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Frequent Detection of Human Rhinoviruses, Paramyxoviruses, Coronaviruses, and Bocavirus During Acute Respiratory Tract Infections

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Viruses are the major cause of pediatric acute respiratory tract infection (ARTI) and yet many suspected cases of infection remain uncharacterized. We employed 17 PCR assays and retrospectively screened 315 specimens selected by season from a predominantly pediatric hospital-based population. Before the Brisbane respiratory virus research study commenced, one or more predominantly viral pathogens had been detected in 15.2% (n = 48) of all specimens. The Brisbane study made an additional 206 viral detections, resulting in the identification of a microbe in 67.0% of specimens. After our study, the majority of microbes detected were RNA viruses (89.9%). Overall, human rhinoviruses (HRVs) were the most frequently identified target (n = 140) followed by human adenoviruses (HAdVs; n = 25), human metapneumovirus (HMPV; n = 18), human bocavirus (HBoV; n = 15), human respiratory syncytial virus (HRSV; n = 12), human coronaviruses (HCoVs; n = 11), and human herpesvirus-6 (n = 11). HRVs were the sole microbe detected in 37.8% (n = 31) of patients with suspected lower respiratory tract infection (LRTI). Genotyping of the HRV VP4/VP2 region resulted in a proposed subdivision of HRV type A into sublineages A1 and A2. Most of the genotyped HAdV strains were found to be type C. This study describes the high microbial burden imposed by HRVs, HMPV, HRSV, HCoVs, and the newly identified virus, HBoV on a predominantly paediatric hospital population with suspected acute respiratory tract infections and proposes a new formulation of viral targets for future diagnostic research studies. ***J. Med. Virol. 78:1232–1240, 2006.*** © 2006 Wiley-Liss, Inc.

KEY WORDS: virus; respiratory infection; epidemiology; RT-PCR; diagnostics

INTRODUCTION

Acute respiratory tract infection (ARTI) is a frequent cause of paediatric morbidity and mortality and a common reason for both outpatient visits and hospitalizations. Among humans, RNA viruses are the most frequent agent to cause the common cold; usually resulting from a self-limiting upper respiratory tract infection (URTI; [Heikkinen and Järvinen, 2003]). Viruses are also frequently associated with lower respiratory tract infections (LRTI) among children [Klig and Shah, 2005] which may necessitate assisted ventilation. Human rhinoviruses (HRVs), respiratory syncytial virus (HRSV) and parainfluenza viruses (HPIVs) are among the most common traditionally diagnosed viruses while human metapneumovirus (HMPV) and recent members of the genus *Coronavirus* are now understood to contribute significantly to acute

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respiratory tract infections [Hayden, 2004; Fouchier et al., 2005; van der Hoek et al., 2005]. Because of increasing reports of an association between HRVs and lower respiratory tract infections, these viruses should be common diagnostic targets yet they, along with the classical human coronaviruses (HCoVs) are frequently absent from diagnostic research studies. Similarly, studies describing newly identified viruses (NIVs) belonging to the family *Coronaviridae* indicate that there is considerably greater potential to cause illness within the genus than once thought [Heikkinen and Järvinen, 2003; van der Hoek et al., 2004].

HRSV, HPIV-1, -2, and -3, influenza virus A and B (IFAV and IFBV) and human adenoviruses (HAdV) are the principal diagnostic targets during laboratory investigations of acute respiratory tract infections. However this panel, even when augmented by common bacterial respiratory pathogens, only accounts for 30%–60% of suspected infections [Ireland et al., 1993; Nicholson et al., 1997; Syrmis et al., 2004]. Therefore any report of a newly identified virus associated with respiratory tract infection should be investigated actively to determine if an expanded diagnostic paradigm could improve the number of clinically relevant microbial diagnoses.

The presence of some viruses among patients presenting to hospital with acute respiratory tract infection has not been extensively examined using PCR. These viruses include human bocavirus (HBoV), human parechovirus (HPEV), HPIV-4, the HCoVs HKU1, NL63, 229E and OC43, influenza C virus (IFCV), HHV-6, Mimivirus and Mossman virus. Furthermore, epidemiological studies addressing the role of these and other viruses in acute respiratory infection using PCR often sacrifice sensitivity and specificity by combining the rapid molecular technique with laborious and subjective culture and serological assays. Studies are also frequently limited by their focus on particular disease entities or because they only test viruses that fulfill the classical role of agents of acute respiratory infection, both of which lead to the under-reporting of co-detections.

