

Adenovirus Species C Is Associated With Chronic Suppurative Lung Diseases in Children

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Background. The role of human adenoviruses (HAdVs) in chronic respiratory disease pathogenesis is recognized. However, no studies have performed molecular sequencing of HAdVs from the lower airways of children with chronic endobronchial suppuration. We thus examined the major HAdV genotypes/species, and relationships to bacterial coinfection, in children with protracted bacterial bronchitis (PBB) and mild bronchiectasis (BE).

Methods. Bronchoalveolar lavage (BAL) samples of 245 children with PBB or mild (cylindrical) BE were included in this prospective cohort study. HAdVs were genotyped (when possible) in those whose BAL had HAdV detected (HAdV⁺). Presence of bacterial infection (defined as $\geq 10^4$ colony-forming units/mL) was compared between BAL HAdV⁺ and HAdV negative (HAdV⁻) groups. Immune function tests were performed including blood lymphocyte subsets in a random subgroup.

Results. Species C HAdVs were identified in 23 of 24 (96%) HAdV⁺ children; 13 (57%) were HAdV-1 and 10 (43%) were HAdV-2. An HAdV⁺ BAL was significantly associated with bacterial coinfection with *Haemophilus influenzae*, *Moraxella catarrhalis*, or *Streptococcus pneumoniae* (odds ratio [OR], 3.27; 95% confidence interval, 1.38–7.75; $P = .007$) and negatively associated with *Staphylococcus aureus* infection ($P = .03$). Young age was related to increased rates of HAdV⁺. Blood CD16 and CD56 natural killer cells were significantly more likely to be elevated in those with HAdV (80%) compared with those without (56.1%) ($P = .027$).

Conclusions. HAdV-C is the major HAdV species detected in the lower airways of children with PBB and BE. Younger age appears to be an important risk factor for HAdV⁺ of the lower airways and influences the likelihood of bacterial coinfection.

Keywords. bronchiectasis; protracted bacterial bronchitis; respiratory bacteria; respiratory viruses; children.

The burden (prevalence, cost, and importance) of protracted bacterial bronchitis (PBB) and bronchiectasis (BE) are increasingly appreciated [1–4]. PBB is the most common cause of chronic cough in children presenting to pediatric pulmonologists in some series [1, 5]. Over the last 2 decades, the diagnosis of BE in

children and adults has increased [6, 7]. Both conditions are characterized by chronic wet cough, lower airway endobronchial suppuration with bacterial infection, intense airway neutrophilia, and upregulation of inflammatory and immune markers [5, 8–10]. It is hypothesized, but remains unproven, that PBB and BE represent a clinical continuum based upon degree of severity, sharing common triggers and/or pathophysiology [11].

To date, research into the microbiology of chronic suppurative lung diseases (PBB and BE) in children has largely focused on bacterial pathogens, with a paucity of research into viral contributors. Viral–bacterial coinfection is associated with heightened neutrophilic inflammation of the lower airways of children [12]. Human adenovirus (HAdV) detection is associated with lower

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airway neutrophilic inflammation in children with chronic respiratory symptoms, [13] and is significantly more likely to be present in the lower airways of children with PBB, compared with controls [14]. HAdVs (particularly types 1–5, 7, 14, and 21; members of HAdV species B, C, and E) are known to be associated with future small airways dysfunction and bronchiectasis [15, 16]. However, no studies, to our knowledge, have examined the HAdV genotypes in the lower airways of children with chronic endobronchial suppuration to investigate a possible role in pathogenesis.

Thus, we studied the bronchoalveolar lavage (BAL) of 245 children with PBB and mild BE. We aimed to identify (1) the prevalence of HAdV; (2) the diversity of genotypes/species using sequence analysis, and (3) whether presence of HAdV increased the odds of bacterial coinfection. We hypothesized that, in the lower airways of children with PBB and mild BE, certain HAdV genotypes/species would predominate, and that presence of HAdV increases the risk of bacterial coinfection.

