mie, Japan

[§]National Institute of Infectious Diseases, Tokyo, Japan

Correspondence to: Takao Fujisawa, MD, PhD

357 Osato-kubota, Tsu, Mie 514-0125, Japan

Fax: +81-59-232-5994

Tel.: +81-59-232-2531

E-mail: fujisawa@mie-m.hosp.go.jp

Shortend title: Adenovirus species epidemiology in children

Abstract

Background: Human adenoviruses (HAdVs) cause severe inflammatory respiratory infections, but previous epidemiological studies lacked analysis of the characteristics of the inflammation.

Methods: Consecutive patients <13 years old with acute febrile illness during a 2-year period were tested. HAdV strains were isolated from nasopharyngeal swabs, and molecular identification was performed by hexon, fiber and species-specific PCR methods. Blood inflammatory markers, including the white blood cell (WBC) count, CRP and 29 cytokines, were measured.

Results: A total of 187 patients were enrolled, and HAdV types were identified from 175 patients (93.5%). Species C (types 2, 1, 5 and 6, in order of frequency) was most common at 37.1%, followed by B (type 3) at 30.9% and E (type 4) at 26.9%. Species C was detected predominantly in 1-year-olds, whereas B and E were in older ages. Species C and B had seasonal circulation patterns, but E was found in only one season during the 2-year study period. The WBC count was highest in patients with species C. Eleven of the 29 tested serum cytokines were detected. Seven kinds, including G-CSF, IL-6 and TNF-α, were elevated in species C infections, whereas IL-10 was lowest in species C.

Conclusion: Species differences in inflammatory responses, especially regarding serum cytokines were described in common pediatric HAdV infections. Species C causes the strongest inflammatory responses in young children. (221 words)

Key Words: Adenovirus, Epidemiology, Seasonal incidence, Inflammation,

Cytokine/Chemokine

Introduction

Family Adenoviridae, genus Adenovirus, human adenovirus types (HAdVs) are known to cause strong inflammatory responses, such as elevation of the white blood cell (WBC) count and C-reactive protein (CRP), which sometimes leads to overuse of antibiotics.[1] Differences in the immune responses to the various species of HAdV have not been well studied. Links between epidemiology and immune responses may provide clues to understanding the mechanisms underlying HAdVs' circulation patterns.

Although HAdVs are one of the most common pathogens in young children, accounting for 10% to 29% of upper respiratory infections,[2,3] the diseases they cause are believed to be mild and self-limiting.[4] However, HAdVs sometimes cause outbreaks of severe disease not only in children,[5-8] but also in adults.[9] Understanding the circulation patterns of and inflammatory responses to HAdV in healthy children might have epidemiological significance since virus circulating in the normal population may serve as a reservoir[10] for outbreaks. There have been, however, no reports that investigated community-based epidemiology, including inflammatory testing for common HAdV respiratory disease in children.

To address the above issue, we conducted a prospective study aimed at clarifying the circulation patterns and clinical/laboratory features of respiratory HAdV infections in children at a primary care clinic in a small community. Their inflammatory responses were studied in detail, including production of cytokines.

METHODS

Study Design and Participants

A prospective cross-sectional study was performed. The target population was

pediatric outpatients <13 years old with HAdV respiratory infection confirmed by a rapid test for adenovirus antigen (ImmunoAce Adeno, Tauns Laboratories Co., Shizuoka, Japan). To avoid selection bias, the eligible patients were surveyed consecutively at a pediatric clinic that looked after the health care of most of the children in a small community. Adenovirus rapid tests were routinely performed using an immunochromatography kit (ImmunoAce Adeno) with a nasopharyngeal swab for patients with febrile respiratory illness. Laboratory tests were also routinely performed to measure the WBC count and CRP. For this study, we obtained all nasopharyngeal swabs that gave a positive rapid test result as well as the residual sera from the routine blood testing performed during the study period, i.e., October 1st, 2013 to September 30th, 2015. Consecutive nasopharyngeal samples from patients with laboratory-defined common HAdV respiratory infections were thus surveyed for 2 years. Nasopharyngeal swabs were placed in 5-mL tubes containing 1-2 ml of normal saline and frozen at –80°C until use. The residual sera were similarly stored.

Ethical Approval

The study protocol was approved by the Ethics Committee of Mie National Hospital (#25-16). Informed consent was obtained from all the parents or caregivers of the enrolled patients.

Adenovirus Detection by the Immunochromatography Kit

The manufacturer of the kit states that it detects HAdV-1, 2, 3, 4, 5, 6, 7, 8, 11, 19 and 37. We confirmed that it also detected HAdV-14, 16, 21, 34, 35 and 57 from clinical isolates with similar sensitivity at 10^5 to 10^7 copies, as for the other types. Thus, the kit covered all respiratory HAdV types except for HAdV-50, which is very rare and has never been detected in Japan.

Adenovirus Culture

To isolate infective HAdV from the nasopharyngeal samples, we performed adenovirus culture in a respiratory epithelial cell line, A549 (ATCC CCL-185). Each frozen tube of saline extract was thawed. The contents were filtered using a disposable syringe filter having a pore size of 0.20 μm (Asahi Glass Co., Ltd., Tokyo, Japan). Each filtrate was tested for presence of virus in the A549 cell line. A549 cells were cultured in minimum essential medium (MEM) supplemented with 10% inactivated fetal bovine serum (FBS) in 50-ml Falcon® tissue culture flasks (Corning, Corning, NY) and grown to 80% confluence. The culture medium was then removed, and 1 ml of each filtrate was inoculated into a flask. After 1 h of incubation, the inoculated sample solution was removed, MEM containing 1-2% inactivated FBS was added to the flask, and culture was continued at 37°C in 5% CO_2 for 7 to 21 days.[11] The cells were observed daily with

an inverted microscope for signs of cytopathic effects (CPE), and adenovirus culture was considered positive when characteristic, round, “cluster of grapes” CPE was observed for over ≥25% of the grown area.[12] The cells and medium were then collected and freeze–thawed at least three times. After centrifugation at 1300 rpm for 10 min, the supernatant was collected and stored at -80°C until PCR assay.

Identification of Adenovirus Type and Species

Molecular identification of isolated HAdVs was performed using multiple PCR methods.[13-16] The primers for hexon, fiber and species PCR are listed in Tables 1A, 1B and 1C, respectively. The PCR products in hexon-PCR were sequenced directly using a kit (BigDye Terminator Cycle Sequencing Kit; Applied Biosystems, Foster City, CA, USA) and a genetic analyzer (3100-Avant; Applied Biosystems). The nucleotide sequences of reference strains of each HAdV type were obtained from the GenBank nucleotide database.

Measurement of Serum Cytokine Concentrations

A MILLIPLEX MAP Human Cytokine/Chemokine Kit (Millipore, Billerica, MA, USA) was used on a Luminex 100™ analyzer (Luminex, Tokyo, Japan) to measure the serum levels of 29 cytokines: interferon α2 (IFN-α2), IFN-γ, interleukin 2 (IL-2), IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17, IFN-γ-

inducible protein 10 (IP-10), tumor necrosis factor α (TNF-α), TNF-β, IL-1 receptor antagonist (IL-1ra), IL-1α, IL-1β, eotaxin, epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), granulocyte-colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α) and MIP-1β.

Statistical Analysis

Descriptive statistics were generated. Continuous variables were presented as mean values with standard deviations (SDs), and categorical variables were presented as numbers and percentages. Comparisons between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). P-values of < 0.05 were considered significant.