It is hypothesized that an expanded diagnostic panel could improve the number of laboratory diagnoses made among patients with acute respiratory tract infection presenting to hospitals. At a time when the possibility of an influenza pandemic is high, development of rapid and specific tools to aid the differential laboratory diagnosis of acute respiratory infection is essential and improved characterization of all respiratory viruses must be a priority. We aimed to apply molecular diagnostics to investigate the role of characterized viruses and NIVs in suspected acute respiratory tract infection.

METHODS

Study Population

The primary Brisbane respiratory virus research study population comprised 315 specimens obtained from people with suspected ARTI who had presented to major metropolitan and regional hospitals in the state of Queensland, Australia during 2003 and 2004. Non-consecutive specimens for the Brisbane study were selected by season, without prior knowledge of patient details or microbiological status. Specimens were selected from winter (n = 63), spring (n = 87), summer (n = 150) and autumn (n = 96; . 3). The subjects ranged in age from 1 day to 80.3 years (mean = 7.7 years, median = 1.2 years), with children 5 years of age or younger comprising 78.9% of the study population. An additional 81 consecutively collected summer specimens (January to February 2003) formed a secondary population for the study of HRV incidence and strain variation. Specimens were predominantly nasopharyngeal aspirates (NPA; 92.4%) collected either at presentation or following hospital admission.

Once specimens were selected, basic data were collected to determine the previous test results for microbial respiratory pathogens. Previous laboratory assays included culture-amplified direct fluorescent assay and RT-PCR to detect HRSV, HAdV, HPIV-1, 2, and 3, IFAV and IFBV [Syrmis et al., 2004] and all four sublineages of HMPV [Maertzdorf et al., 2004]. PCR assays were also used to detect *Mycoplasma pneumoniae* [Bernet et al., 1989] and *Bordetella pertussis* [Kosters et al., 2002] when requested by a clinician. Selective culture media were used to isolate non-viral pathogens including *Pseudomonas aeruginosa*, *Streptococcus species*, *Haemophilus influenzae*, *Neisseria species*, *Staphylococcus species*, *Legionella pneumophila*, and *Candida albicans*. No microbes had been detected in 267 of 315 specimens (84.8%) while 80 suspected pathogens were identified from 48 of the 315 specimens (15.2%).

Lower respiratory tract infection was defined in patients with one or more of the following clinical features upon consultation; bronchiolitis, pneumonia, asthma, lower respiratory tract infection, wheezing, pneumonitis or laryngotracheobronchitis (croup) or among those requiring ventilation without an underlying physiological disease.

Polymerase Chain Reaction

Research assays to detect HRVs, HCoVs (NL63, HKU1, OC43 and 229E), Mossman virus, HPEV, IFAV, IFBV, IFCV, HPIV-1, 2, 3, and 4 and a fragment of the human RNA polymerase II gene consisted of a single-tube RT-PCR amplification (OneStep RT-PCR kit, QIAGEN, Australia) using the primers listed in Table I to examine 1 μL of purified RNA. Reaction mixtures including 0.3 μM of each primer were subjected to a 20 min incubation at 50°C followed by 15 min at 95°C. PCR was performed for 45 cycles of 94°C for 20 sec, 55 to 58°C for 20 sec and 72°C for 30 sec.

Single round PCR assays to detect Mimivirus, HAdV, HHV-6, parvovirus B19 and HBoV and the second round of nested PCR assays to detect HCoV-NL63, IFAV, IFBV, and IFCV included 0.38 μM of each primer and were subjected to a 15 min incubation at 95°C. PCR

