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Detection of a Broad Range of Human Adenoviruses in Respiratory Tract Samples Using a Sensitive Multiplex Real-Time PCR Assay

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Human adenoviruses (hAdVs) are associated with acute respiratory tract infections in pediatric populations and have been identified as a cause of outbreaks in institutional settings. Rapid diagnosis of hAdV infection is critical for appropriate and timely management. This study reports the design and validation of a sensitive and specific multiplex real-time PCR for the detection of a broad range of hAdV serotypes in respiratory samples. The assay targets the conserved region of the hAdV hexon gene and utilizes hydrolysis probes for the detection of amplified products. The assay was evaluated using retrospectively (n = 864) and prospectively (n = 11,451) collected samples from November 2005 to July 2006. Seasonality studies and analysis of outbreaks was conducted over a 2-year period from January 2005 to December 2006 (n = 33,067 samples). The assay gave a hAdV positive rate of 7.1% (n = 811) for specimens tested prospectively and was able to detect a broad range of hAdV serotypes with good sensitivity and specificity. A high rate of co-infection was noted (21.7%). Adenovirus infections were more prevalent in the young with a median age of 24 months for positive patients. Sequence analysis of hAdV positives showed that serotype 7 was the most prevalent followed by serotypes 2 and 3. Association of hAdVs with respiratory outbreaks was low at 2.3% (6 of 266 outbreaks tested) and no seasonal variation was observed for hAdV infections during the 2-year study period. This assay can improve the detection of hAdVs in respiratory samples and can be used to provide valuable epidemiological information. ***J. Med. Virol. 80:856–865, 2008.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: human adenoviruses; respiratory virus; serotype; co-infection; outbreak

INTRODUCTION

Human adenoviruses (hAdVs) cause a broad spectrum of diseases including respiratory tract infections, acute conjunctivitis, cystitis, gastroenteritis and systemic infections in immunocompromised patients [Brown, 1990; Lion et al., 2003; Adhikary et al., 2004; Banik et al., 2005; Casas et al., 2005; Ebner et al., 2006; Ison, 2006; Wade, 2006]. Human adenoviruses are responsible for up to 10% of lower respiratory tract infections in pediatric populations [Chen et al., 2004; Jennings et al., 2004; Arden et al., 2006; Moura et al., 2007]. An outbreak of adenovirus infection in a long term assisted care center was associated with significant costs [Piednoir et al., 2002].

Fifty-one serotypes of hAdV have been recognized based on their biological, physiochemical and genetic properties, and these are divided into six species (A through F) [Davison et al., 2003; Ebner et al., 2005a]. Species B (types 3, 7, 11, and 16), and species C (types 1, 2, 5, and 6) are found predominantly in children [Kidd et al., 1996; Kim et al., 2003; Adhikary et al., 2004]. Species B and E (serotype 4) have been reported to cause acute respiratory tract infections in military camps [Erdman et al., 2002; Kolavic-Gray et al., 2002; Echavarria et al., 2003; Heim et al., 2003; Blasiole et al., 2004; Chmielewicz et al., 2005a; Kajon et al., 2007]. Traditionally, diagnosis of hAdV infection utilizes viral culture with serotype determination based on neutralization or haemagglutinin inhibition with type-specific antisera. Direct fluorescent antigen (DFA) methods for the detection of hAdVs are insensitive and viral culture is time consuming [Coyle et al., 2004; Moura et al., 2007]. Sensitive and specific hAdV assays

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with rapid turn-around are needed as treatment may be considered for hAdV infections in immunocompromised hosts.

This study describes the development and evaluation of a multiplex real-time PCR for the rapid detection of a range of hAdV serotypes. The epidemiology of hAdV infections including infection rate, age distribution, seasonality and the role of hAdVs in respiratory outbreaks is reported.

MATERIALS AND METHODS

Clinical Samples and Adenovirus Control Strains

The PCR designed and optimized for this study was validated retrospectively using stored specimens from two pediatric studies (n = 720) and from patients with community acquired pneumonia (n = 144) collected during 2003–2004. The assay was further evaluated using respiratory specimens (n = 11,451) submitted for virus testing to the Provincial Laboratory for Public Health (ProvLab) in Alberta between November 2005 and July 2006 (Table I).

According to the ProvLab diagnostic testing algorithm, nasopharyngeal specimens analyzed in the prospective study were first screened by DFA for influenza A and B (IFVA and IFVB), respiratory syncytial virus (RSV) and parainfluenzavirus (PIV). The DFA negative specimens were subjected to a panel of nucleic acid amplification tests (NAT) for the detection of respiratory viruses including IFVA, IFVB, PIV, RSV, human metapneumovirus (hMPV) and hAdV. All other respiratory sample types (non-nasopharyngeal) included in the prospective analysis; such as throat swab, auger suction, bronchoalveolar lavage and endotracheal tube samples were tested by the NAT panel only.

The seasonality of adenoviral infections was evaluated for samples collected between January 2005 and December 2006 (n = 33,067). Respiratory outbreaks are defined by public health professionals based on epidemiologically linked symptomatic cases in the province of Alberta. These were evaluated for evidence of hAdV infection over the same time period (n = 266 outbreaks).

TABLE I. Sample Types Analyzed Prospectively for Detection of hAdVs by Multiplex Real-Time PCR

Sample type[a]	Number tested (% of total)[b]	hAdV PCR positive (% of each sample type)
Upper respiratory tract	8,381 (73.2)	651 (7.8)
Lower respiratory tract	2,839 (24.8)	144 (5.1)
Others	231 (2.0)	16 (6.9)
All samples	11,451	811

[a]Upper respiratory tract specimens analyzed were nasopharyngeal swabs or aspirates, and throat swabs. Lower respiratory tract specimens included bronchial and tracheal samples such as endotracheal tube suctions, bronchoalveolar lavage, and auger suctions. Other sample types tested included pleural biopsy, lung biopsy, pericardial fluid, plasma, Cerebral spinal fluid, urine, and eye swabs.
[b]Samples included in this analysis were collected between November 2005 and July 2006 (n = 11,451).

