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Human adenovirus in nasopharyngeal and blood samples from children with and without respiratory tract infections

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ABSTRACT

Background: Human adenovirus (HAdV) is a double-stranded DNA virus associated with respiratory tract infections (RTI) in children. Using polymerase chain reaction (PCR) tests, HAdV often is detected together with other virus species, even in healthy controls.
Objectives: The aim of this study was to compare molecular detection of HAdV with culture, and to examine the associations of various methods to RTI.
Study design: Nasopharyngeal aspirates (NPA) were collected from 4319 children admitted with RTI and from 361 controls. The NPAs were examined for 23 viral and bacterial pathogens, using inhouse real-time PCR-assays based on TaqMan probes, in addition to bacterial and viral culture. HAdV concentration was evaluated semi-quantitatively from the Ct-value and quantitatively by use of ADENOVIRUS R-gene®.
Results: HAdV-DNA was detected in 6.1% patient samples and in 10.5% controls ($p < 0.001$). Compared to controls, patients had an OR of 3.8 (95% CI 1.4–10.3) for mono-detection of HAdV DNA, and an OR of 5.1 (95% CI 2.0–13.4) for HAdV-positive samples grew adenovirus by culture. HAdV DNA loads from children with RTI consisted of two clusters: one cluster with high viral loads (Ct < 30 and > 106 copies/ml) and one cluster with low viral loads, whereas among the controls, nearly all had low viral loads (OR 7.8, 95% CI 2.2–27.1). In 61 available plasma samples, 16.4% were positive for HAdV DNA, all were from patients.
Conclusion: The detection of HAdV DNA per se by qualitative PCR is not useful as a diagnostic test. Detection of HAdV by use of viral culture and a high viral HAdV DNA load are the two methods most strongly associated with RTI in children.

1. Background

Human adenovirus (HAdV) is a double-stranded DNA virus and a common pathogen among children and adults. HAdV belongs to the *Adenoviridae* family and the *Mastadenovirus* genus. The link between HAdVs and respiratory tract infections (RTIs) is well established [1–3], especially in children and military recruits. They may also cause epidemic keratoconjunctivitis and gastroenteritis. More rarely, HAdV infections may lead to haemorrhagic cystitis [4,5]. Severe disease in immunodeficient patients, children and elderly has also been documented [6–8]. Infections with adenovirus may occur sporadically or in epidemics and can be detected throughout the year. However, it is a well-known phenomenon that asymptomatic shedding can occur [9,10]. Until now more than 52 HAdV types have been identified and further classified into seven species (A–G). The different types were initially defined by serum neutralisation methods as serotype, but are now identified based on molecular methods and named as genotypes. New genotypes are still identified [8,11–14].

Modern molecular methods, such as polymerase chain reaction (PCR), allows for rapid and sensitive identification of HAdV nucleic acids compared to antigen detection methods (e.g. immunoflourescence) and culture [6,8,15,16]. However, several studies have shown that HAdV DNA frequently is detected together with other viruses [1,17,18], and HAdV may even be detected in healthy children [19,20]. Hence, the clinical value of PCR-based diagnostic tests for HAdV may be questioned.

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2. Objectives

Our primary aim was to compare the results of molecular HAdV detection methods with the results of viral culture. Our secondary aim was to compare the results of both methods in children hospitalized with RTI and a control group of children without RTI.

3. Material and methods

The study was performed at Department of Medical Microbiology in cooperation with Children's Department at St. Olavs Hospital, Trondheim University Hospital, Norway. St. Olavs Hospital, Trondheim University Hospital is the regional hospital for Mid-Norway covering a population of 711 000.

Nasopharyngeal aspirates (NPA) were collected from children under the age of 16 years who were admitted to the Children's Department with respiratory tract infections (RTI), from November 13, 2006 to December 31, 2012 (n = 4319).

A control group (n = 361) was included prospectively during the same period. The controls were children admitted to elective surgery whose parents claimed that the child had no RTI symptoms during the last two weeks before specimen collection. Most controls had surgery for cryptorchidism, hernia repair or benign skin tumours, and none for ear, nose and throat surgery.

2819 of 4680 children were boys (60.2%), 58.7% in the patient group (n = 4319) and 78.7% in the control group (n = 361).