TABLE I. Oligonucleotides Used in this Study

Oligonucleotide name	Oligonucleotide sequence	Target gene	Amplicon length (bp)	Origin
B19 INT-F	AATGAAAACTTTCCATTTAATGA	Non-structural	591	Carrière et al. [1993]
B19 INT-I	TCCTGAACTGGTCCCGGGGATGGG			
FluAC 01.1	GAACTCRTYCYWWATSWCAAWGRRGAAAT	Nucleoprotein	738	Coiras et al. [2003]
FluABC 02.1	ATKGCGCWYRAYAMWCTYARRTCTTCAWAIGC			
FluC 01.2	AAATTGGAATTTGTTCCTTTCAAGGGACA		111	
FluAC 02.2	TCTTCAWATGCARSWSMAWKGCATGCCATC			
HAdV 01.1	GCCACGGTGGGGTTTCTAAACTT	Hexon	132	Heim et al. [2003]
HAdV 02.1	GCCCCAGTGGTCTTACATGCACATC			
HBoV 01.2	TATGGCCAAGGCAATCGTCCAAG	NP1	291	This study.
HBoV 02.2	GCCGCGTGAACATGAGAAACAGA			
HCoV-HKU1	Patrick C Y Woo, Personal communication			
HCoV NL63 01.1	GTGATGCATATGCTAATTTG	1b	237	van der Hoek et al.
HCoV NL63 02.1	CTCTTGCAGGTATAATCCTA			van der Hoek et al.
HCoV NL63 01.2	TTGGTAAACAAAAGATAACT		169	
HCoV NL63 02.2	TCAATGCTATAAACAGTCAT			
HCoV NL63 01.3	CTTTTGATAACGGTCACTATG	1a	839	
HCoV NL63 02.3	CTCATTACATAAAACATCAAACGG			
HCoV NL63 01.4	GGTCACTATGTAGTTTATGATG		525	
HCoV NL63 02.4	GGATTTTTCATAACCACTTAC			
HCoV OC43 01.2	CGATGAGGCTATTCCGACTAGGT	Nucleocapsid	257	van Elden et al. [2004]
HCoV OC43 02.3	CTTGCTGAGGTTTAGTGGCAT			This study.
HCoV 229E 01.3	GAACCACAACGTGGTCGTCAGGGT	Nucleocapsid	326	This study.
HCoV 229E 02.3	CGCCTAACACCGTAACCTGT			
HPEV 01.2	GGCCCCAGATCAGATCCA	5′ NTR	301	This study.
HPEV 02.1	GGCCCCAGATCAGATCCA			Corless et al. [2002]
HRV 01.3	TACTTTGGGTGTCCGTGTTTC	5′NTR	648-HEV	Arola et al. [1996]
HRV 02.3	GGCAACTTCCACCACCACCC	VP2	533-HRV	
HRV 02.5	TTTGAGGATGTGTCTGGATGAGTT	VP2		This study
HRV 02.6	TCCAAATCTTACTTTCTAGTGT R TA			
HRV 02.7	GCCATGTTTTACT W TCCAATGT			
MHExt6s	CTCACGTCTGCCCGATTCTGAA	DNA polymerase	440	Mackay et al. [2003]
MHExt6as	ATTTATGGTGATACGGATAGC			
HPIV1 01.2	GGAAGAAGCATGGAAACTAGCAG	Phosphoprotein	223	This study.
HPIV1 02.2	GGCTGGTTTTGGTGTTGTGGTCTT			
HPIV2 01.2	TAGAGAAAGAGCAAGAGGCAAC	Phosphoprotein	296	This study.
HPIV2_02.2	CGCCTTGAGACTAGTCATTTCA			
HPIV_3 01.3	TGCGACAATCTGCAGATGTT	Phosphoprotein	378	This study.
HPIV_3 02.3	TTTGCCCCTTTGTGTCGGTG			
HPIV_4 01.4	GAAAGAGGCTTGGGTTACACA	Phosphoprotein	637	Hibbitts et al. [2003]
HPIV_4 02.5	GCTCTTATCACAGTCTCCAAA			
MIM_02.1	TAATTACCATACGCAATTCCTG	Unknown	298	La Scola et al. [2005]
MIM_01.1	TTATTGGTCCCAATGCTACTC			
MOSS_01.1	AGGAAGGTTGCTGGAGGTTA	Nucleoprotein	309	Linfa Wang
MOSS_02.2	AAGTTGGCCATCGCTCTATC			
RPII_01.1	GCACCACGTCCAATGACAT	Human RNA polymerase II	632 (gDNA)	Radonic et al. [2004]
RPII_02.1	GTGCGGCTGCTTCCATAA		270 (mRNA)	

(HotStarTaq, QIAGEN, Australia) was performed for 45 cycles of 94°C for 20 sec, 55 to 58°C for 20 sec and 72°C for 30 sec.