METHODS

Children included in this study were a subset of a larger prospective, longitudinal cohort study on chronic cough in children [12]. Ethical approval was obtained from The Queensland Children's Health Services Human Research Ethics Committee (HREC/03/QRCH/17). Written informed consent was obtained from the parents/guardians.

Children were recruited between March 2008 and September 2013, covering 6 winter seasons. Of 398 children undergoing flexible bronchoscopy and BAL for clinical indication, 245 were diagnosed with PBB or BE, and were therefore eligible for inclusion. Children were divided into 2 groups: BAL positive for HAdV (HAdV⁺) or BAL negative for HAdV (HAdV⁻).

Key demographics and cough characteristics were obtained via completion of a standardized clinical questionnaire. Prospective follow-up of participants was undertaken with daily cough diaries and monthly contact via email or telephone.

Basic immune function tests were performed on peripheral blood, including full blood examination, immunoglobulins (IgG, IgA, IgM, IgE), IgG subclasses, specific antibody (IgG) responses to *Haemophilus influenzae* type b and *Clostridium tetani*, and lymphocyte subsets (including CD16⁺ and CD56⁺ natural killer [NK] cells). Bronchoscopy and BAL were performed as described previously [13], in accordance with European Respiratory Society guidelines [17]. Quantitative bacteriology and cellularity on BAL specimens were undertaken as outlined in a prior study [8].

Microbiology

Standardized semiquantitative culture of BAL fluid was undertaken using routine laboratory techniques, as described previously [8]. Infection with major respiratory pathogens (*H. influenzae*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*,

and *Staphylococcus aureus*) was defined by bacterial load of $\geq 10^4$ colony-forming units (CFU)/mL BAL, using standard culture techniques described elsewhere [8].

Real-time polymerase chain reaction (PCR) [18–21] was used to detect a conventional panel of respiratory viruses in all, and an extended panel in a random subset of, children. Viruses included HAdVs, respiratory syncytial virus, human influenza A virus, human influenza B virus, human parainfluenza viruses 1–3 (HPIV1-3), human metapneumovirus (standard panel), human rhinoviruses, human bocavirus, human coronaviruses (NL63, OC43, 229E, HKU1), WU polyomavirus, and KI polyomavirus (extended panel).

HAdV PCR testing was undertaken on all BAL samples at a clinical laboratory (Pathology Queensland, Royal Brisbane and Women's Hospital, Brisbane, Australia), using a previously described method [22], to identify HAdV⁺ participants. A random subset of HAdV isolates, based upon sample availability, from 26 HAdV⁺ on BAL children, underwent nucleotide sequencing at a research laboratory (Queensland Paediatric Infectious Diseases Laboratory, Brisbane, Australia).

HAdV genotyping, based on the hexon gene hypervariable regions 1–6, was conducted as previously described [23]. In brief, 2 μ L of nucleic acid extract was amplified in a nested PCR reaction. Round 1 contained primers (0.38 μ M; AdHexF1-TICTTTGACATTCGIGGIGTICTIGA and AdHexR12-CTGTCTCAGCCTGRTCCACA), magnesium chloride (4.75 μ M), buffer, and MyTaq HS DNA polymerase (Bioline kit, Australia) and was incubated for 1 minute at 94°C followed by 35 cycles of 94°C for 1 minute, 45°C for 1 minute, 72°C for 2 minutes, and a final incubation at 72°C for 1 minute. Round 2 used 2 μ L of the Round 1 PCR product as template, the same conditions but different primers (AdHexF2- GGYCCYAGYTTTAAARCCCTAYTC and AdHexR2- GGTTCGTCTCICCCAGAGARTCIAGCA). Round 2 PCR products were checked by agarose gel electrophoresis, purified, and subjected to nucleotide sequencing (Australian Equine Genetics Research Centre, UQ) results were analyzed to determine the HAdV genotype (Geneious Pro version 6.1).