A blood sample was collected from included children, when possible, and HAdV-positive children were tested for HAdV DNAviremia (n = 61).

The NPAs were analyzed for HAdV, human bocavirus (HBoV), coronavirus (OC43, 229E and NL63), enterovirus, human metopneumovirus, influenza A and B virus, parainfluenzavirus type 1–4, parechovirus, respiratory syncytial virus (RSV), rhinovirus, *Bordetella pertussis*, *Chlamydia pneumoniae* and *Mycoplasma pneumoniae*. All were identified by using real-time PCR-assays (RT-PCR) based on TaqMan probes.

An in-house RT-PCR was used for the detection of HAdV [15]. Viral nucleic acid was extracted from 200 µl sample using NucliSENS easyMAG extractor (BioMérieux) and eluted into 55 µL of buffer. Five µL of the eluate from each sample was transferred to 15 µL PCR mix and subjected to RT-PCR using CFX96™ Real-Time System (C1000™ Thermal Cycler, Bio-Rad) [21].

The test results were recorded semi-quantitatively based on the cycle threshold value (C_t-value) and grouped in two categories (high and low viral load). Based on a ROC analysis comparing C_t-value with growth of HAdV in viral culture, the breakpoint was set to C_t 30, corresponding to 10^6 copies/mL. PCR-products with C_t -values below 40 were sequenced. The PCR-product (212 bp) was sequenced directly. Sequencing analysis was performed on an ABI 3130xl DNA Sequencer (Applied Biosystems) and the results were compared to known adenovirus sequences in GenBank (National Center for Biotechnology Information). Our typing method covers all the 51 available genotypes [22].

A quantification kit from Argene, ADENOVIRUS R-gene®, was used to quantify HAdV in the NPAs. Ten µL eluate from each sample was transferred to 15 µL amplification premix and subjected to RT-PCR using CFX96™ Real-Time System (C1000™ Thermal Cycler, Bio-Rad). Amplification conditions consisted of 1 cycle at 95 °C for 15 min, followed by 45 cycles at 95 °C for 10 s and 60 °C for 40 s.

The NPAs were collected in ordinary virus transport media with no antibiotics added. The samples were cultured for viruses in standard cell lines (HE, TMG-1, LLC-MK2). All samples were also cultured for bacteria using standard methods. Growth of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Moraxella catarrhalis* were recorded.

Statistical analysis was done by Pearson Chi-square test for categorical data, and Student's *t*-test or Wilcoxon test for continuous data. To evaluate the association between HAdV and RTI, we used multiple logistic regression analysis. The odds ratio (OR) is reported with 95% confidence interval (95% CI) and the corresponding p-value as a measure of the strength of the association. All analyses were performed using SPSS software version 21 (Statistical Package of Social Science Inc.).

Informed consent was obtained from the children's parents. The study was approved by the Regional Committee for Medical and Health Research Ethics in Mid-Norway.

4. Results

4.1. Demographic characteristics and adenovirus occurrence

In all, 301 out of the 4680 (6.4%) NPA samples were positive for HAdV DNA. Among HAdV positive children, less patients than controls were boys (149 of 263 patients (56.7%) vs. 30 of 38 controls (78.9%), $p < 0.009$). The patients were younger than the controls (mean 27 months, SD 27 months vs mean 35 months, SD 21 months, $p = 0.085$).

The occurrence of HAdV DNA was lower in the patient group (263 of 4319) compared to the control group (38 of 361, 6.1% vs 10.5%, $p = 0.001$), and this difference persisted as trend after adjusting for age, gender and presence of other commonly detected viruses (HBoV, enterovirus, rhinovirus and RSV) (OR = 0.69, 95% CI: 0.47–1.01, $p = 0.056$).

HAdV DNA-positive samples were found evenly throughout the year, and there were no clear outbreaks within the time period from 2006 to 2012.

Of the 301 HAdV DNA-positive samples, 192 were genotyped. Genotypes 1, 2 and 3 were the most common adenovirus genotypes. The genotype distribution is presented in Table 1.