Previously positive clinical or cultured material was used as positive control and to validate the molecular assays.

Nucleotide Sequencing and Phylogenetic Analysis

Amplicon was purified directly from the reaction mixture (QIAquick, QIAGEN, Australia) and nucleotide sequencing reactions were performed on both amplicon strands using the ABI PRISM™ BigDye cycle sequencing kit (Perkin Elmer Applied Biosystems Division). Sequences were determined using an Applied Biosystems 377 DNA sequencer.

Nucleotide sequences were aligned using BioEdit v7.0.0 and presented in a topology tree prepared in MEGA 3. Nucleotide sequences deposited into GenBank as a result of this study include those for HRV (DQ190473 to DQ190503), HAdV (DQ145514 to DQ145525), and HBoV (DQ200648).

RESULTS

Viral Detections

In an attempt to improve upon previous laboratory testing of our study population, additional 17 PCR assays were employed including seven modified from the literature or previously undescribed, and retested the specimens. The new assays found 207 additional instances of a virus (Table II), raising the detection figure to 67.0% of specimens (n = 211) encompassing 287 total identifications. The majority of newly detected viruses contained an RNA genome (n = 162; 78.3%). A single microbe was detected in 46.7% of study specimens (n = 147), two microbes in 13.3% of specimens (n = 42) and three or more microbes in 4.8% (n = 15).

Employing primers described by Arola et al. permitted the differentiation of HRV (533 bp) from HEV (648 bp) by amplicon size. HRV comprised 67.6% of the new detections (n = 140; 44.4% of all specimens) followed in frequency by HAdV, HBoV, HHV-6, HEV, HCoV-NL63, HCoV-OC43, HPEV, and HCoV-229E. Almost a third of HRV-positive specimens (n = 44; 31.4%) contained one to three additional microbes. HRV was the most frequently co-detected microbe present in 77.2% of all co-detections. HRV detections occurred in all seasons of the year. A secondary population of 81 summer specimens was screened retrospectively to examine the incidence of HRV in a continuous specimen population and 26 (32.1%) were found to be positive. In contrast, 47.8% of specimens tested in the summer months of the primary respiratory virus study population were HRV-positive.

Testing for HAdV had already been performed on our population; however an adaptation of a more recently described real-time HAdV PCR assay [Heim et al., 2003], was used and succeeded in detecting an additional 19 positive specimens, increasing the detection of HAdV four-fold. The new assay failed to identify one instance of HAdV detected by original testing.

TABLE II. Results of Microbial Testing Using Previous Laboratory Assays and Newly Employed Molecular Assays

Microbe	Pre-study (% of study population)	New detections (% of study population)
HRSV	12 (3.8)	NP
HPIV-1, 2, and 3	9 (2.9)	0
HadV	6 (1.9)	19 (6.0)
IFAV	3 (1.0)	NP
IFCV	NP	0
EBV	1 (0.3)	NP
HHV-6	NP	11 (3.5)
VZV	2 (0.6)	NP
HMPV	18 (5.7)	NP
HRV	NP	140 (44.4)
HEV	NP	8 (2.5)
HPEV	NP	2 (0.6)
HboV	NP	15 (4.8)
HCoV-OC43	NP	4 (1.3)
HCoV-229E	NP	1 (0.3)
HCoV-NL63	NP	6 (1.9)
Bacteria	29 (6.0)	NP
Detections	80	206
Total Specimens	315	

EBV, Epstein–Barr virus; HcoV, human coronaviruses; HHV-6, human herpesvirus 6; HAdV, human adenoviruses; HEV, human enteroviruses; HBoV, human bocavirus; HMPV, human metapneumovirus; HPEV, human parechoviruses; HRSV, human respiratory syncytial virus; HPIV, human parainfluenzaviruses; HRV, human rhinoviruses; IFAV, influenza A virus; IFCV, influenza C virus. NP, not performed.