Relationships between HAdV genotypes, sequenced from 24 HAdV⁺ study children, were illustrated in a phylogenetic tree (Figure 1) constructed using the neighbor-joining method (with evolutionary history inferred) in MEGA5 [24] after sequence alignment in Geneious version 6.1 [25]. Additional sequences were included from GenBank (labeled with genotype and accession number) to define the species according to a previously described approach [23].

Clinical Definitions

BE was diagnosed based on radiological criteria [26] in children with clinical symptoms consistent with BE [27]. All had evidence of mild (cylindrical) BE on high-resolution computed tomography (CT) scan (reconstructed from a multidetector CT scan). A

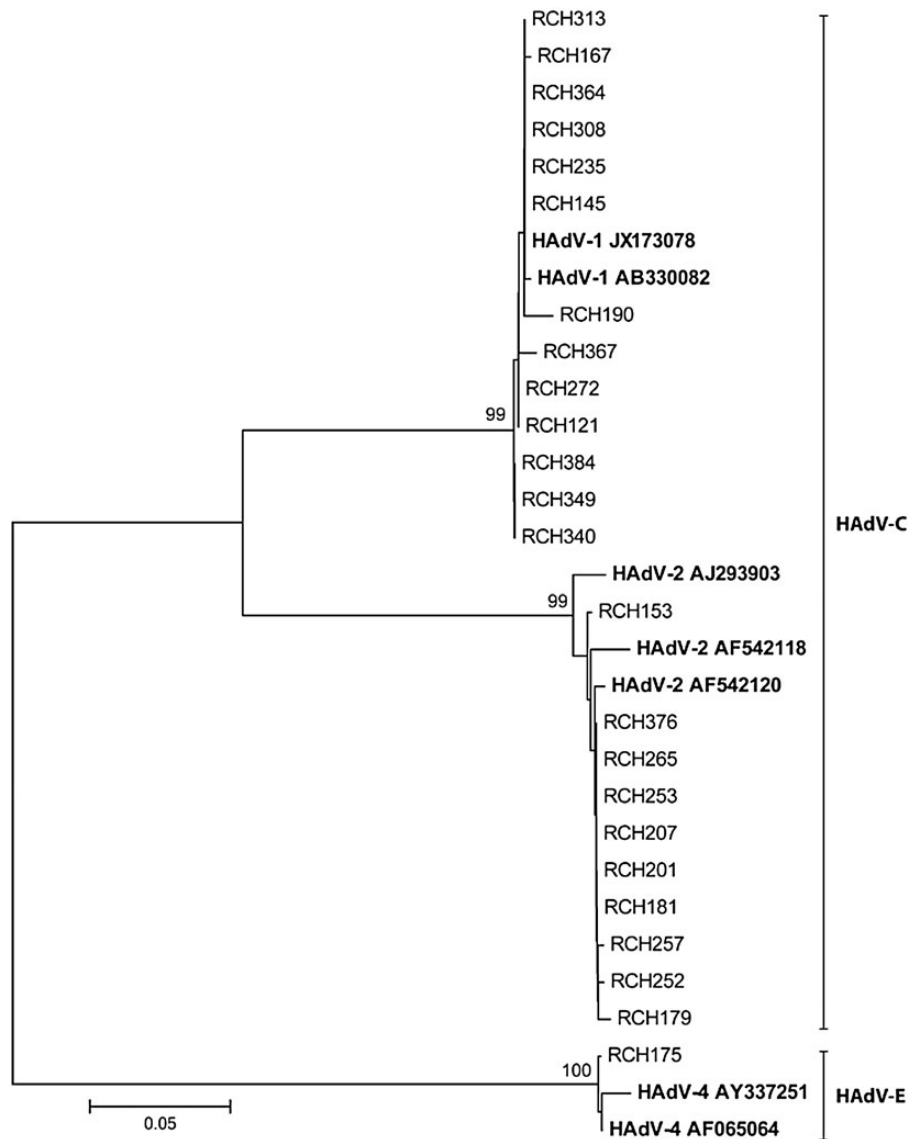


Figure 1. A neighbor-joining method of phylogenetic analysis of human adenovirus (HAdV) genotypes and their assigned species. The 24 nucleotide sequences (approximately 800 nt long) from the hexon gene of HAdV-positive samples from study patients are prefixed with Royal Children’s Hospital (RCH). The evolutionary distances were computed using the maximum composite likelihood method [23] and are in the units of the number of base substitutions per site. Additional sequences for speciation are included from GenBank (labeled with genotype and accession number in bold). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the major branches.

diagnosis of PBB was made in children who fulfilled the following criteria: (1) history of chronic (≥ 4 weeks) wet cough, (2) prospective evidence (supported by cough diaries) of response to 2 weeks of amoxicillin-clavulanate antibiotics, and (3) absence of clinical pointers suggesting an alternative cause for cough [5].

Statistical Analysis

Descriptive statistics were used to summarize baseline patient characteristics. Median and interquartile range (IQR) were

reported as data were nonnormally distributed. Univariate analyses were performed using Pearson χ^2 (or Fisher exact test) for categorical variables. Mann-Whitney U test was used for 2-group comparisons and Kruskal-Wallis test for >2 -group comparisons of continuous variables. Binary logistic regression was used to calculate odds ratios and P values. A 2-tailed P value of $<.05$ was considered statistically significant. Statistical analyses were performed using IBM SPSS version 20.0 (IBM SPSS, Armonk, New York).