RESULTS

Frequency of the Isolated HAdVs

Within the 2-year study period, 187 children were enrolled, and 177 (94.7%) viral strains were isolated. The HAdV species and types were identified for 175 of the 177 isolates. They included 3 species and 6 types, i.e., species B (type 3), species C (types 2, 1, 5 and 6, in the order of frequency), and species E (type 4). Species C was the most common (37.1%), followed by species B (30.9%) and species E (26.9%) (Fig. 1). Multiple types/species were co-detected in 13 samples (5.1%) (Table S1).

Temporal Distribution of the Isolated HAdVs

The time trends of virus detection by species (Fig. 2A-C) and types (Table S2) during the 2-year study period showed that HAdV circulated from early spring to early fall, less frequently in winter. Multiple types/species circulated concurrently, but the circulation pattern appeared to differ with the species. The incidence of species B was high in summer and early fall (Fig. 2A), whereas species C circulated year-round, but more in late spring and summer, less in winter (Fig. 2B). In contrast to the yearly circulation patterns seen for species B and C, species E circulated in only one season, i.e., early spring in 2013, but in only one case each in 2014 and 2015 (Fig. 2C).

Gender and Age Distributions of the Isolated HAdVs

The male/female ratios of the patients infected with species B, C and E were 27/27, 37/24 and 25/23, respectively, indicating no gender preference by species (chi-square test, P=0.48). The mean age of all patients was 3.2 ± 2.7 y. Patients infected with species C were significantly younger than those infected with species B and E: 1.4 ± 1.3, 3.3 ± 2.1 and 5.3 ± 3.0 y, respectively (one way ANOVA; P<0.0001, followed by Tukey's multiple comparisons test; P<0.0001). It is of note that the age distribution of patients infected with species C showed a sharp peak at age 1 (Fig. 2E), in contrast to the broad age distributions of patients infected with species B (0-9 years) and species E (0-12 years) (Fig. 2D, 2F). Table S3 shows the age distributions of the patients with each type.

Clinical and Laboratory Findings

The clinical and laboratory data were compared among the species. The recorded clinical symptoms were similar among all the patients, and none of the children were severe enough to be hospitalized. The mean body temperature (BT) of the subjects was 39.2°C, with no difference in BT among the species (Table 2).

The WBC count and CRP concentration were determined for 148 (89.7%) and 150 (90.9%) of the children, respectively. The WBC count in patients infected with species C was significantly higher than in those with species B and E (Table 2, Figure S1A).

Because the normal WBC count range is higher in infancy, we performed age-matched comparison for subgroups of patients less than 36 months of age (Fig. S1B) and at 36 months or more (Fig. S1C). The WBC count with species C was still higher than the counts with species B and E in both subgroups. Since the WBC count changes rapidly during the course of infection, the sampling date may have influenced the WBC data. However, there was no significant difference in the mean number of days from onset to sampling among the species (data not shown). There was no significant difference in the CRP concentration among the species (Table 2), even when age-matched comparison was performed for the subgroups of less than 36 months of age and 36 months or older (data not shown).

Serum Cytokine Concentrations

The residual serum was obtained from 139 patients (74.3%), and 29 cytokines were measured. Eleven kinds of cytokines were detected in more than 70% of the samples (Table 3). Among them, 7 cytokines, namely, G-CSF, IFN-γ, IL-6, TNF-α, MCP-1, MIP-1β and eotaxin, were significantly elevated in patients with species C (Fig. 3A-G), whereas IL-10 was significantly lower in those with species C (Fig. 3H), than the other species. The serum levels of 3 cytokines, i.e., IL-1ra, IL-8 and IP-10, did not differ among the species groups (Fig. S2).

DISCUSSION

This study aimed to investigate the circulation patterns and clinical/laboratory features of respiratory HAdV infections, particularly the inflammatory responses to HAdV, which have not been reported in previous studies, especially in regard to serum cytokines.

We determined the epidemiology and laboratory characteristics of HAdVs causing common respiratory infections in otherwise healthy children without any underlying disease. We found that species C (types 2, 1, 6 and 5, by order of frequency) was most common at 37.1%, followed by species B (type 3) at 30.9% and species E (type 4) at 26.9 %. Species C predominated at young age, especially at 1 year. It induced stronger inflammatory responses, as evidenced by higher WBC, higher proinflammatory cytokines such as G-CSF and TNF-α, and lower anti-inflammatory IL-10 in the serum, compared with the other 2 species. In contrast, species B and E were widely distributed up to age 9 years and 12 years, respectively, and caused weaker inflammatory responses compared with species C.

There have many reports on outbreaks and severe infections caused by HAdV,[8, 9, 17-20] but few have investigated the epidemiology of common respiratory HAdVs that cause mild disease and circulate regularly. We performed a systematic review of the

epidemiology of HAdVs. We used “adenovirus”, “epidemiology" and “children” as keywords to conduct a PubMed search of original research articles in English, published from 1957 to 2017. This search yielded a total of 1,015 articles. We then excluded 303 studies dealing with gastroenteritis or conjunctivitis and 315 studies that dealt with patients with underlying conditions such as immunocompromised states and post-transplantation. Next, we excluded 103 papers that studied severe HAdV infections and outbreak settings. We also excluded 231 studies of hospitalized children and lower respiratory adenovirus infections, and 45 studies of other viral infections. Finally, we selected 18 articles on the epidemiology of upper respiratory adenovirus infections in normal children, and the main findings are summarized in Table S4. Briefly, the prevalent HAdV species were C and B. Species E occurred sporadically. Young children of < 6 years of age were infected, and species C affected younger children than species B. Seasonality varied depending on the area of the globe, generally from spring to summer in temperate zones and in winter and spring in tropical zones. Our present results correspond well with those earlier epidemiological findings. In terms of inflammatory responses, only WBC and CRP had been compared between species B and C in previous reports.[18; 21] Species differences in serum cytokine levels have not been studied.

An outbreak of HAdV-4 was reported in military trainees, but not in young children.[9] The reason why young, otherwise healthy, adults were susceptible to the virus was unclear. Here, we found that HAdV-4 circulated in only one season during the 2-year study period. In two other epidemiological studies in children, that type was identified only once during a 12-year period.[21, 22] These results suggest that HAdV-4 circulates only in a restricted time period, meaning that it induces little herd immunity in children and leaves young adults "susceptible". In contrast, we and others[4, 23-25] found that species C circulated every year and infected only young children, not older children (Table S4). This suggests that species C readily generates herd immunity in children, and thus older children, possibly even young adults, may be protected.

Our current findings that the WBC count and several inflammatory cytokines were elevated in patients infected with species C may be related to the above-mentioned phenomenon. Higher inflammatory markers may indicate higher innate immune responses leading to protective acquired immunity.[26] However, the species showed no differences in CRP. This may be explained by the differential cytokine responses found here. That is, the patients infected with species C showed significantly elevated levels of G-CSF, a hematopoietic cytokine for granulocytes; TNF-α and IL-6, inflammatory cytokines that activate vascular endothelium to recruit leukocytes; and MCP-1, MIP-1β

and eotaxin, chemokines for monocytes and eosinophils. Those responses may explain the higher WBC count seen with species C. Meanwhile, among the cytokines that induce CRP production in the liver, only IL-6, not IL-1β, was elevated with species C.