HBoV was detected in 4.8% (n = 15) of all specimens and comprised 5.2% of microbial detections. HBoV detection coincided with another microbe in 10 instances (66.7% of HBoV detections) including 5 with HRV alone, one with HRV and HRSV, one with HRV and HPEV, one with HAdV and HHV-6, and one each with HEV and HPEV alone. Coronavirus detection occurred in 3.8% (n = 11) of all specimens and 4.2% of microbial detections. HCoV-NL63 was co-detected with another virus in three instances including one with *Bordetella pertussis* and HMPV, one with HAdV and one with HRV. HCoV-229E was co-detected with HAdV as was one instance of HCoV-OC43 infection. No identifications of Mimivirus, Mossman virus, HPIV-4, IFCV, HCoV-HKU1, or human Parvovirus B19 were made.

The greatest number of HRV, HBoV, HMPV, HHV-6, and HEV detections occurred among subjects aged between 6 and 12 months of age. While this age group also contributed the largest number of specimens (19.0%), the rate of infection (23.3%; viral detections in age group per total virus detections) in this group was higher than in any of the other age groupings. The maximum number of detections of the other viruses constituted less than 9.0% of the specimens from any age group. HRSV detections peaked among subjects aged between 6 and 18 months of age while HPIV-2 was spread equally between 0 and 2 month and 1 and 2 years old. HPIV-3 detections occurred between 0 months and 20 years of age while the three IFAV detections occurred

with no apparent age prevalence. HCoV-229E was detected in a 4 month old while HCoV-NL63 detections occurred mostly among 2 to 5 year olds and HCoV-OC43 mostly in 2 to 3 month olds. We identified 56, 60, 36, and 28 instances of a putative viral pathogen among subjects aged 0 to 6 months (n = 74 specimens tested), 6 to 12 months (n = 60), 12 to 18 months (n = 41) and 18 to 24 months (n = 26) respectively.

While HRV and HBoV were detected throughout the year HPIV-3 did not occur during winter. Most of the HRSV detections (66.7%, n = 8) occurred in winter while 76% (n = 19) of HAdV, 77.8% (n = 14) of HMPV and 87.5% (n = 7) of HEV detections were made throughout winter and spring. All HCoV-NL63 detections occurred during autumn and winter. IFAV, HCoV-OC43, and HCoV-229E were detected solely in spring.

A human RNA polymerase endogenous internal control [Radonic et al., 2004] was employed to investigate the role of specimen quality on negative amplifications. This control provided some insight into the nature of the nucleic acids present in the stored specimens. An amplicon of 270 bp indicated the presence of mRNA whereas a product of 632 bp indicated human genomic DNA (HGD) had been amplified. A quarter of the specimens (n = 79) were tested and HGD alone was detected in 34.2% (n = 27) of those, mRNA alone was detected in 2.5% (n = 2) and both targets were identified in 54.4% (n = 43) of tested specimens. Among 8.9% (n = 7) of specimens tested for the endogenous internal control target neither mRNA nor HGD was amplified. In three of these a microbial target was detected leaving four (5.1%) specimens that were inhibited or did not contain PCR-viable nucleic acid template.

Genotyping

Sequencing was undertaken to genotype 10 HRV strains from each season and as many HAdV strains as possible. The assay primers used for HAdV and HRV amplification as well as HRV02.5, HRV02.6, and HRV02.7 (Table I) were applied to sequencing. A variety of putative HRV lineages were circulating during the study period but most of these clustered within the HRV type A (HRV-A) classification (92.5% of all HRV; n = 37; Fig. 1). HRV were detected during summer (23.7% of HRV detections; n = 33), autumn (29.5%; n = 41), winter (17.3%; n = 24) and spring (29.5%; n = 41). HRV-A could be further divided into 2 previously unidentified sublineages we called HRV-A1 (75.6% of all HRV-A) and HRV-A2. Within sublineages HRV-A1 and HRV-A2, amplicon sequences shared 57.4 to 100% and 68.9 to 100.0% nucleotide identity respectively. Only 56.1% to 67.3% identity was shared between HRV- A1 and HRV-A2, 55.5% to 64.0% between HRV-A2 and HRV-B and 55.2% to 88.2% between HRV-A1 and HRV-B. Eighteen strains identified as HRV by the RT-PCR assay were sequenced from the summer secondary study population; nine were HRV-A1 and nine were HEV-like. The new HRV-A2 sublineage was populated solely by strains detected from our study, mostly occurring during spring