Table 1. Characteristics of the Study Population

Group	No. (%)	Age, mo		Sex	
		Median	(IQR)	M	F
HAdV ⁺	40	19	(13–25)	29	11
PBB	33 (83)	17	(12–22)	24	9
BE	7 (18)	30	(22–65)	5	2
HAdV ⁻	205	37	(18–69)	126	79
PBB	126 (61)	26	(15–56)	78	48
BE	79 (39)	57	(31–90)	48	31

Abbreviations: +/-, positive/negative detection; BE, bronchiectasis; HAdV, human adenovirus; IQR, interquartile range; PBB, protracted bacterial bronchitis.

RESULTS

Study Population

Of the 245 children with PBB or BE, 40 were HAdV⁺ and 205 HAdV⁻. The median age of the children in the study was 30 months (IQR, 17–63 months), 23 months (IQR, 14–47 months) in those with PBB, and 57 months (IQR, 30–87 months) in those with BE. The median age was similar between HAdV⁺ and HAdV⁻ groups in children with BE ($P = .312$), Table 1. In children with PBB, those in the HAdV⁺ group were significantly younger than the HAdV⁻ group ($P = .001$). There were no differences in the sex of the children according to HAdV status within the PBB or BE groups ($P = .248$ and $P = .703$). No seasonal differences in HAdV detection rates were observed ($P = .506$).

CD 16⁺ and CD56⁺ NK cells were elevated above the normal range in 20–25 (80%) of HAdV⁺ and 64 of 114 (56.1%) of HAdV⁻ children ($P = .027$). When HAdV was excluded, similar rates of NK cell elevation were seen in children with or without standard panel respiratory viruses detected in BAL ($P = .601$). Basic immune function tests were otherwise normal.

HAdV Genotypes

HAdV genotyping was performed on a random subset (based upon sample availability) of 26 HAdV⁺ on BAL children. Of 24 typeable HAdV isolates, 23 (96%) were identified as HAdV species C; 13 (57%) as HAdV-1, and 10 (43%) as HAdV-2. A single HAdV⁺ isolate was identified as genotype 4, species E, in a child with low-grade fever and mild upper respiratory tract symptoms at time of bronchoscopy (Figure 1).