4.2. Adenovirus mono-detection and co-detection of other viruses

HAdV DNA was detected alone in 95 of 301 HAdV-positive samples (31.6%). One additional virus was found in 112 of 301 (37%), two in 65 (22%), three in 19 (6%), four in 9 (3%) and five additional viruses were found in one sample (0.3%). Rhinovirus (n = 56, 21%), enterovirus (n = 50, 19%), HBoV (n = 40, 15%) and RSV (n = 32, 12%) were the most commonly co-detected viruses among patients. The corresponding figures among controls were rhinovirus (n = 17, 45%), enterovirus (n = 16, 42%), HBoV (n = 15, 39%) and RSV (n = 0). In univariate analyses, NPAs from children with RTI more often had HAdV DNA mono-detection than controls (90 of 263 (34.2%) vs 5 of 38 (13.2%), $p = 0.009$). Adjusted for gender and age, HAdV DNA mono-detection was associated with RTI (OR = 3. 8, 95% CI 1.4–10.3, $p = 0.010$) (Table 2).

4.3. Adenovirus culture

296 of 301 HAdV DNA-positive NPAs (PCR) were cultured and 142 (48%) were positive by culture. More culture-positive NPAs were from

Table 1
HAdV genotypes detected in patient and control group (n = 192).

Species	Genotype	Patient group[a] (N = 173) (%)	Control group[a] (N = 19) (%)
A	31	0 (0.0)	1 (5.3)
B	3	21 (12.1)	3 (15.8)
	7	2 (1.2)	0 (0.0)
C	1	52 (30.0)	9 (47.4)
	2	80 (46.2)	5 (26.3)
	5	7 (4.1)	1 (5.3)
	6	11 (6.4)	0 (0.0)

[a] HAdV detected with in-house RT-PCR, N = 192.

Table 2
The associations between HAdV in patients and controls, as determined by various methods.

Diagnostic approach	Patient-group	Control-group	OR[a] (95% CI)	*p*-value
HAdV mono-detection (n = 301)	34.2% (90/263)	13.2% (5/38)	3.8 (1.4-10.3)	0.010
HAdV Ct < 30 (> 10^6 copies/mL)[b] (n = 301)	42.2% (111/263)	7.9% (3/38)	7.8 (2.2-27.1)	0.001
Growth of HAdV (n = 296)	52.7% (136/258)	15.7% (6/38)	5.1 (2.0-13.4)	< 0.001
Growth of HAdV in viral culture and mono-detection of HAdV[b] (n = 142)	38.7% (54/136)	16.7% (1/6)	2.9 (0.3-25.4)	0.348
Growth of HAdV in viral culture and HAdV Ct < 30 (> 10^6 copies/mL)[b] (n = 142)	75.7% (103/136)	33.3% (2/6)	7.4 (1.1-50.3)	0.042

[a] Adjusted for age and gender.
[b] In-house RT-PCR.

children with RTI than controls (136 of 258 (52.7%) vs. 6 of 38 (15.7%), p < 0.001). This finding remained significant after adjusting for gender, age and co-detected viruses, (OR: 5.1, 95% CI: 2.2–14.7, $p <$ 0.001). Among culture-positive samples, more had high (> 10^6 copies/mL) than low viral loads in NPA (105 of 142 (73.9%) vs. 37 of 142 (26.1%), p < 0.001) (Table 2), and more had HAdV DNA mono-detection (Table 2).

4.4. Exact measurements of adenovirus concentrations

227 of the 301 NPAs positive for HAdV DNA were available for exact virus quantification by use of the ADENOVIRUS R-gene® test kit. The median HAdV concentration in the HAdV culture positive samples was higher compared to the HAdV culture-negative samples ($1.2*10^8$ copies/mL vs. $5.0*10^4$ copies/mL, $p <$ 0.001, n = 223) (Fig. 1). Children with RTI had higher HAdV DNA concentrations compared to controls (n = 195, median $8.6*10^5$ copies/mL vs n = 32, $1.6*10^5$ copies/mL, $p =$ 0.021, n = 227).

4.5. Semi-quantitative estimations of adenovirus concentrations

Using viral culture as reference standard in a ROC analysis, we found that a C_t-value of 30 had the highest discriminatory power. A subset of the samples was further analysed with the quantitative assay, and we found that a C_t-value of 30 corresponded to 10^6 copies/mL NPA.