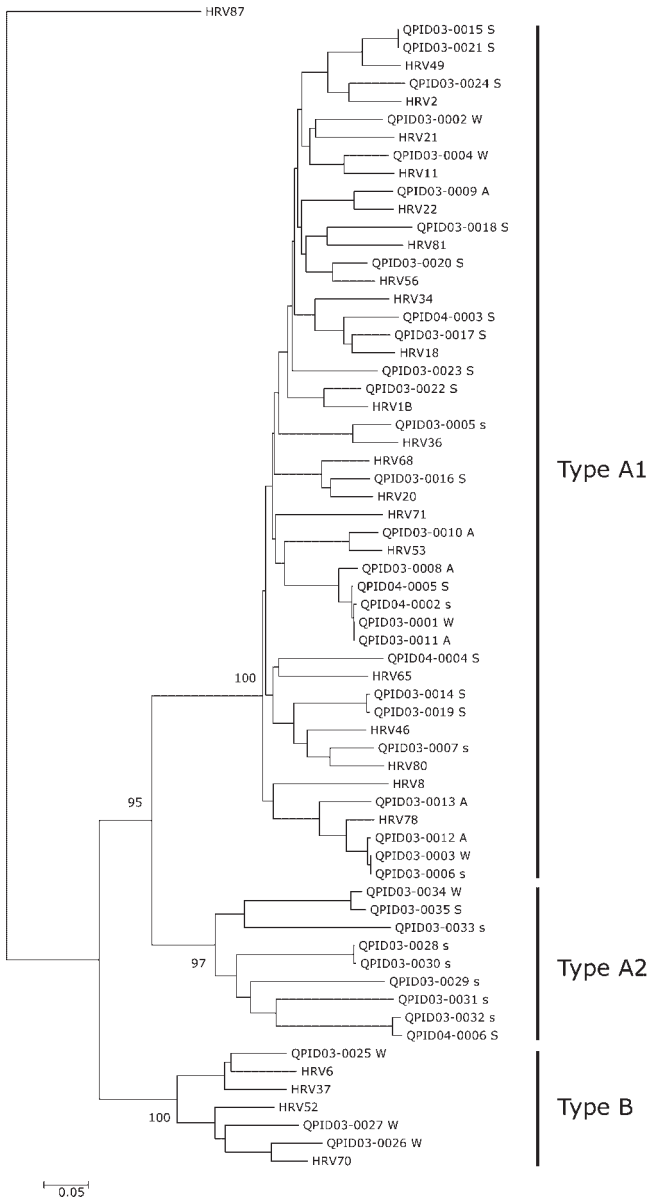

Fig. 1. Phylogenetic analysis of HRV strains detected in Queensland (QPID) presented on a topology tree prepared in MEGA 3 and compared to type strains obtained from GenBank. A 331 bp nucleotide alignment (nucleotide 623 to 953 of the VP4/VP2 region from GenBank accession number D00239) was prepared using BioEdit v7.0. The nucleotide distance matrix was generated using the Kimura 2-parameter estimation. Nodal confidence values indicate the results of boot strap resampling (n = 1,000). Sequences for the HRV comparative phylogenetic analysis were obtained from GenBank including D00239 (HRV 1b), X02316 (HRV 2), AY016402 (HRV 6), AF343594 (HRV 8), AF343623 (HRV 11), AY016407 (HRV 18), AF343644 (HRV 20), AF343626 (HRV 21), AF343628 (HRV 22), AF343634 (HRV 34), AF343588 (HRV 36), AY016401 (HRV 37), AY040235 (HRV 46), AF343598 (HRV 49), AY016398 (HRV 52), AF343592 (HRV 53), AF343610 (HRV 56), AF343586 (HRV 65), AF343591 (HRV 68), AF343646 (HRV 70), AF343587 (HRV 71), AY016408 (HRV 78), AF343593 (HRV 80), AF343606 (HRV 81), AY040243 (HRV 87). Human rhinovirus serotypes (A and B), proposed sublineages (A1 and A2) and seasonal occurrence (S, summer; A, autumn; W, winter; s, Spring) are indicated. HRV 87 was used as the outgroup.