Bacterial Codetection With HAdV

The major bacterial pathogens detected in both HAdV⁺ and HAdV⁻ groups were *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*. HAdV⁺ status showed positive association with each major bacterial pathogen (Table 2). *Staphylococcus aureus* was

Table 2. Univariate Logistic Regression Showing Relationships Between Human Adenovirus Status and Bacterial Infection on Bronchoalveolar Lavage

Bacterial Infection	HAdV ⁺ (n = 40)	HAdV ⁻ (n = 205)	OR (95% CI)	P Value
<i>Haemophilus influenzae</i>	27 (68%)	96 (47%)	2.36 (1.15–4.83)	.019
<i>Moraxella catarrhalis</i>	14 (35%)	38 (19%)	2.37 (1.13–4.96)	.022
<i>Streptococcus pneumoniae</i>	14 (35%)	46 (22%)	1.86 (.90–3.85)	.094
<i>Staphylococcus aureus</i>	0 (0%)	21 (10.2%)030 ^a

Abbreviations: +/-, positive/negative detection; CI, confidence interval; HAdV, human adenovirus; OR, odds ratio.

^a Calculated using Fisher exact test.

the fourth most common bacterium causing lower airway infection; however, a significant negative association with HAdV⁺ status was observed ($P = .03$).

Lower airway infection with 1 or more of the 3 major bacteria showed significant positive association with HAdV detection (odds ratio [OR], 3.273 [95% confidence interval {CI}, 1.382–7.748]; $P = .007$). After adjustment for age, using multiple logistic regression, this association was no longer significant (OR, 2.383 [95% CI, .980–5.794]; $P = .055$), whereas the relationship between bacterial infection and age remained significant (OR, 0.987 [95% CI, .980–.994]; $P < .001$).

Although there were slightly more detections of HAdV-1 compared to HAdV-2, no further associations between individual genotypes and bacterial coinfection were observed (data not shown).

The HAdV⁺ group was more likely to have lower airway infection with multiple (2 or more) major bacterial species (excluding *S. aureus*), compared with the HAdV⁻ group (OR, 2.471 [95% CI, 1.228–4.969]; $P = .011$). However, on age adjustment, using multiple logistic regression, this association was no longer significant (OR, 1.674; [95% CI, .807–3.475], $P = .166$).

Other Pathogens

Mycoplasma pneumoniae detection rates (using PCR) were similar between HAdV⁻ and HAdV⁺ groups (4 in HAdV⁻ and 1 in HAdV⁺ groups; $P = .580$). Similarly, standard panel respiratory viruses were detected at similar rates in HAdV⁻ and HAdV⁺ children. There were 3 (1.5%) and 0 detections of influenza virus ($P = 1.00$) and 10 (4.9%) and 2 (5%) detections of HPIV ($P = 1.00$) in the HAdV⁻ and HAdV⁺ groups, respectively. Respiratory syncytial virus was detected in 11 (5.4%) and 0 ($P = .22$), and human metapneumovirus was detected in 5 (2.4%) and 0 ($P = .594$) children in the HAdV⁻ and HAdV⁺ groups, respectively.

Of 52 (41 HAdV⁻ and 11 HAdV⁺) participants who had extended viral panel testing of BAL, approximately a quarter of HAdV⁻ children were human rhinovirus positive on BAL (n = 10 [24.4%]) compared with more than half of HAdV⁺ children (n = 6 [54.5%]; *P* = .054). Likewise, human bocavirus was detected less commonly in HAdV⁻, compared to HAdV⁺ children (1 of 41 [2.4%] vs 3 of 11 [27.3%]; *P* = .026). Human coronavirus (OC43) was detected in 1 HAdV⁻ participant and no HAdV⁺ participants (*P* = 1.00).