We believe several points characterize our study. First, we identified HAdV types by multiple methods, i..e, screening with an immunochromatography kit, virus culture and 3 PCR methods (fiber, hexon and species). Especially, we confirmed that the immunochromatography kit (ImmunoAce Adeno) detected all the respiratory HAdVs with sufficient sensitivity except for HAdV-50, which is very rare and has never been detected in Japan. Second, we employed a prospective "near" population-based method by surveying consecutive children diagnosed with febrile respiratory illnesses at a single pediatric clinic that looked after the health care of most children living in a small community. In addition, we employed laboratory-defined criteria for inclusion of patients using the rapid ImmunoAce Adeno test. This serial inclusion based on definitive criteria minimized selection bias.

Some limitations should also be mentioned. The study population was recruited at only one clinic. A multi-center population-based study would better delineate the epidemiology. However, such a study might not be feasible due to the logistics of investigating the large number of patients contracting this very infectious disease. Thus,

we intentionally used a single site that provided care to most of the children in the small community. We think that the present results accurately reflect the actual HAdV epidemic patterns in that community.

In conclusion, this study elucidated the temporal and age-related epidemiology and inflammatory responses of HAdV respiratory infections in children. A detailed understanding of the epidemiology may help in planning future strategies for prevention of common, sometimes lethal, HAdV infections.

ACKNOWLEDGMENTS

This research was supported by a grant-in-aid from the Japan Agency for Medical Research and Development (AMED) and a medical research grant from the Association of Pediatricians in Mie. The authors would like to thank Ms. Manami Negoro for her excellent technical assistance, and Dr. Toshiaki Ihara for his advice regarding the research and valuable comments on this study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

1. Van Lierde S, Corbeel L, Eggermont E. Clinical and laboratory findings in children with adenovirus infections. Eur J Pediatr 1989;148:423-425.
2. Kim JK, Jeon JS, Kim JW, Rheem I. Epidemiology of respiratory viral infection using multiplex rt-PCR in Cheonan, Korea (2006-2010). J Microbiol Biotechnol 2013;23:267-273.
3. Chonmaitree T, Revai K, Grady JJ, Clos A, Patel JA, Nair S, Fan J, Henrickson KJ. Viral upper respiratory tract infection and otitis media complication in young children. Clin Infect Dis 2008;46:815-823.
4. Sakamoto M, Yazaki N, Katsushima N, Mizuta K, Suzuki H, Numazaki Y. Longitudinal investigation of epidemiologic feature of adenovirus infections in acute respiratory illnesses among children in Yamagata, Japan (1986-1991). Tohoku J Exp Med 1995;175:185-193.
5. Moura PO, Roberto AF, Hein N, Baldacci E, Vieira SE, Ejzenberg B, Perrini P, Stewien KE, Durigon EL, Mehnert DU, Harsi CM. Molecular epidemiology of human adenovirus isolated from children hospitalized with acute respiratory infection in Sao Paulo, Brazil. J Med Virol 2007;79:174-181.
6. Lee WJ, Jung HD, Cheong HM, Kim K. Molecular epidemiology of a post-influenza pandemic outbreak of acute respiratory infections in Korea caused by human adenovirus type 3. J Med Virol 2015;87:10-17.
7. Li Y, Zhou W, Zhao Y, Wang Y, Xie Z, Lou Y, Tan W. Molecular typing and epidemiology profiles of human adenovirus infection among paediatric patients with severe acute respiratory infection in China. PLoS ONE 2015;10:e0123234.

8. Lin YC, Lu PL, Lin KH, Chu PY, Wang CF, Lin JH, Liu HF. Molecular Epidemiology and Phylogenetic Analysis of Human Adenovirus Caused an Outbreak in Taiwan during 2011. PLoS ONE 2015;10:e0127377.
9. Barraza EM, Ludwig SL, Gaydos JC, Brundage JF. Reemergence of adenovirus type 4 acute respiratory disease in military trainees: report of an outbreak during a lapse in vaccination. J Infect Dis 1999;179:1531-1533.
10. Ashford RW. When is a reservoir not a reservoir? Emerg Infect Dis 2003;9:1495-1496.
11. Enomoto M, Fujimoto T, Konagaya M, Hanaoka N, Chikahira M, Taniguchi K, Okabe N. Cultivation for 21 days should be considered to isolate respiratory adenoviruses from samples containing small numbers of adenoviral genomes. Jpn J Infect Dis 2010;63:338-341.
12. Ahluwalia GS, Scott-Taylor TH, Klisko B, Hammond GW. Comparison of detection methods for adenovirus from enteric clinical specimens. Diagn Microbiol Infect Dis 1994;18:161-166.
13. Adhikary AK, Inada T, Banik U, Numaga J, Okabe N. Identification of subgenus C adenoviruses by fiber-based multiplex PCR. J Clin Microbiol 2004;42:670-673.
14. Banik U, Adhikary AK, Suzuki E, Inada T, Okabe N. Multiplex PCR assay for rapid identification of oculopathogenic adenoviruses by amplification of the fiber and hexon genes. J Clin Microbiol 2005;43:1064-1068.
15. Okada M, Ogawa T, Kubonoya H, Yoshizumi H, Shinozaki K. Detection and sequence-based typing of human adenoviruses using sensitive universal primer sets for the hexon gene. Arch Virol 2007;152:1-9.
16. Xu W, McDonough MC, Erdman DD. Species-specific identification of human

adenoviruses by a multiplex PCR assay. J Clin Microbiol 2000;38:4114-4120.

17. Mitchell LS, Taylor B, Reimels W, Barrett FF, Devincenzo JP. Adenovirus 7a: a community-acquired outbreak in a children's hospital. Pediatr Infect Dis J 2000;19:996-1000.

18. Cheng CC, Huang LM, Kao CL, Lee PI, Chen JM, Lu CY, Lee CY, Chang SY, Chang LY. Molecular and clinical characteristics of adenoviral infections in Taiwanese children in 2004-2005. Eur J Pediatr 2008;167:633-640.

19. Lewis PF, Schmidt MA, Lu X, Erdman DD, Campbell M, Thomas A, Cieslak PR, Grenz LD, Tsaknardis L, Gleaves C, Kendall B, Gilbert D. A community-based outbreak of severe respiratory illness caused by human adenovirus serotype 14. J Infect Dis 2009;199:1427-1434.

20. Ghanaiem H, Averbuch D, Koplewitz BZ, Yatsiv I, Braun J, Dehtyar N, Wolf DG, Mandelboim M, Engelhard D. An outbreak of adenovirus type 7 in a residential facility for severely disabled children. Pediatr Infect Dis J 2011;30:948-952.

21. Wang YF, Shen FC, Wang SL, Kuo PH, Tsai HP, Liu CC, Wang JR, Chi CY. Molecular Epidemiology and Clinical Manifestations of Adenovirus Respiratory Infections in Taiwanese Children. Medicine (Baltimore) 2016;95:e3577.

22. Wang SL, Chi CY, Kuo PH, Tsai HP, Wang SM, Liu CC, Su IJ, Wang JR. High-incidence of human adenoviral co-infections in taiwan. PLoS One 2013;8:e75208.

23. Adrian T, Wigand R, Knocke KW, Schafer G, Grundmann M. Genome type analysis of adenoviruses: isolates from one year from the Hannover area. Arch Virol 1989;105:89-101.

24. Moro MR, Bonville CA, Suryadevara M, Cummings E, Faddoul D, Kobayaa H, Branigan PJ, Domachowske JB. Clinical features, adenovirus types, and local

production of inflammatory mediators in adenovirus infections. Pediatr Infect Dis J 2009;28:376-380.

25. Rojas LJ, Jaramillo CA, Mojica MF, Escalante MP, Delgado P. Molecular typing of adenovirus circulating in a Colombian paediatric population with acute respiratory infection. Epidemiol Infect 2012;140:818-822.