(83.3%; n = 5). No characterized prototype HRV strains clustering with this subgroup were found. All the Queensland HRV-B strains (2.2% of HRV detections; n = 3) were detected from specimens collected in winter months. Among those subjects with suspected lower respiratory tract infection and infected solely by an HRV (n = 31), ten were genotyped and seven classified as HRV-A1 while the remainder were HRV A2.

HAdV PCR positive specimens for which extracted nucleic acids remained (n = 12) were also sequenced. The majority of these strains clustered together with the HAdV type C (83.3%; n = 10; Fig. 2) and occurred during

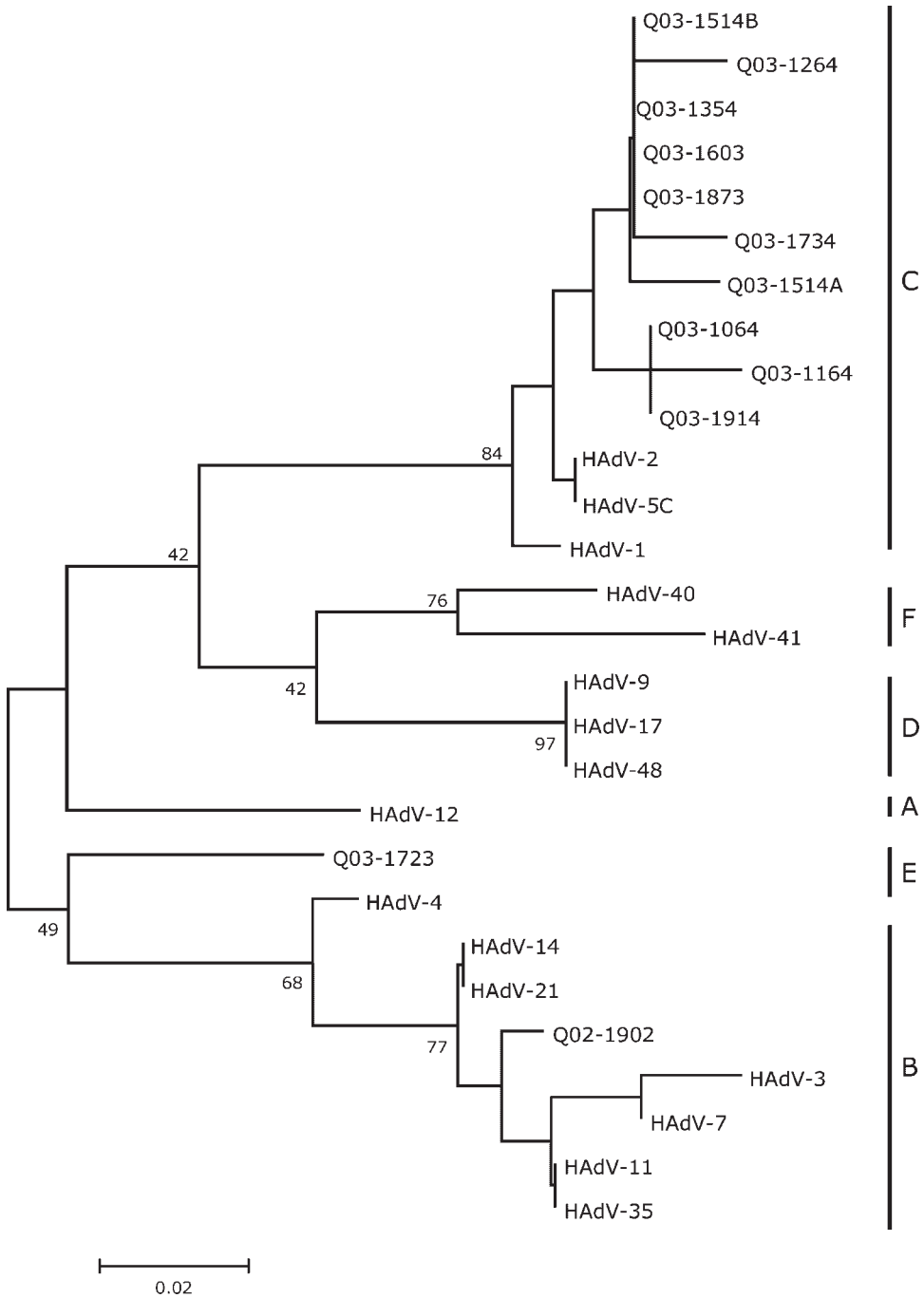

Fig. 2. Phylogenetic analysis of HAdV strains detected in Queensland (Q) presented on a topology tree prepared in MEGA 3 and compared to type strains obtained from GenBank. An 81 bp nucleotide alignment (nucleotide 51 to 131 of hexon gene from GenBank accession number AJ854486) was prepared using BioEdit v7.0. The nucleotide distance matrix was generated using the Kimura 2-parameter estimation. Nodal confidence values indicate the results of bootstrap resampling (n = 500). Sequences for the HAdV comparative phylogenetic analysis were obtained from GenBank and included AC_000017, J01917, AJ250780, NC_003266, AC_000008, AC_000018, AJ854486, NC_004001, X73487, AJ250783, AF108105, AY008279, AC_000019, L19443, D13781, U20821, respectively. Adenovirus serotypes are indicated.