DISCUSSION

This is the first study to examine the HAdV genotypes detectable in the lower airways of children with chronic endobronchial suppuration (PBB and bronchiectasis). We found HAdV species C (genotypes 1 and 2) to be the major HAdV species in the BAL of these children, irrespective of season. We have also shown that lower airway bacterial infection, with *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae*, but not *S. aureus*, are increased in HAdV⁺ children. Younger age is an independent predictor of infection with HAdV and common respiratory bacteria, and age increases the odds of viral-bacterial coinfection. Our finding of elevated CD16 and CD56 NK cells in the blood of 80% of HAdV⁺ children provides indirect evidence of a systemic immune response to HAdV in the airways of these children.

The HAdV-C species, comprising genotypes 1, 2, 5, 6, and 57, is one of the most frequent HAdV species known to infect the respiratory tract of children [28]. Infection can result in a range of clinical manifestations, from uncomplicated upper respiratory infection to severe pneumonia. Most primary HAdV-C infections occur within the first 2 years of life [29]. The highest levels of HAdV DNA are detected in adenotonsillar tissue of 2-year-old children undergoing routine adenoidectomy or tonsillectomy, and the amount of HAdV DNA decreases with increasing age [30]. HAdVs demonstrate the ability to establish latent infection within lymphocytes [30], tonsillar tissue [31], and the lung [32] and are capable of evading host innate immune responses via multiple mechanisms [33].

Several studies have demonstrated HAdV's propensity to establish latent and/or persistent infection within the upper and lower respiratory tract [34, 35]. In a recent longitudinal study of children with chronic upper respiratory infections, 13 had repeated HAdV detection; 8 carried an identical HAdV genotype on successive occasions, suggesting chronic rather than repeated infection [34]. Using repeated lower airway sampling, Macek and colleagues demonstrated persistence of HAdV in the lower airways of 9 of 11 children with persistent asthma [35]. In adults, Matsuse and colleagues showed increased presence of latent HAdV DNA in the lung tissue of patients with chronic obstructive pulmonary disease (COPD), compared with those

without COPD [32]. No studies to date, however, have genotyped HAdV in lower airways of children with chronic endobronchial suppuration.

A higher prevalence of HAdV in the airways of patients with chronic respiratory diseases has led to speculation that "smoldering HAdV at the site of lung inflammation" [30] contributes to the pathogenesis of chronic lung diseases, including asthma in children [35] and COPD in adults [32, 36]. Marin et al showed that HAdV was detected in 78.4% of nasopharyngeal samples of asthmatic children during symptom-free periods, vs 5% of healthy controls [37]. Furthermore, we have recently shown that HAdV is significantly more likely to be detected in BAL fluid of children with PBB (23%) compared with controls (4%) [14]. It is possible that chronic inflammatory diseases of the respiratory tract predispose to HAdV persistence in the respiratory tract, or that HAdVs induce an inflammatory response that may be a key factor in chronic respiratory disease pathogenesis. If the latter is correct, the association of HAdV with bacterial infection of the lower airways is likely to be a relevant and significant finding.

Childhood lower respiratory infections are known to cause bronchiectasis. Nontypeable *H. influenzae* (NTHi) is the most common bacterium in the lower airways of children with PBB and other chronic lung diseases [5, 38, 39]. Recently, De Schutter and colleagues showed that NTHi is also the commonest cause of (nonresponsive or recurrent) community-acquired pneumonia in children [40]. Similarly, compared with controls, elevated rates of *H. influenzae*, *M. catarrhalis*, and *S. pneumoniae* infection are detected in the lower airways of children with PBB [14].

The propensity for codetection of HAdV with bacterial pathogens (eg, NTHi), has been documented in studies on human respiratory diseases [41, 42]. However, the most convincing evidence for microbial synergy arises from an animal study on otitis media. In a chinchilla model of experimental otitis media due to HAdV-1 (species C) and NTHi, Suzuki et al demonstrated a synergistic effect of the 2 pathogens. They found that inflammation was greatest in the presence of both HAdV and NTHi, compared with HAdV or NTHi alone. Furthermore, timing of inoculation was important. HAdV inoculation prior to NTHi inoculation resulted in the greatest tympanic membrane inflammation and mucosal dysfunction [43]. We have previously shown that neutrophilic lower airway inflammation is maximal in the presence of viral-bacterial coinfection of the airways of children [12]. It is indeed plausible that HAdV-C and *H. influenzae* may also play a synergistic role in the initiation and/or exacerbations of chronic suppurative lung diseases in children, and further research is needed.