Some adenovirus isolates were obtained from the National Collection of Pathogenic Viruses (NCPV, www.hpa.org.uk/ncpv). Serotypes 2 (NCPV #00213), serotype 4 (NCPV #180), serotype 10 (NCPV #294), serotype 31 (NCPV #128) and serotype 40 (NCPV #183) of known titer were provided by the University of Wales College of Medicine (Cardiff, UK). Additional virus isolates (serotypes 1, 3, 5, 6, 7, 8, 19, 41) were obtained from ProvLab, Calgary, Alberta. The serotyping of these viruses was performed by neutralization assay with type-specific antiserum.

Isolation of DNA

Viral DNA was extracted from cultured virus stocks of known titer using the QIAamp® DNA Mini Kit (Qiagen, Inc., Mississauga, Ontario, Canada) according to the manufacturer's instructions using an input volume of 50 μl and elution volume of 100 μl. All respiratory specimen types were pretreated with 25 μl of 1 AU/ml protease (Qiagen) at 56°C in a thermomixer (Eppendorf, Westbury, New York) for 10 min. Viscous lower respiratory tract specimens were incubated further to liquefy the sample. Extraction of total nucleic acid from the pretreated samples was performed using the nucliSENS® and easyMAG® automated extraction systems (bioMérieux Canada, Inc., Québec, Canada) following the manufacturer's instructions with an input volume of 200 μl and elution volume of 110 μl.

Primer/Probe Design and Assay Optimization

Partial or full-length hAdV hexon gene sequences from GenBank were aligned and analyzed using the MegAlign module from Lasergene 6 (DNAstar, Madison, WI). The alignment included the 35 most common hAdV serotypes: types 1–8, 10–11, 13–14, 16, 18–19, 21–26, 30–31, 34–35, 37, 40–41, and 45–51 and more than 100 GenBank sequence submissions. Two sets of primers and probes (Adv-2 and Adv-4) were designed with one probe being degenerate to facilitate detection of genetically diverse hAdV serotypes. The alignment of 19 representative hAdV serotype sequences is provided in Figure 1. Primer and probe design was performed using the Primer Express™ software [version 2.0.0, Applied Biosystems (ABI), Foster City, CA]. The hydrolysis probes were labeled with 6-carboxyfluorescein (FAM) at the 5′ end and carboxytetramethyl-rhodamine (TAMRA) as a quencher at the 3′ end. Sequences of the designed oligonucleotides and the amplified product sizes are given in Table II.

Optimal primer concentrations were determined by checkerboard titrations from 50 to 900 nM using positive virus controls as templates. The primers were synthesized at the Core DNA services, University of Calgary (Alberta, Canada) and probes were purchased from ABI. Different hAdV serotypes (1, 2, 3, 4, 5, 6, 7, 8, 10, 19, 31, 40, and 41) were tested with individual primer and probe sets and in combination (multiplex format) to ensure detection of the different serotypes.

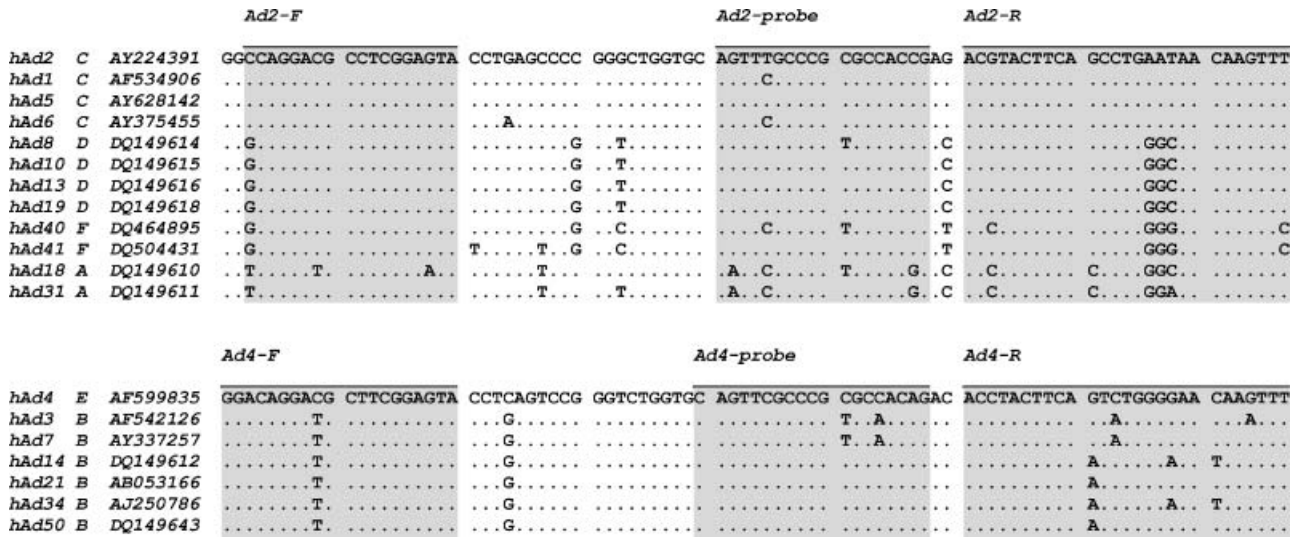

Fig. 1. Alignment of the partial hexon gene sequences from representative prototype strains for primer and probe design.