26. Zheng S, De BP, Choudhary S, Comhair SA, Goggans T, Slee R, Williams BR, Pilewski J, Haque SJ, Erzurum SC. Impaired innate host defense causes susceptibility to respiratory virus infections in cystic fibrosis. Immunity 2003;18:619-630.

Figure Legends

Figure 1 Frequency of species and types of isolated HAdVs.

Of 177 virus isolates, HAdV species and types were identified for 175 samples. Identified types are listed in the right column and the pie chart indicates proportion of the types and species.

Figure 2 Temporal and age distribution of the isolated HAdVs during the 2-year study period.

Sample collection in this study started in October 2013 and ended in September 2015. Number of isolates identified in each month for species B, species C and species E are shown in 2A, 2B and 2C, respectively. Data for the first year (from October 2013 to September 2014) are depicted as solid columns and the second year (from October 2014

to September 2015) as open columns. Number of isolates by age for species B, species C and species E are depicted in 2D, 2E and 2F, respectively.

Figure 3 Serum cytokine/chemokine levels by HAdV species.

Of 11 cytokines detected in more than 70% of the samples, 7 cytokines (A-G), were significantly elevated and IL-10 (H) was significantly lower in patients with species C than those with the other species.

Dunn's multiple comparisons test; * P<0.05, ** P<0.01, *** P<0.005, **** P<0.0001

Legends for supplementary figures

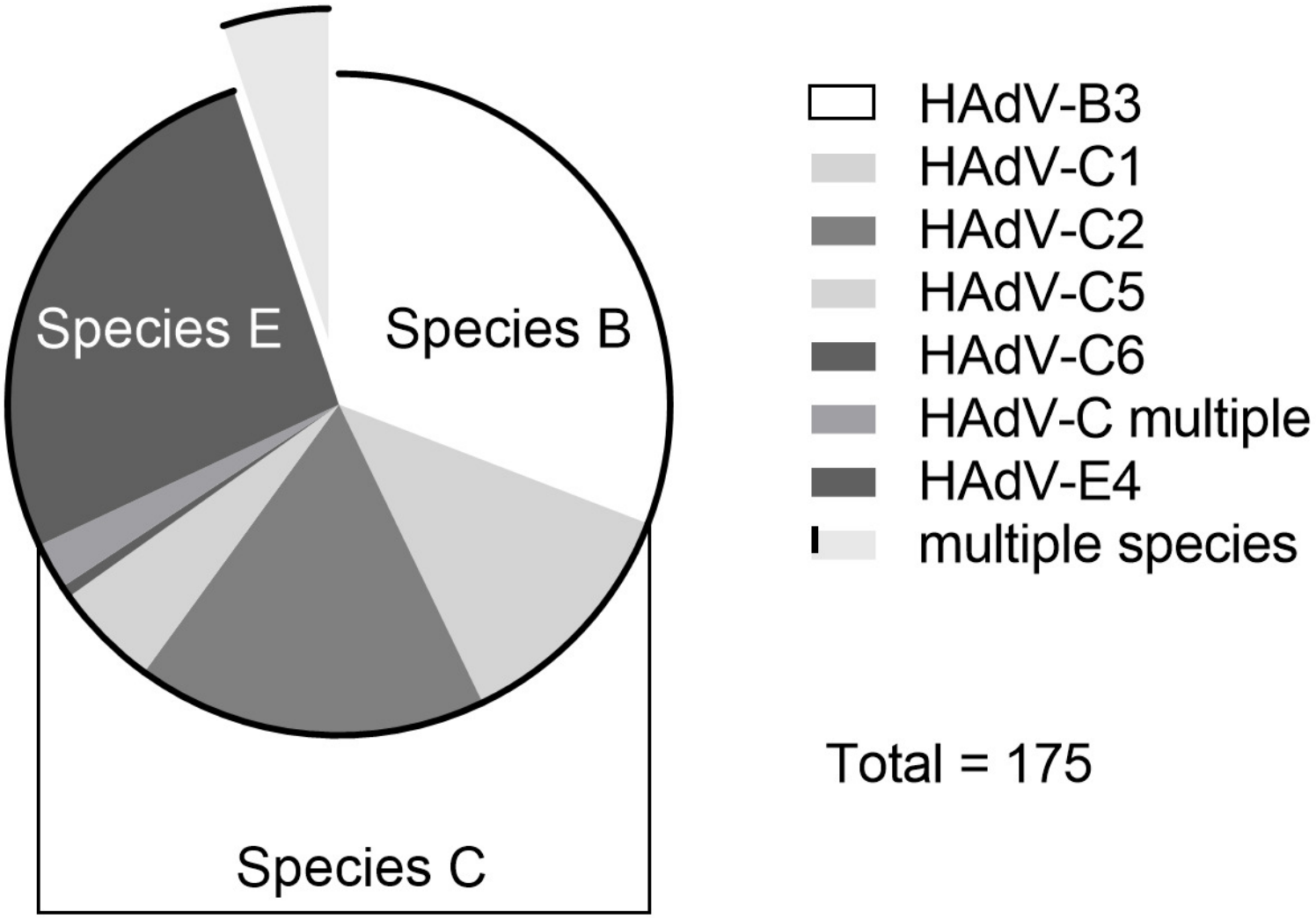
Figure S1 White blood cell count by HAdV species in all subjects (A), patients less than 36 months of age (B) and patients at 36 monthsor more (C).

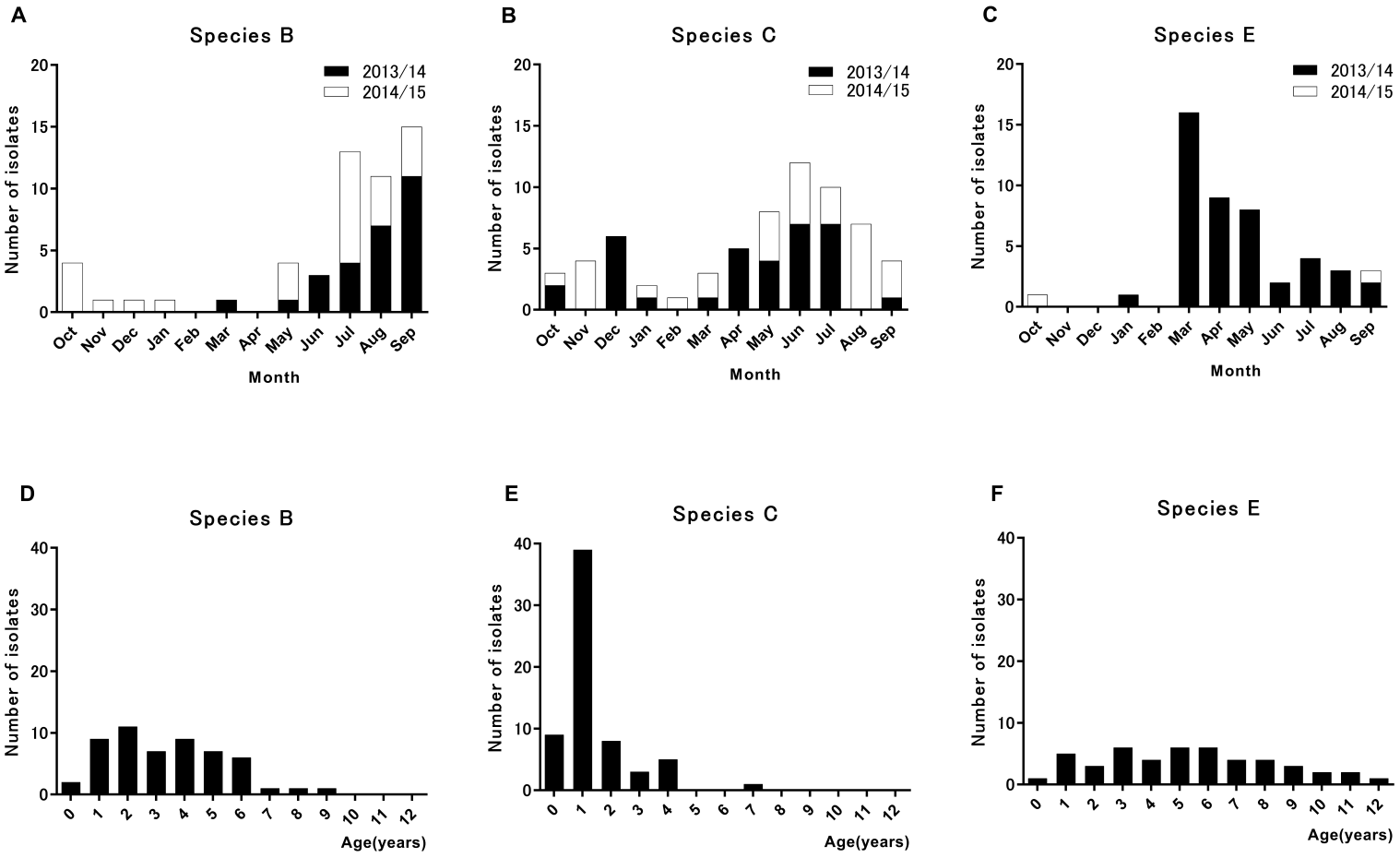
Dunn's multiple comparisons test; * P<0.05, ** P<0.01, **** P<0.0001