The negative association of HAdV with *S. aureus* in the lower airways observed in the present study may be due to the increased presence of other bacterial pathogens (eg, *H. influenzae*) in HAdV⁺ compared with HAdV⁻ children. Negative associations

between *S. pneumoniae* and *S. aureus* [44–48] and between *H. influenzae* and *S. aureus* [44, 45] are well described in studies of the upper respiratory tract. These interactions are believed to reflect natural competition between colonization with these organisms [48].

Hence, our findings in relation to HAdV in the lower airways of children with PBB and BE are likely to have clinical relevance and may represent an important clue to the underlying pathogenesis of these disease processes. Our hypothesis is based upon several key findings. These include (1) a predominance of HAdV-C in the airways of these children, with a plausible mechanism for disease; (2) the presence of elevated NK cell levels in HAdV⁺ children (however, not in those with other standard panel respiratory viruses), indicating a systemic immune response to HAdV in the lung; (3) previous research linking HAdV (in the nasopharynx and/or lung) to heightened neutrophilic inflammation and lymphopenia of the lower airways (suggestive of active viral replication) [13]; and (4) the demonstrated association between HAdV⁺ status and lower airway bacterial infection (the latter being a key feature of PBB and BE). It is therefore unlikely that HAdV is an innocent bystander, as early epidemiologic studies suggested.

Although we have described 2 novel and important findings, several limitations to our study merit discussion. First, although our cross-sectional data highlights important associations, we cannot attribute causality. Repeated lower airway sampling would be needed to establish the temporal sequence of lower airway infection and determine a cause-and-effect relationship. However, subjecting children to repeated general anesthesia for lower airway sampling, solely for research, would be unethical. Second, we did not include HAdV serology in our blood test panel. This ideally requires repeat venipuncture, which was not feasible in our context as many children in the study reside outside the Brisbane area. With regards to methods of HAdV detection, although culture detects infectious virus, PCR is more sensitive and is the current clinical standard [16]. Although a positive PCR for HAdV does not readily distinguish infectious from noninfectious virus, the identification of HAdV DNA indicates that infection has occurred at some stage. Further, HAdV's association with a systemic immune response (ie, elevated NK cells) supports the notion that HAdV may play a clinically relevant role. Third, we detected few conventional respiratory panel viruses (other than HAdV) and only performed extended viral panel analyses on a subset, limiting our ability to explore other possible viral–bacterial and viral–viral interactions. Last, the strong relationship between age and HAdV status, and the increased odds of bacterial coinfection with HAdV in younger children, is likely to be significant. This finding raises the question of whether younger children are predisposed to polymicrobial infection, via immature immune system processes, rather than a true viral–bacterial interaction.

Timing of HAdV acquisition, with respect to age, may therefore be important in further evaluating the link between HAdV and future development of PBB and/or BE.

We conclude that HAdV-C genotypes 1 and 2 are the dominant HAdV species infecting the lower airways of young children with chronic endobronchial suppuration. The significant association between HAdV and lower airway bacterial infection suggests a possible role of HAdV-C in the pathogenesis of chronic suppurative lung diseases in young children. Our findings may have implications for targets in the prevention of chronic suppurative lung diseases; however, further research to definitely establish causality would be required.

Notes

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Potential conflicts of interest. I. M. M. has received payment for consultancy services from Firebrick Pharma. J. W. U. has served on the advisory board for Novartis and has previously received payments for lectures or served on speakers' bureaus for AstraZeneca, GSK, Novartis, and Boehringer. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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