Plasmid Preparation for Use as DNA Controls

Degenerate primers hex1deg-F and hex2deg-R [Allard et al., 2001] were used to amplify a 301 bp fragment of the hexon gene including the PCR target regions (Table II). The amplified fragments were cloned into plasmid vector pCRII-TOPO® and electroporated into One Shot TOP-10 F′ competent *Escherichia coli* cells (Invitrogen, Carlsbad, CA). The plasmids, with cloned hAdV fragments, were purified using the QIAprep® Kit (Qiagen) and quantified by UV spectroscopy (DU® 530, Beckman Coulter, Inc., Fullerton, CA) to calculate the copy number. The cloned plasmids were serially diluted in a background of carrier RNA (poly A) to maintain plasmid integrity during storage [Jothikumar et al., 2005].

Real-Time Amplification and Detection

Amplification of the target was carried out in a final volume of 25 μl with 5 μl of extracted DNA as template, 12.5 μl of TaqMan® Universal Master Mix (ABI), 400 nM of each primer and 200 nM of each probe. Amplification and detection was performed using the 7000 or 7500 SDS real-time PCR systems (ABI). The amplification conditions were: Incubation at 50°C for 2 min (UNG), *Taq* activation for 10 min at 95°C, followed by 45 cycles of amplification comprising denaturation for 15 sec at 95°C and annealing and primer extension for 1 min at 60°C.

Assay Specificity, Sensitivity, and Reproducibility

The specificity of the assay is inherent in the design and sequence searches undertaken to ensure no cross-reaction with other nucleic acid. However, specificity was also evaluated experimentally using high titer extracted DNA/RNA from IFVA and IFVB, PIV (types 1, 2, 3, 4A, and 4B), RSV (A and B), human coronaviruses (229E, OC43, NL63, and HKU1), hMPV (lineages 1 and 2), rhinovirus 1B, human bocavirus, *Chlamydophila pneumoniae*, *Legionella pneumophila*, and *Mycoplasma pneumoniae*.

The limit of detection (LOD) for different hAdV serotypes was determined using tenfold serial dilutions of stock cultures from prototype strains. In addition, the

TABLE II. Primer and Probes Sequences Used for This Study

	Oligonucleotide name	Oligonucleotide sequence (5′–3′)	Amplicon (bp)	Location
Sequencing and cloning primers[a]	hex1deg-F	GCC SCA RTG GKC WTA CAT GCA CAT C	301	21–45[b]
	hex2deg-R	CAG CAC SCC ICG RAT GTC AAA		301–321[b]
Adv-2 set	Ad2-F	CCA GGA CGC CTC GGA GTA	85	51–68[b]
	Ad2-R	AAA CTT GTT ATT CAG GCT GAA GTA CGT		135–109[b]
	Ad2-probe	FAM-AGT TTG CCC GCG CCA CCG-TAMRA		89–106[b]
Adv-4 set	Ad4-F	GGA CAG GAC GCT TCG GAG TA	81	49–68[c]
	Ad4-R	CTT GTT CCC CAG ACT GAA GTA GGT		129–106[c]
	Ad4-probe	FAM-CAG TTC GCC CGY GCM ACA G-TAMRA		85–103[c]

[a]Primers from Allard et al. [2001].
[b]Nucleotide numbering based on the hexon region of hAdV serotype 2 of GenBank accession no. AY224391.
[c]Nucleotide numbering based on the hexon region of hAdV serotype 4 of GenBank accession no. AF599835.

analytical sensitivity of the real-time PCR was investigated by testing replicate serial dilutions of known copy number plasmids (n = 8 for each dilution). The assay reproducibility was assessed by testing serial dilutions of the hAdV2 and hAdV4 plasmids and four clinical samples in triplicate on three different days.

Sequence Analysis of Positives Samples

Thirty-seven positive clinical samples were selected randomly for sequencing. The 301 bp PCR fragment was amplified using the primers hex1deg-F and hex2deg-R [Allard et al., 2001], and the amplified product was purified using the QIAquick® PCR Purification Kit (Qiagen). Sequencing was performed for both strands using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI) in the ABI PRISM® 3100-Avant Genetic Analyzer. Sequencing analysis utilized the Data Collection Software v2.0 (ABI). Serotype designation was undertaken by comparison of 253–255 bases of sequence with prototype sequences obtained from GenBank. Nucleotide sequences were aligned using ClustalV and the phylogenetic tree was constructed using the neighbor-joining method in the MegAlign module from Lasergene 6.

Statistical Analysis

The SPSS software v14.0 was utilized for statistical analysis of data. Probit analysis was used to determine assay limit of detection (with 95% CI). The McNemar test was used for comparison of results obtained by culture and PCR during the retrospective evaluation of the assay. The crossing threshold (Ct) values of specimens positive for hAdV only were compared with those for mixed infections using the Mann–Whitney *U*-test. The statistical significance of hAdV infections in different age groups and the co-infection rate were analyzed using Pearson Chi-squared (χ^2) analysis. In all cases, $P < 0.05$ was utilized to denote a statistically significant difference between parameters compared. Assay variation was expressed as coefficient of variation (standard deviation/mean expressed as %CV). Kappa statistic was used to assess the concordance between methods.