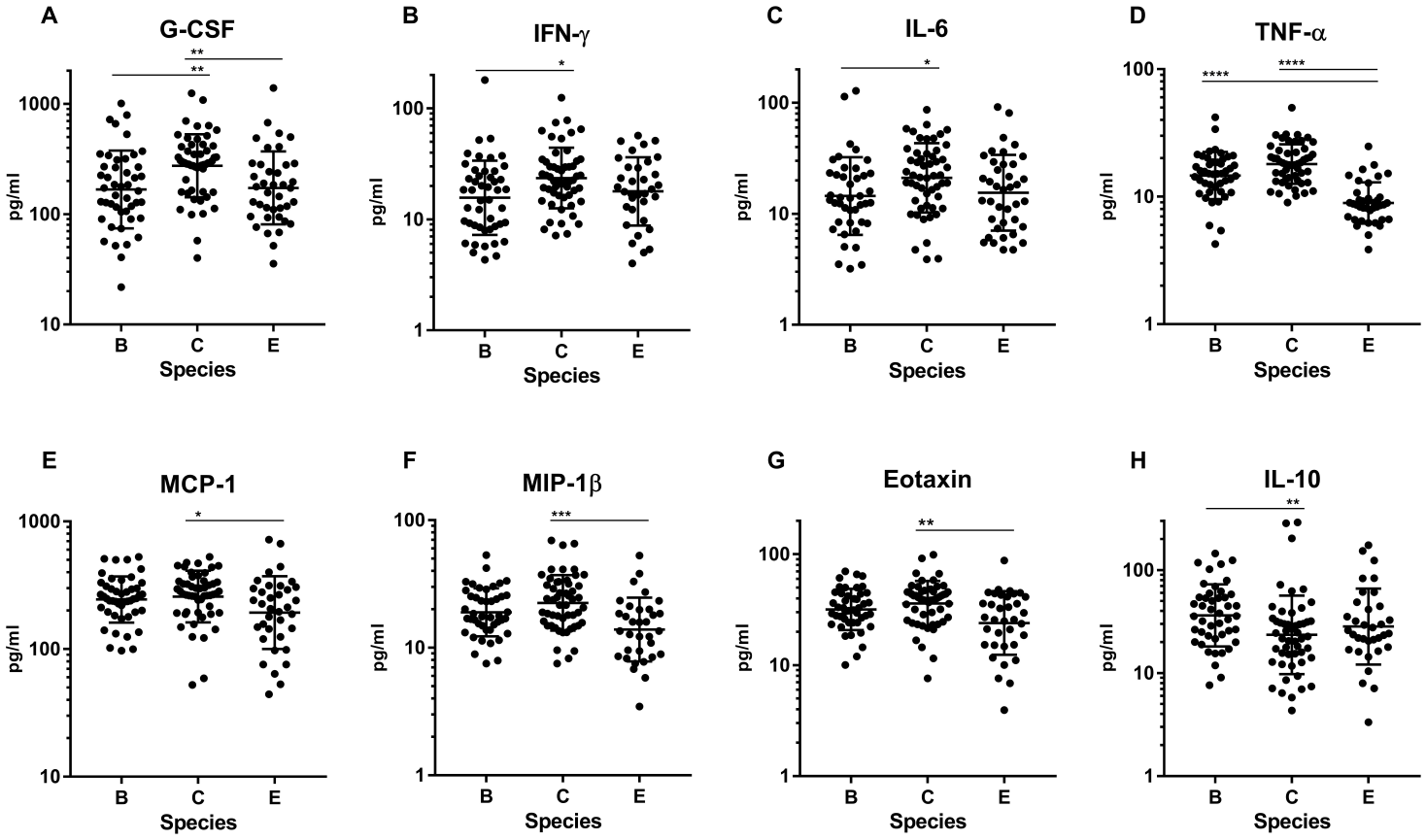
Figure S2 Serum cytokine/chemokine levels by HAdV species.

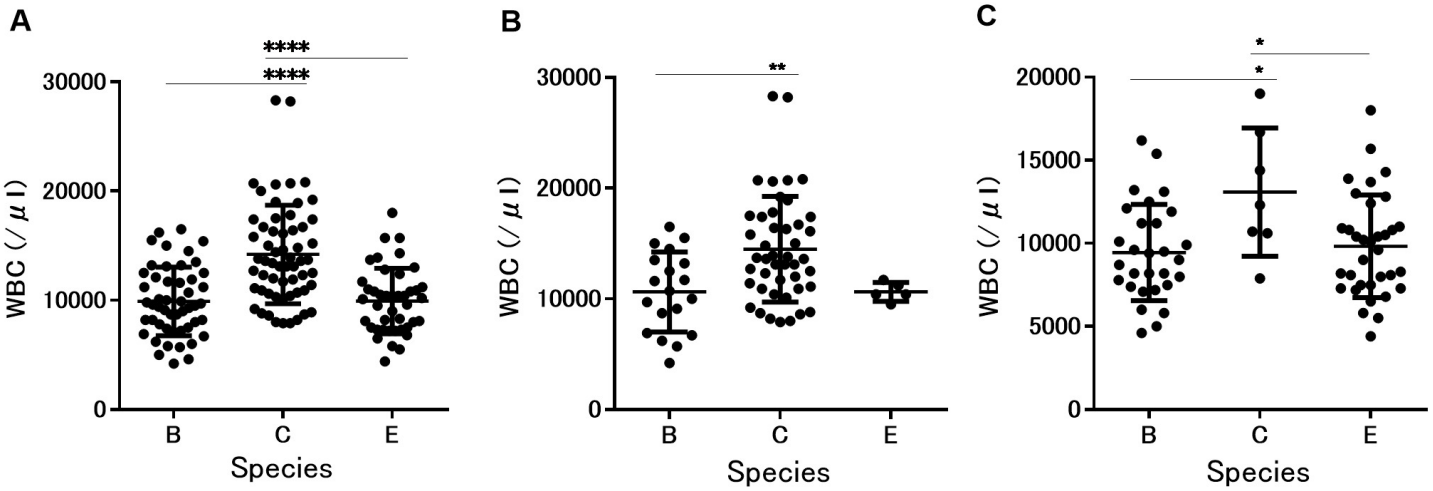
IL-1ra (A), IL-8 (B) and IP-10 (C)