A high HAdV DNA load, as defined by $C_t < 30$, was found in 111 of 263 (42.2%) patient samples and 3 of 38 (7.9%) controls (p < 0.001). Adjusted for age, gender and presence of other viruses, a logistic regression analysis showed that $C_t < 30$ was associated with RTI (OR: 7.8, 95% CI: 2.2–27.1, $p =$ 0.001) (Table 2). A graphic presentation showed

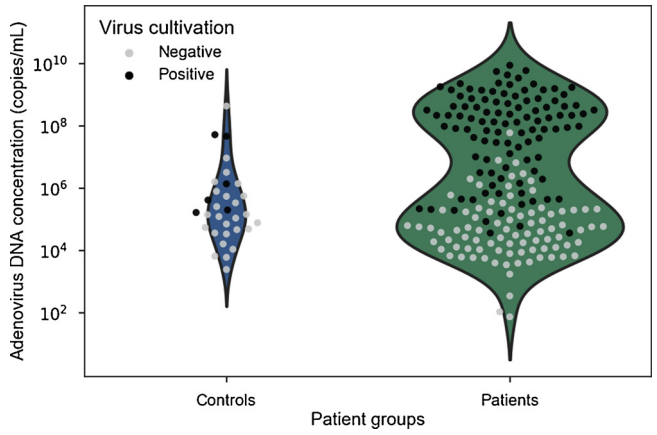

Fig. 1. Distribution of HAdV loads in 223 samples available for quantitative measurements of adenovirus concentrations with (n = 100) and without (n = 123) growth of HAdV.

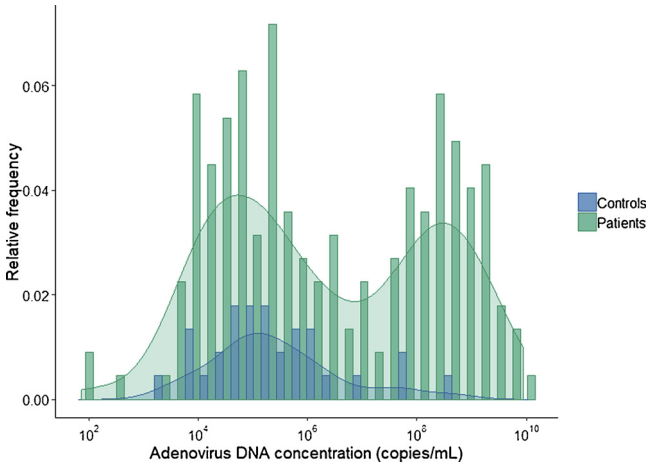

Fig. 2. HAdV loads (copies/mL) distribution in patients (n = 195) and controls (n = 32). A threshold of 10^6 copies/mL NPA corresponds to a Ct value of 30, as measured in the in-house RT-PCR. Samples from children with RTI clustered in two groups, the controls clustered below this threshold.

that the samples from children with RTI clustered in two groups with concentrations in NPA either above or below C_t 30. In contrast, the controls clustered below this threshold value (Fig. 2). There were no differences in mono-detection rates of HAdV DNA from infected children with high ($Ct < 30$) and low ($Ct > 30$) HAdV DNA load (data not shown).

4.6. Adenovirus viremia

61 plasma samples were available from 301 HAdV DNA-positive children. 10 plasma samples (16%) were HAdV DNA-positive by PCR and all were from children with RTI.

4.7. Bacterial culture and adenovirus concentrations

211 of 301 samples were cultured aerobically for bacteria. Of the 211 samples, 197 (93,3%) had bacterial findings. 153 samples grew either *Streptococcus pneumoniae*, *Haemophilus influenzae* or *Moraxella catarrhalis*. 93 samples had growth of only one of the three bacteria and 55 samples had two of the bacteria. Five samples grew all three. There were no significant differences between the patient and control groups, and there were no relations to the presence of HAdV DNA (data not shown).

5. Discussion

The main findings of our study are that a positive HAdV culture in nasopharyngeal aspirate and high levels of HAdV DNA > 10^6 copies/

mL were strongly associated with RTI in hospitalized children. Many infected children and even more healthy controls had low levels of HAdV DNA. Hence, the detection of HAdV DNA per se by PCR was not associated with RTI, and a qualitative method is not useful for diagnostic purposes.