RESULTS

Evaluation of the PCR for Detection of a Range of hAdV Serotypes

The Adv-2 and Adv-4 primer and probe sets were evaluated for the detection of different hAdV serotypes in singleplex and multiplex formats. The Adv-2 primer and probe set detected serotypes 1, 2, 5, 6, 7, 8, 10, 19, 40, and 41 but not serotypes 3, 4, and 31. The Adv-4 primer and probe set detected serotypes 1, 3, 4, 5, 6, 7, and 40 but not serotypes 2, 8, 10, 19, 31, and 41. All hAdV serotypes were detected using the multiplex assay except serotype 31. Serotype 31 can be detected upon reduction of the annealing temperature from 60 to 57°C (data not shown).

PCR Specificity, Sensitivity, and Reproducibility

There was no cross-reaction with high copies of other respiratory pathogens showing the multiplex real-time PCR to be specific for the detection of hAdVs. Serial dilution and amplification of hAdV serotypes 2, 4, 10, and 40 titrated stocks showed the LOD for the assay to be 0.62, 1.01, 0.84, and 3,600 $TCID_{50}$/ml, respectively. Plasmids with cloned hAdV2 and hAdV4 fragments of known copy number were serially diluted and tested. Probit analysis confirmed that 33 and 22 copies of hAdV2 and hAdV4 per reaction, respectively, could be detected in the multiplex PCR (95% CI, Fig. 2). Based on serial dilutions of the plasmids, the linear range of the assay was determined to be between 9.1×10^0 to 9.1×10^7 and 3.8×10^0 to 3.8×10^7 copies/reaction for hAdV2 and hAdV4, respectively (Fig. 3). A standard curve could be established in this range if quantification was thought to be useful for further clinical studies.

The intra-assay coefficient of variation ranged from 0.22% to 1.93% for the hAdV2 plasmid and 0.18% to 1.81% for the hAdV4 plasmid. The inter-assay variability ranged from 0.57% to 2.22% and 0.72% to 1.27% for the hAdV2 and hAdV4 plasmid dilutions, respectively. The intra- and inter-assay coefficient of variation for the four clinical samples tested in triplicate on three independent runs was $\leq$2.04% and $\leq$1.69%, respectively.

Validation of the Real-Time PCR Using Stored Clinical Samples

Retrospective validation was performed on a total of 864 samples collected during 2003–2004. No hAdV positives were detected in a subset of 144 samples collected from adults with community acquired pneumonia by DFA/culture. However, 2 hAdV positives were detected by PCR increasing the positive rate in this subset by 1.4%. A dataset of 720 samples from two pediatric studies was also tested retrospectively for hAdV using the PCR. Positive results were confirmed by PCR for 89 of the 91 positives detected by DFA/culture methods (Kappa = 0.87). Nineteen additional hAdV positive samples were identified by PCR only in this sample set, increasing the positive rate by 3.0%. Two DFA/culture positive samples could not be confirmed by PCR and the likely reasons for this discrepancy are discussed below. Overall, from the retrospective analysis, the enhanced sensitivity of hAdV PCR was apparent with 21 additional samples identified and only two culture positive samples not confirmed by PCR ($P < 0.05$, McNemar analysis).

Prospective Evaluation of the hAdV Real-Time PCR

Over a 9-month period from November 2005 to July 2006, a total of 11,451 specimens were tested using the hAdV multiplex real-time PCR. All samples types tested are summarized in Table I with the majority being from the upper respiratory tract. The overall positive rate for adenoviral infections was 7.1% (n = 811) with a similar rate among the different sample types. The Ct values for