List of supplementary tables


Species E
Species B
Species C
HAdV-B3
HAdV-C1
HAdV-C2
HAdV-C5
HAdV-C6
HAdV-C multiple
HAdV-E4
multiple species
Total = 175


A
Species B
2013/14
2014/15
Number of isolates
Month
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Jul
Aug
Sep
B
Species C
C
Species E
D
Species B
Age(years)
E
Species C
F
Species E


A
G-CSF
**
**
1000
100
10
pg/ml
B
C
E
Species
B
IFN-γ
*
100
10
1
pg/ml
B
C
E
Species
C
IL-6
*
100
10
1
pg/ml
B
C
E
Species
D
TNF-α
****
****
100
10
1
pg/ml
B
C
E
Species
E
MCP-1
*
1000
100
10
pg/ml
B
C
E
Species
F
MIP-1β
***
100
10
1
pg/ml
B
C
E
Species
G
Eotaxin
**
100
10
1
pg/ml
B
C
E
Species
H
IL-10
**
100
10
1
pg/ml
B
C
E
Species


A
****
****
30000
20000
10000
0
WBC (/μl)
B
C
E
Species
B
**
30000
20000
10000
0
WBC (/μl)
B
C
E
Species
C
*
*
20000
15000
10000
5000
0
WBC (/μl)
B
C
E
Species

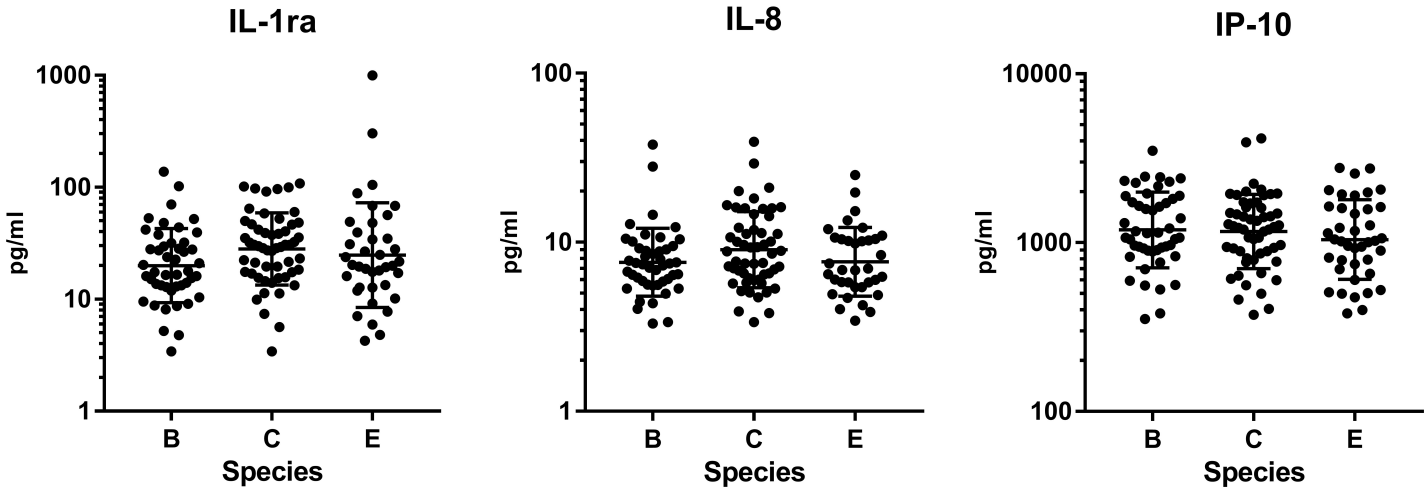

IL-1ra
1000
100
10
1
pg/ml
B
C
E
Species
IL-8
100
10
1
pg/ml
B
C
E
Species
IP-10
10000
1000
100
pg/ml
B
C
E
Species

Table 1A. Primers for hexon PCR

Primer name	Sequence (5`→3`)	Target	Amplicon size (bp)
Adhex-GT3F Adhex-GT2R	CSGGNCAGGAYGCYTCGGRGTA CACCCATGTTRCCWGTNCTGTT	HAdV hexon	1029 (1st PCR)
Adhex-GT2F Adhex-GT1R	AAYAARTTTAGRAAYCCCAC TTRTCYCTRAADSCAATGTARTT	HAdV hexon	905 (2nd PCR)

Table 1B. Primers for fiber PCR

Primer name	Sequence (5`→3`)	Target	Amplicon size (bp)
AdCF	TGCTTGCGCTHAAAATGGGCA	HAdV C fiber	*
Ad1R	CGAGTATAAGACGCCTATTTACA	HAdV-1 fiber	630
Ad2R	CGCTAAGAGCGCCGCTAGTA	HAdV-2 fiber	204
Ad5R	ATGCAAAGGAGCCCCGTAC	HAdV-5 fiber	455
Ad6R	CTTGCAGTCTTTATCTGAAGCA	HAdV-6 fiber	929
AdnB1	TACCCYTATGAAGATGAAAGC	HAdV B fiber	*
Adn3t	TGTTTTTAAATAAGGTGTTAACG	HAdV-3 fiber	517
Adn7t	GCCATTATTTGACAGTTGGCAGT	HAdV-7 fiber	404
Adn4F	ACCAGTAGTACAGAAACAGGAG	HAdV-4 fiber	*
Adn4t	CTTGAGCACTGCTTACTGTGCC	HAdV-4 fiber	295
Ad57F1	ATCCGCGCCCCTTAAAGTTGT	HAdV-57 fiber	808 *

* Forward primer reference 19, 20

Table 1C. Primers for species PCR

Primer name	Sequence (5`→3`)	Target	Amplicon size (bp)
AdA1 AdA2	GCTGAAGAAMCWGAAGAAAATGA CRTTTGGTCTAGGGTAAGCAC	HAdV A fiber	1444－1537
AdB1 AdB2	TSTACCCYTATGAAGATGAAAGC GGATAAGCTGTAGTRCTKGGCAT	HAdV B fiber	670–772
AdC1 AdC2	TATTCAGCATCACCTCCTTTCC AAGCTATGTGGTGGTGGGGC	HAdV C fiber	1988–2000
AdD1 AdD2	GATGTCAAATTCCTGGTCCAC TACCCGTGCTGGTGTAAAAATC	HAdV D fiber	1205–1221
AdE1 AdE2	TCCCTACGATGCAGACAACG AGTGCCATCTATGCTATCTCC	HAdV E fiber	967
AdF1 AdF2	ACTTAATGCTGACACGGGCAC TAATGTTTGTGTTACTCCGCTC	HAdV F fiber	541–586

Table 2. Body temperature, white blood cell count and CRP of patients infected with each species