Occurrences of HAdV between 2–10% in children with RTI have been reported in many studies based on molecular methods [1,15,16]. With an occurrence of 6.1%, our findings were in line with these studies, but a higher occurrence among healthy children (10.5%) in this study, was not expected. Previously, a few studies reported that HAdV DNA is commonly detected among healthy children, but they still reported a higher occurrence in children with RTI [1,20,23]. Further studies are needed to address these findings.

In our study, HAdV did not have a particular seasonal pattern, and this has been described before [24]. The HAdV genotypes we detected have all been characterised previously and have been reported to cause RTI. However, the numbers of each genotype were limited. Hence, it was not feasible to study the relation to clinical outcomes.

As shown in previous studies [25,26], we found that co-detection of other viruses is a very common phenomenon in many children with HAdV DNA positive NPAs. As many as two-thirds of the HAdV DNA-positive samples were positive for other viruses, in particular rhinovirus, enterovirus and HBoV. HAdV mono-detection was associated with RTI in children in both univariate analyses, and after adjusting for age and gender differences. However, the OR was lower compared to the presence of a positive HAdV culture and high HAdV DNA levels (Table 2).

Growth of HAdV in cell culture was clearly associated with RTI. Viral culture is labour intensive and does not provide a fast answer for clinical decision-making, but we think that it is a useful laboratory tool for the evaluation of nucleic-acid based methods. Approximately half of the HAdV DNA positive NPAs collected from children with RTI were also positive by virus isolation, and we suggest that this indication of live viral particles support that active adenoviral infection was present. In addition, many of these samples with HAdV growth had high viral loads. More than 9 of 10 NPA samples with high viral load above 10^6 copies/ml, were collected from children with RTI. Hence, a positive HAdV culture result and the detection of high HAdV level above 10^6 copies/ml s are good indicators of RTI in children, whereas a positive HAdV PCR qualitative test result alone does not indicate RTI. Indeed, we showed that nasopharyngeal samples from children with RTI consisted of two clusters: one cluster with high viral loads ($Ct < 30$) and one cluster with low vial loads ($Ct > 30$) whereas among the controls, nearly all had low viral loads and clustered below this threshold value. It has been reported previously that HAdV may be excreted for long time or even may increase excretion again in association with a new infection [10,20,27], and these phenomena may explain the findings of low HAdV levels in many infected as well as in many healthy children included in our study.

Duration of viremia for HAdV is unknown and the sensitivity of this parameter when used as a diagnostic test is uncertain. The low percentage of patients with viremia (16%) indicates at a lower sensitivity of viremia compared to viral culture of NPA (48%). Viremia could be a marker for severe disease, such as lower RTI, but this question has not been addressed in this study. It is noteworthy that no controls had viremia.

A strength of our study is the large sample size, and the fact that all samples were examined for 16 viruses and 3 bacteria, using the same in-house PCR's during the entire study period. Due to the inclusion of a control group of healthy children, we were also able to correlate viral findings with RTI. However, due to the cross-sectional design, we didn't evaluate clinical sensitivity exactly. A longitudinal study with repeated samplings from each child would also be better to study viral concentrations in relation to various clinical manifestations. Furthermore, the study was hospital based, and hence the included children do not represent the entire spectrum of adenoviral infections. There were significant age and gender differences between the patient and control groups, but this was controlled for in the analyses.

In conclusion, our results show that HAdV DNA detection in NPA is commonly seen both in children with RTI and healthy controls. Adenovirus positive NPA culture indicates RTI in hospitalized children, but a high HAdV DNA level > 10^6 copies/ml is a more useful clinical test.

Authorship declaration

We certify that we have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest the subject matter or materials discussed in this manuscript.

CRediT authorship contribution statement

Hans-Johnny Schjelderup Nilsen: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Project administration. **Svein Arne Nordbø:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Sidsel Krokstad:** Methodology, Investigation, Resources, Writing - original draft. **Henrik Døllner:** Conceptualization, Methodology, Writing - original draft, Writing - review & editing, Supervision, Funding acquisition. **Andreas Christensen:** Conceptualization, Methodology, Formal analysis, Writing - original draft, Writing - review & editing, Supervision, Project administration.

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