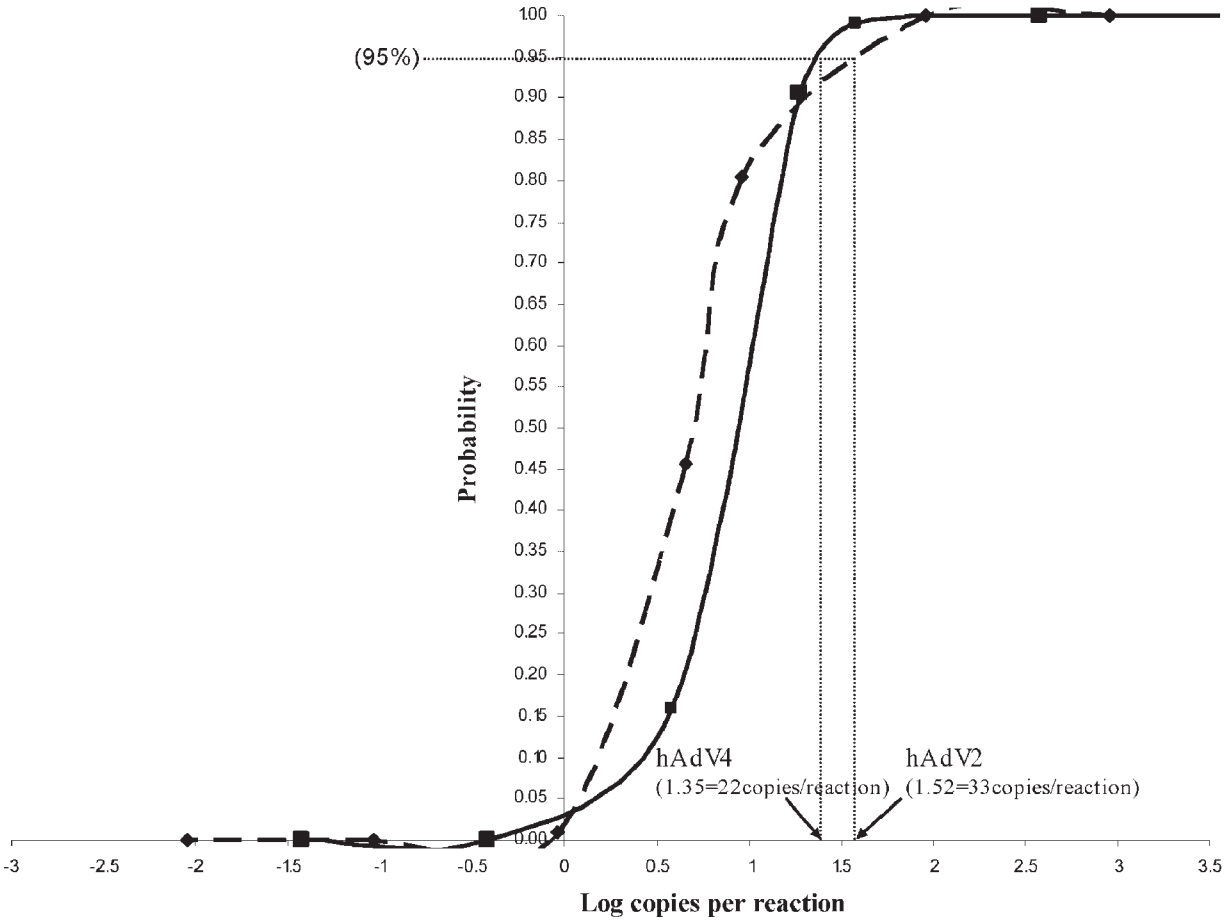

Fig. 2. Analytical sensitivity of the hAdV real-time multiplex PCR. Sensitivity calculations are based on hAdV2 and hAdV4 standards which are cloned plasmids of known copy numbers. Each data point represents eight replicates.

the positive specimens ranged from 14.7 to 45.0 with a median value of 32.9 showing a variation in viral load for the positive samples. There was a significant difference between median Ct values for specimens containing adenovirus only (Median Ct = 32.1, 14.7–45.0) compared with those positive for more than one virus (Median Ct = 35.6, 15.5–43.1) ($P < 0.01$, Mann–Whitney U-test).

Age Distribution for hAdV PCR Positive Patients

The patients tested in the prospective study ranged in age from 1 day to 106 years with a median age of 23 months. The positive patients ranged in age from 16 days to 94 years with a median of 24 months. The highest rate of hAdV infection was observed in the age group of 6 months to 2 years at 17.0%, compared with 5.0% positive for patients >2 years of age (χ^2, $P < 0.001$). Figure 4 shows the number and percentage of positive cases among the different age groups.

Co-Infection Rate for hAdV Positive Samples

A high rate of co-infection was observed with 21.7% of the hAdV positive specimens (n = 176/811) having a positive result for one or more additional respiratory viruses. Parainfluenza virus was the most common co-infecting virus, accounting for 42.5% of the mixed infections; followed by hMPV, RSV, IFVB, and IFVA at 27.3%, 18.2%, 4.5%, and 1.7%, respectively. Two different respiratory viral targets were detected in addition to hAdV in 10 specimens (1.2%). The highest co-infection rate was observed in the age groups of 6 months to 2 years and 2 to 6 years at 28.8% and 29.7%, respectively compared with 10.6% for patients >7 years of age (χ^2, $P < 0.001$).

Seasonality and Outbreak Investigations

The monthly distribution of hAdV positive specimens is shown in Figure 5. The percentage of hAdV positives during the 24 months varied from 0.7% to 15.1% with no identifiable seasonal trends.

A total of 1,245 specimens from 266 respiratory outbreaks were tested for hAdVs from January 2005 to December 2006. Positive hAdV PCR results were obtained for specimens from six outbreaks (2.3%); four in schools and two in long term and assisted care facilities. Except for one of the school outbreaks, five of