	Species B	Species C	Species E	P value‡
Body temperature (°C) †	39.2 ± 0.5 (38.0-40.2)	39.2 ± 0.6 (37.8-40.4)	39.4 ± 0.6 (38.2-40.8)	0.2782
WBC (/μl) †	9975 ± 3163 (4200-16500)	14219 ± 4707* (7900-28300)	10024 ± 2884 (4400-18000)	<0.0001
CRP (mg/dl) †	2.9 ± 2.3 (0.2-10.3)	3.7 ± 3.3 (0.2-12.4)	4.3 ± 2.9 (0.7-12.1)	0.134

† Mean ± SD (range) ‡ ANOVA * Tukey's multiple comparisons test P<0.0001

Table 3. Number (percentage) of patients with detected serum cytokines (>3 pg/ml)

Infected HAdV species / Cytokine	Species			Total
	B	C	E	
	n=54	n=65	n=47	n=166
EGF	27 (50)	39 (60)	27 (57.4)	93 (56)
Eotaxin †	45 (83.3)	54 (83.1)	35 (74.5)	134 (80.7)
G-CSF †	46 (85.2)	53 (81.5)	39 (83)	138 (83.1)
GM-CSF	13 (24.1)	28 (43.1)	10 (21.3)	51 (30.7)
IFN-α2	9 (16.7)	18 (27.7)	3 (6.4)	30 (18.1)
IFN-γ †	47 (87)	54 (83.1)	34 (72.3)	135 (81.3)
IL-10 †	45 (83.3)	51 (78.5)	34 (72.3)	130 (78.3)
IL-12p40	15 (27.8)	33 (50.8)	6 (12.8)	54 (32.5)
IL-12p70	6 (11.1)	10 (15.4)	5 (10.6)	21 (12.7)
IL-13	4 (7.4)	9 (13.8)	6 (12.8)	19 (11.4)
IL-15	5 (9.3)	10 (15.4)	6 (12.8)	21 (12.7)
IL-17	11 (20.4)	14 (21.5)	9 (19.1)	34 (20.5)
IL-1ra †	46 (85.2)	54 (83.1)	38 (80.9)	138 (83.1)
IL-1α	13 (24.1)	21 (32.3)	9 (19.1)	43 (25.9)
IL-1β	1 (1.9)	5 (7.7)	3 (6.4)	9 (5.4)
IL-2	1 (1.9)	5 (7.7)	2 (4.3)	8 (4.8)
IL-3	0 (0)	2 (3.1)	0 (0)	2 (1.2)
IL-4	10 (18.5)	14 (21.5)	10 (21.3)	34 (20.5)
IL-5	1 (1.9)	2 (3.1)	0 (0)	3 (1.8)
IL-6 †	42 (77.8)	53 (81.5)	39 (83)	134 (80.7)
IL-7	13 (24.1)	27 (41.5)	7 (14.9)	47 (28.3)
IL-8 †	45 (83.3)	54 (83.1)	35 (74.5)	134 (80.7)
IP-10 †	47 (87)	54 (83.1)	40 (85.1)	141 (84.9)
MCP-1 †	47 (87)	52 (80)	36 (76.6)	135 (81.3)
MIP-1α	9 (16.7)	21 (32.3)	2 (4.3)	32 (19.3)
MIP-1β †	44 (81.5)	52 (80)	33 (70.2)	129 (77.7)
TNF-α †	45 (83.3)	52 (80)	38 (80.9)	135 (81.3)
TNF-β	5 (9.3)	9 (13.8)	5 (10.6)	19 (11.4)
VEGF	17 (31.5)	34 (52.3)	12 (25.5)	63 (38)

N: number of patients tested for serum cytokines.

†Cytokine was detected in more than 70% of patients.

Table S1. List of isolates co-detected by different PCR methods

ID	Age (y)	PCR method						
		Hexon	Fiber				Species	
M6	0	HAdV-C1	HAdV-C1	HAdV-C2			C	
M14	0	HAdV-C2	HAdV-B3	HAdV-C2			B	C
M10	0	HAdV-C5	HAdV-C2	HAdV-C5	HAdV-E4		C	E
M85	1	HAdV-C1	HAdV-C1	HAdV-C2			C	
M174	1	HAdV-C1	HAdV-C1	HAdV-C2			C	
M70	1	HAdV-C2	HAdV-C2	HAdV-C5			C	
M5	1	HAdV-E4	HAdV-C2	HAdV-E4			C	E
M162	2	HAdV-B3	HAdV-B3	HAdV-C1	HAdV-C2		B	C
M13	3	HAdV-B3	HAdV-B3	HAdV-E4			B	E
M87	3	HAdV-B3	HAdV-C2	HAdV-B3	HAdV-C5		B	C
M23	4	HAdV-B3	HAdV-B3	HAdV-E4			B	E
M86	4	HAdV-C5	HAdV-C1	HAdV-C2	HAdV-C5	HAdV-E4	C	E
M15	5	HAdV-C1	HAdV-C1	HAdV-E4			C	E

Table S2. Temporal distribution of viral types

Year	Month	Species B	Species C				Species E	Dual detection
		HAdV-3	HAdV-1	HAdV-2	HAdV-5	HAdV-6	HAdV-4	
2013	Oct		1		1			
	Nov							
	Dec		1	4				
2014	Jan					1	1	
	Feb							
	Mar	1	1	1			17	
	Apr	1		2	2		9	
	May	1	2	2			8	
	Jun	3		4	2		2	1
	Jul	4	2	4			4	
	Aug	7					3	
	Sep	11		1			2	
	Oct	4		1			1	
	Nov	1	2	2				
	Dec	1						
2015	Jan	1		1				
	Feb		1					
	Mar		1	1				
	Apr							
	May	3	1	2	1			
	Jun		2	3				
	Jul	9	3					
	Aug	3	3	3				
	Sep	4	1		2		1	1

Table S3. Viral types detected at each patient age

Age (y)	**Species B**	**Species C**				**Species E**
	HAdV-3	HAdV-1	HAdV-2	HAdV-5	HAdV-6	HAdV-4
0	2	3	3	2	1	1
1	10	16*	18*#	5#		5
2	11		6	2		3
3	7		3			6
4	8	3	2			5
5	7					6
6	6					6
7	1		1			4
8	1					4
9	1					3
10						2
11						2
12						1

Dual detection was observed in 2 patients: *HAdV-1 and 2; #HAdV-2 and 5.

Table S4. Summary of findings for respiratory HAdV epidemiology in healthy children

Reference	Zone	Presenting symptom	Age range	Study period	Number of samples	Detection methods	Predominant type/species	Age distribution	Seasonality
[1]	Tm	F, R	<7 y	6 y (1974-1981)	116	Culture/ NT	1 (34.7%) 2 (45.9%), C(86.7%)	Peak in 12-17 m rare < 6 m,	83% in fall to spring
[2]	Sa	F, R	<5 y	15 y (1967-1981)	298	Culture/ NT	1 (39.3%) 2 (25.5%) 5 (17.1%) C (92%)		1: fall and winter, 2: winter and spring
[3]	Tm	R, G	<15 y	1 y (1981)	63	Culture/ NT/ PCR	1 (11.1%), 2 (33.3%), 5 (15.9%), C (60%)	C: < 1 year	Year-round
4	Tm	F, R	<15 y	6 y (1986-1991)	989	Culture	1 (17.2%)	C: most < 2 y	Year-round
[5]	Tm	F, R	<22 y	17 mon (2000-2002)	143	Culture/ DFA	-	90% < 5y	Winter and spring