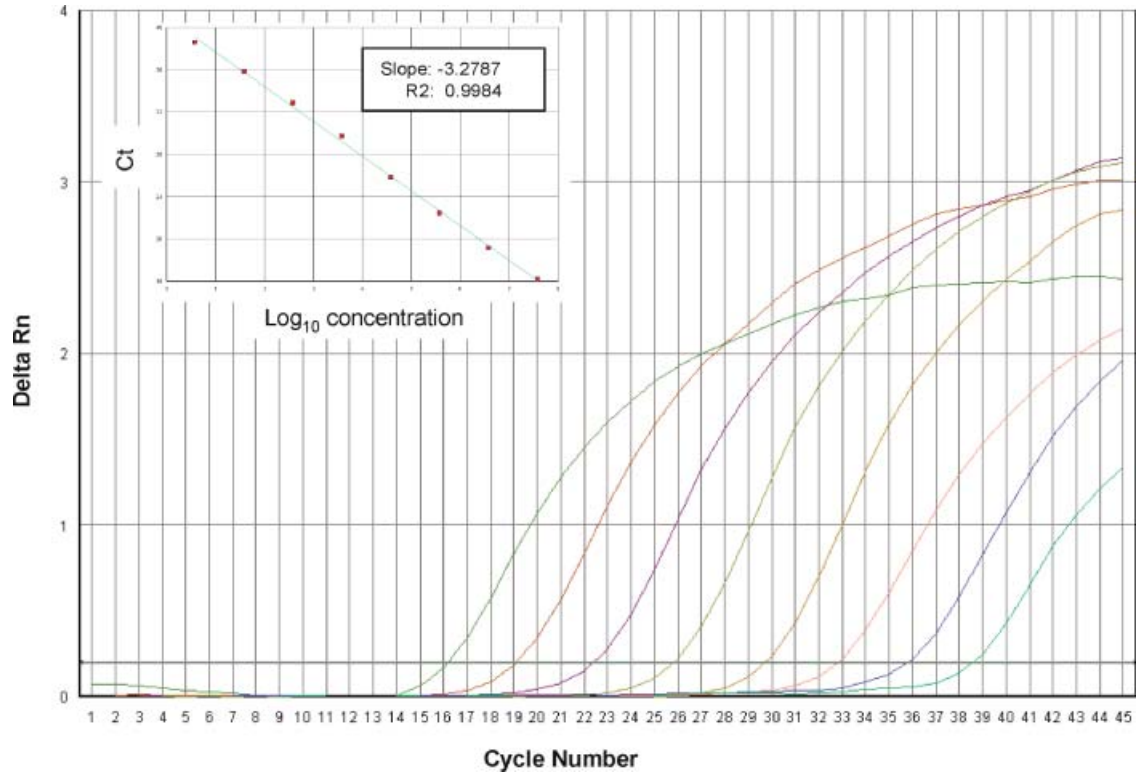

Fig. 3. Dynamic range of hAdV detection. Amplification curves (Delta Rn vs. Cycle) and standard curve (Ct vs. $\log_{10}$ concentration) for serial dilutions of the hAdV4 plasmid showing the assay dynamic range (3.8×10^0 copies/reaction to 3.8×10^7 copies/reaction).

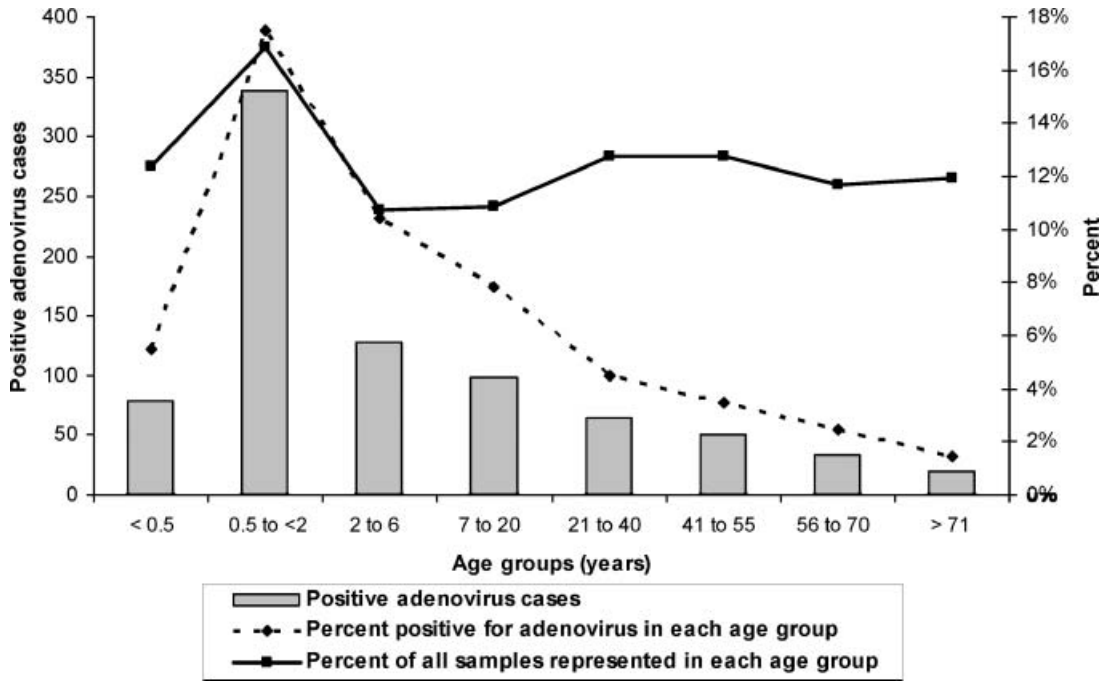

Fig. 4. Adenovirus infection in different age groups. Samples were collected from November 2005 to July 2006 (n = 11,451).

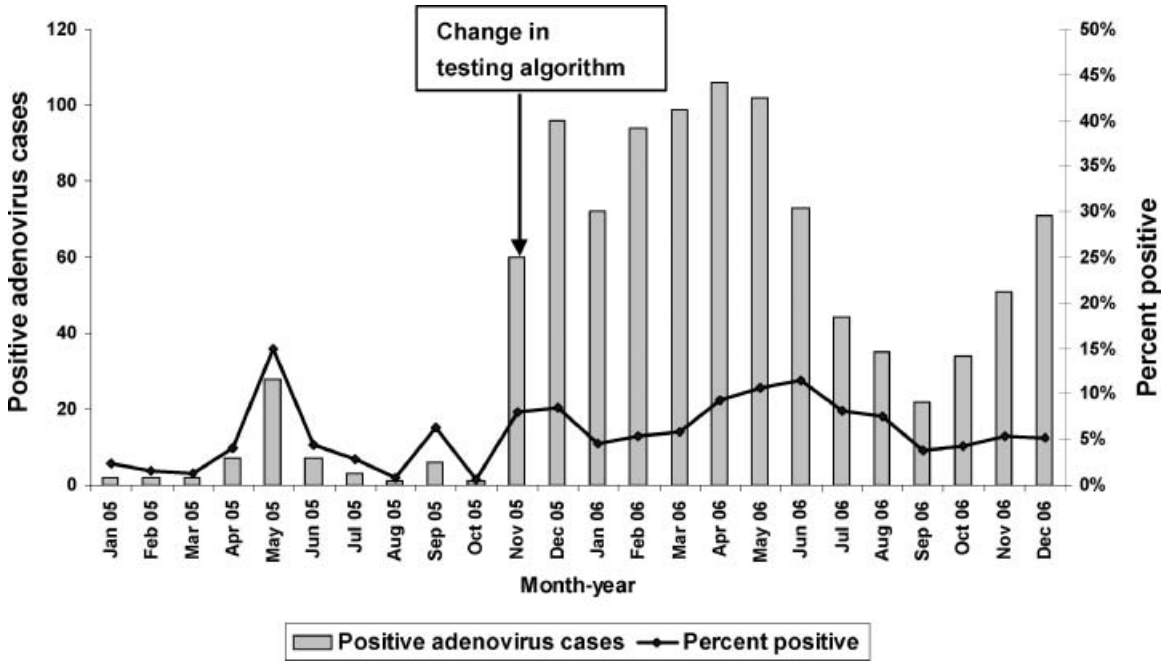

Fig. 5. Seasonality of adenovirus infections. Data shown is from January 2005 to December 2006 (n = 33,067). In November 2005, the testing algorithm was changed to test DFA negative nasopharyngeal samples and all other specimen types by a nucleic acid test panel for respiratory viruses (see Materials and Methods Section).

the six outbreaks tested had other co-infecting respiratory pathogens identified. The Ct values for specimens from the school outbreak that only had positive results for hAdV ranged from 21.8 to 31.4, while the Ct values from the specimens for the other 5 outbreaks were $\geq$37.9.

Sequence Analysis of hAdV Positive Samples

Partial sequence of the hAdV hexon gene was obtained from a variety of specimen types from 37 patients. The sequences were identified as serotype 1 (n = 2), serotype 2 (n = 8) and serotype 6 (n = 2) belonging to species C, serotype 3 (n = 8) and serotype 7 (n = 15) belonging to species B and serotype 4 (n = 2) belonging to species E. Figure 6 shows the relationship between sequences for the clinical samples compared with the relevant prototype sequences from GenBank. The sequences corresponding to each serotype were identical to those from GenBank except for the specimen VC13 which had a single base change when compared with prototype sequence AY224391. The novel sequence of sample VC13 was submitted to GenBank (accession number = EU074851).

DISCUSSION

This study describes the development of a multiplex real-time PCR for the detection of hAdV in respiratory tract specimens, allowing for rapid diagnosis and management of hAdV cases. Real time nucleic acid amplification based protocols for detection of multiple serotypes of hAdV have been described previously based on the DNA polymerase, fiber and hexon genes [Gu et al., 2003; Heim et al., 2003; He and Jiang, 2005; Chmielewicz et al., 2005b; Ebner et al., 2005b]. The assay described here targets the hexon gene and shows good analytical sensitivity with a wide linear dynamic range. An improvement in positive rate by 4.2% was noted when compared to DFA and culture in the validation data set. Good concordance was noted between viral culture and this multiplex PCR (with the PCR being more sensitive). During the retrospective study, only two hAdV culture positive samples were not confirmed by NAT, which could be related to the degradation of nucleic acid in stored samples over time.

The positive rate of 7.1% in the prospective analysis could be an underestimate because DFA positive NP samples were not be tested for hAdV according to the ProvLab testing algorithm. The data presented here shows a high hAdV infection rate among children between the ages of 6 months and 6 years which supports previous findings that most children infected with hAdVs are under 2 years of age [Castro-Rodriguez et al., 2006]. Co-infecting viruses were detected in a high proportion of hAdV positive samples; this confirms other similar reports [Coyle et al., 2004; Jennings et al., 2004; Lee et al., 2006; Choi et al., 2006b]. In this study, the higher Ct values for hAdV in samples with mixed infection may indicate ongoing low-level shedding which may not lead to hAdV related disease. The co-infection rate reported here could also be an underestimate as a result of the testing algorithm.