[6]	Tr	F, R	6-12 y	6 mo (2005)	23	Culture PCR (Hexon)	C (2,5): 100%	No difference in age	Peak in winter and spring
[7]	Tm	F, R	0-13 y	7 mo (2006-7)	25	PCR (Hexon)	-	44%, 7m-2y: 44% 3-5 y:	Peak in spring
[8]	Sa	F, R	1M-14y	15 y (1998-2007)	78	PCR (Hexon)	1 (34.6%), 2 (32.1%), 3 (15.4%), C (70.5%)	51% <1 y, C: 65.4% <1 y	-
[9]	Tm	F, R	0-15y	11 y (2000-2010)	581	Culture PCR (Hexon)	C: 75.6%		No obvious seasonality
[10]	Tm	F, R	not reported	1 y (2010-2011)	171	PCR (Hexon)	3 (70.2%), 7 (9.4%), B (79.6%) C (12.9%)	96.5% < 7 y	Peak in spring to early summer
[11]	Tr	F, R	1M-3y	1 y (2008)	52	IFA/ PCR (Hexon)	3 (46%), 7 (40%), B (85.4%) C (12.5%)	65.4% < 1 y	-

[12]	Tm	F, R	<14 y	2 y (2006-2008)	25	PCR (Hexon)	B3 (43.5%), C1&C2 (34.8%), E4 (21.7%)	No obvious age distribution	Peak in summer
[13]	Sa	F, R	0-17 y	1.5 y (2008-2010)	73	Culture/ PCR (Hexon)	B3 (38.4%), C1 (19.2%), C2 (38.4%), C: 61.6%	76.7% <4 y	No obvious seasonality
[14]	Tm	F, R	<10y	10 y (2003-2012)	884	Culture/ IFA/ PCR (Hexon)	3 (53.2%), 7 (36.8%), B: 90%, C: 7%	Peak at 0-3 y	-
[15]	Tm	F, R	0-168M	3 y (2006-2009)	74	PCR-sequence	3 (78.4%)	Peak at 3-4 y	No obvious seasonality
[16]	Tr	F, R	1M～14y	4 y (2009-2012)	71	PCR-sequence	3 (32%), 1 (31%), 2 (13%), 5 (12%), C: 56%	Peak at 0-3 y (54.9%)	Peak in winter and early summer
[17]	St~Tr	F, R	8M-9.3y	10 y (2002-2011)	126	Culture/ PCR (Hexon, Fiber)	3 (64.3%), 2 (23.8%)	Not described	Peak in winter and early summer

[18]	St~ Tr	F, R	not reported (Mean age 3.7y)	12 y (2002-2013)	175	Culture/ PCR (Hexon)	3 (50.6%), 2 (23.6%)	39%< 3 y. B older than C.	-
This study	Tm	F, R	0-13y	2 y (2013-2015)	187	IC/ Culture /PCR (Hexon, Fiber, Species)	C (37.1%) B (30.9%) E (26.9%)	C: peak w/in 1 yearB: 1-6 years E: 1-12 years	C: spring and summer, B: summer and early fall. E: sporadic

Sa: subarctic, Tr: tropical, St: subtropical, Tm: temperate

F: fever; R: respiratory symptoms; G: gastrointestinal symptoms

NT: neutralization assay; DFA: direct fluorescent assay; IFA: immunofluorescence assay; IC: immunochromatography

References

1. Edwards KM, Thompson J, Paolini J, Wright PF. Adenovirus infections in young children. Pediatrics 1985;76:420-424.

2. Pacini DL, Collier AM, Henderson FW. Adenovirus infections and respiratory illnesses in children in group day care. J Infect Dis 1987;156:920-927.

3. Adrian T, Wigand R, Knocke KW, Schafer G, Grundmann M. Genome type analysis of adenoviruses: isolates from one year from the Hannover area. Arch Virol 1989;105:89-101.

4. Sakamoto M, Yazaki N, Katsushima N, Mizuta K, Suzuki H, Numazaki Y. Longitudinal investigation of epidemiologic feature of adenovirus infections in acute respiratory illnesses among children in Yamagata, Japan (1986-1991). Tohoku J Exp Med 1995;175:185-193.

5. Rocholl C, Gerber K, Daly J, Pavia AT, Byington CL. Adenoviral infections in children: the impact of rapid diagnosis. Pediatrics 2004;113:e51-56.

6. Rosete DP, Manjarrez ME, Barron BL. Adenoviruses C in non-hospitalized Mexican children older than five years of age with acute respiratory infection. Mem

Inst Oswaldo Cruz 2008;103:195-200.

7. Fabbiani M, Terrosi C, Martorelli B, Valentini M, Bernini L, Cellesi C, Cusi MG. Epidemiological and clinical study of viral respiratory tract infections in children from Italy. J Med Virol 2009;81:750-756.
8. Moro MR, Bonville CA, Suryadevara M, Cummings E, Faddoul D, Kobayaa H, Branigan PJ, Domachowske JB. Clinical features, adenovirus types, and local production of inflammatory mediators in adenovirus infections. Pediatr Infect Dis J 2009;28:376-380.
9. Ampuero JS, Ocana V, Gomez J, Gamero ME, Garcia J, Halsey ES, Laguna-Torres VA. Adenovirus respiratory tract infections in Peru. PLoS One 2012;7:e46898.
10. Gao XQ, Jin Y, Xie ZP, Gao HC, Xie LY, Zhang J, Duan ZJ. [The epidemiological study of adenovirus in children with respiratory tract infections in Nanjing area from 2010 to 2011]. Bing Du Xue Bao 2012;28:531-535.
11. Rojas LJ, Jaramillo CA, Mojica MF, Escalante MP, Delgado P. Molecular typing of adenovirus circulating in a Colombian paediatric population with acute respiratory infection. Epidemiol Infect 2012;140:818-822.
12. Zou L, Zhou J, Li H, Wu J, Mo Y, Chen Q, Fang L, Wu D, Wu J, Ke C. Human adenovirus infection in children with acute respiratory tract disease in Guangzhou, China. APMIS 2012;120:683-688.
13. Abbas KZ, Lombos E, Duvvuri VR, Olsha R, Higgins RR, Gubbay JB. Temporal changes in respiratory adenovirus serotypes circulating in the greater Toronto area, Ontario, during December 2008 to April 2010. Virol J 2013;10:15.
14. Deng J, Qian Y, Zhao LQ, Zhu RN, Sun Y, Tian R. [Identification and typing of adenovirus from acute respiratory infections in pediatric patients in Beijing from 2003 to 2012]. Bing Du Xue Bao 2013;29:615-620.
15. Jin Y, Zhang RF, Xie ZP, Yan KL, Gao HC, Song JR, Yuan XH, Hou YD, Duan ZJ. Prevalence of adenovirus in children with acute respiratory tract infection in Lanzhou, China. Virol J 2013;10:271.
16. Sriwanna P, Chieochansin T, Vuthitanachot C, Vuthitanachot V, Theamboonlers A, Poovorawan Y. Molecular characterization of human adenovirus infection in Thailand, 2009-2012. Virol J 2013;10:193.
17. Wang SL, Chi CY, Kuo PH, Tsai HP, Wang SM, Liu CC, Su IJ, Wang JR. High-incidence of human adenoviral co-infections in taiwan. PLoS One 2013;8:e75208.
18. Wang YF, Shen FC, Wang SL, Kuo PH, Tsai HP, Liu CC, Wang JR, Chi CY. Molecular Epidemiology and Clinical Manifestations of Adenovirus Respiratory Infections in Taiwanese Children. Medicine (Baltimore) 2016;95:e3577.