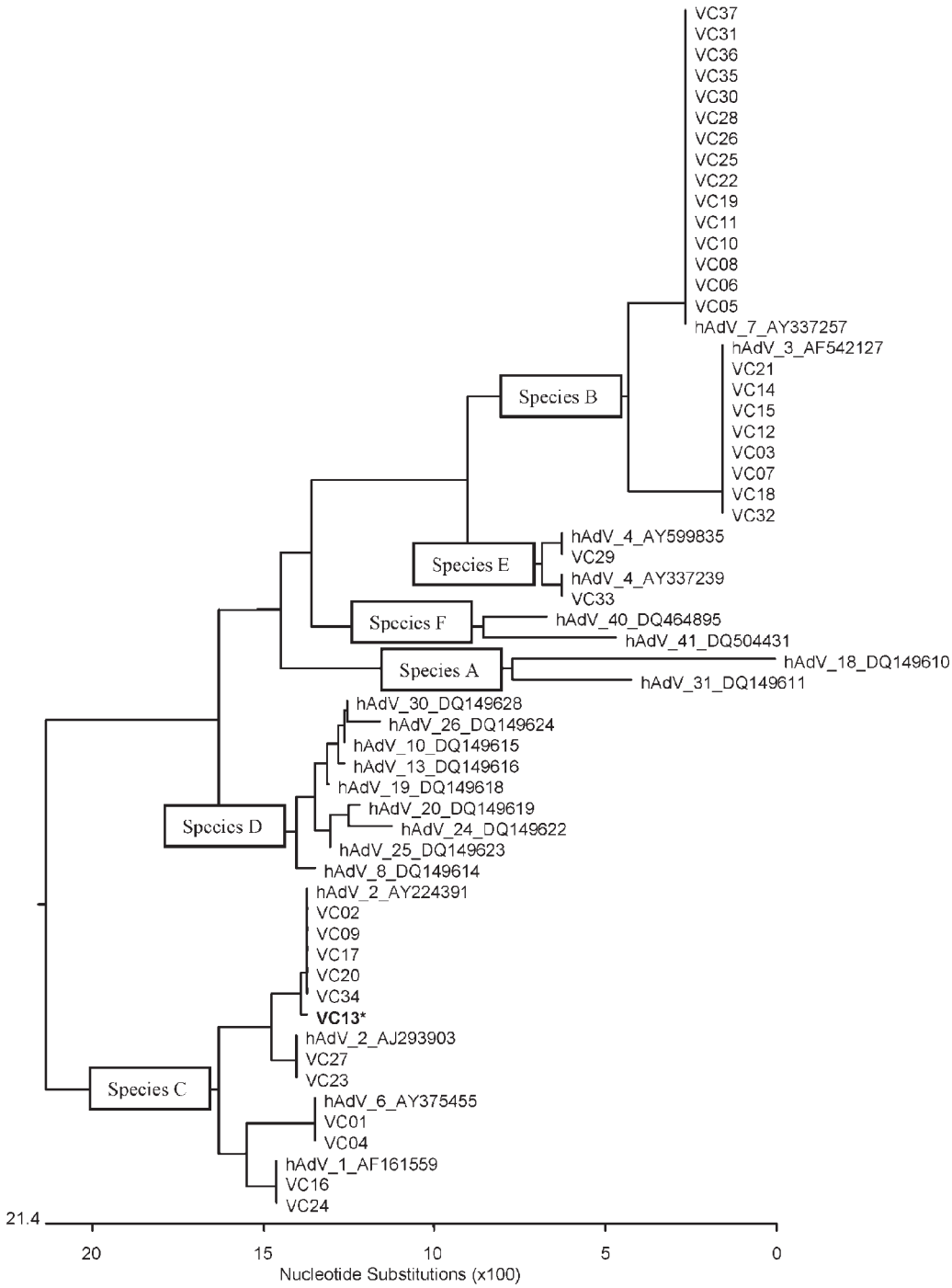

Fig. 6. Comparison of partial hexon gene sequences for clinical samples with hAdV prototype sequences from GenBank. (*VC13 GenBank number = EU074851). The scale shows the nucleotide substitution for every 100 base pairs.

No obvious hAdV seasonality was noted during the prospective testing period in Alberta, Canada. In one long term study, no seasonal pattern was reported for hAdV infections over a 10-year period from 1967 to 1977 in North America [Schmitz et al., 1983]. The majority of outbreak samples with detectable hAdV sequences had a low viral load and other co-infecting respiratory viruses were identified. Thus, it is unlikely that hAdV played an important role in the outbreaks reported here.

The low viral load for hAdVs when a co-infecting pathogen is present suggests that quantitative data may be useful for assessment of clinical relevance. The multiplex real-time PCR described had good linearity

over a wide dynamic range and could be adapted for this purpose (data not shown). Quantitative hAdV assays have been reported previously [Heim et al., 2003; Faix et al., 2004; He and Jiang, 2005; Jothikumar et al., 2005; Leung et al., 2005; Shike et al., 2005; Chmielewicz et al., 2005b; Ebner et al., 2005b] and may be particularly useful for studies of systemic infection and response to therapy in blood samples of immunocompromised individuals [Watzinger et al., 2004; Ebner et al., 2006; Humar et al., 2006].

Serotype specific PCR methods have targeted the hexon gene of hAdV serotype 4 [Houng et al., 2002; Faix et al., 2004] and serotypes 40 and 41 (species F) [Jothikumar et al., 2005; Logan et al., 2006]. Genotyping of individual hAdVs species has been reported using six pairs of hybridization probes on the LightCycler platform [Chmielewicz et al., 2005b]. Such assays for serotype specific detection of hAdV may be a useful adjunct or follow up to the broad spectrum adenovirus screening assay reported here.

Sequencing of positive samples in this study identified serotypes 1, 2, 3, 4, 6, and 7 confirming broad range hAdV detection by the assay described. Although hAdV serotype 31 is detected with low sensitivity using the PCR conditions reported, this serotype, in addition to hAdV 40 and 41, are commonly isolated only from infants with gastroenteritis [Adrian and Wigand, 1989; Brown, 1990]. Serotypes identified in this study are those most often found in the respiratory tract [Chen et al., 2004; Casas et al., 2005]. Pneumonia in children has been associated with serotypes 1, 2, 3, and 7 [Choi et al., 2006a], whereas infections in adults are most often caused by serotypes 4 and 7 [Lin et al., 2004; Moura et al., 2007]. In this study, there was one pediatric fatality associated with a high viral load of hAdV serotype 7 in the respiratory specimen and plasma (data not shown). It has been reported that children suffer more severe clinical manifestations when infected with hAdV serotype 7 having a significantly longer hospital stay [Larranaga et al., 2000; Mitchell et al., 2000; Choi et al., 2006a]. Human adenovirus serotypes 3 and 7 are the two most important etiological agents of pneumonia in China, accounting for 69–100% of the hAdV isolated from patients with pneumonia. The fatality rate reported for infantile pneumonia caused by hAdV serotype 7 was 24%, significantly higher than that for hAdV serotype 3 [Li et al., 1996]. Other studies have reported serotype 7 as the causative agent for severe childhood pneumonia [Choi et al., 2006a] and a nosocomial outbreak at a paediatric hospital [Gerber et al., 2001].

The reported multiplex real-time PCR method has been successfully implemented for diagnosis of hAdV respiratory infections in a routine diagnostic laboratory. This is the first report to describe such a large scale validation of a real-time PCR for detection of hAdV in respiratory specimens. The assay allows for broad spectrum detection of respiratory hAdV serotypes and can be adapted to provide quantitative results for samples such as blood or urine. Sequence analysis of amplified products obtained directly from the sample extract show feasibility of this approach for epidemiological studies and individual case analysis.

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