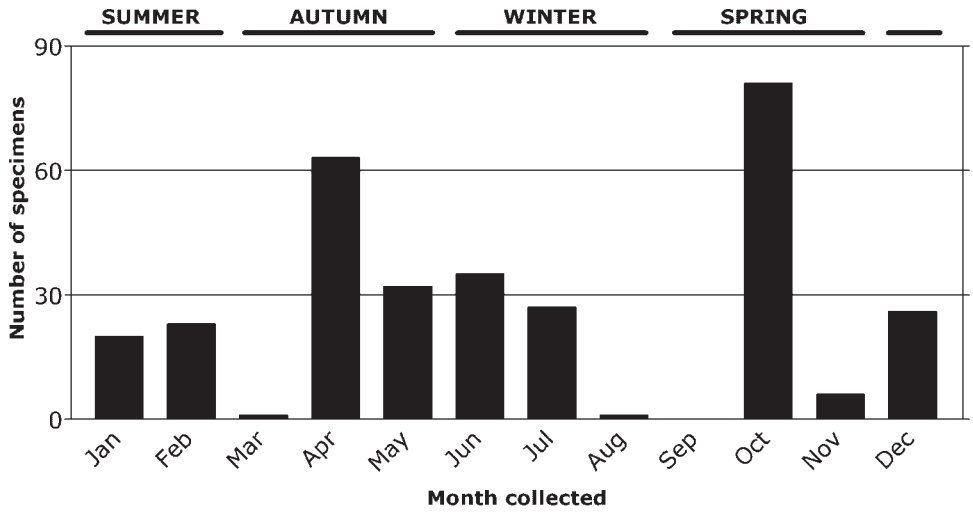

Fig. 3. Monthly distribution of all tested respiratory specimens.

spring. The remainder consisted of one HAdV type E and one HAdV type B. Of those sequenced, six were obtained from new HAdV detections and five of those were type C virus.

Signs and Symptoms upon Presentation

Preliminary studies of the type of illness affecting our study population were performed by examining the available clinical notes made on each patient at presentation. Legible notes accompanied 74.9% of specimens (n = 236). LRTI was suspected in 26.0% (n = 82) of specimens and an HRV was detected in 48.8% (n = 40) of those (Table II). HRV was the sole microbe detected in 37.8% (n = 31) of specimens originating from patients suspected with lower respiratory tract infection. Among other subjects with suspected LRTI for whom notes were available the following microbes were detected by themselves, HMPV (n = 6), HAdV (n = 1), HCoV-OC43 (n = 2), HCoV-NL63 (n = 1), HRSV (n = 1) and HPIV-3 (n = 1). We detected a type E HAdV in one patient with pneumonia in the absence of any other microbe. In three patients with suspected bronchiolitis a type C adenovirus was detected. HHV-6, HRV, and HCoV-229E were co-detected in one of these patients, HHV-6 and HBoV another and a HRV coincided with HAdV in the third. HBoV was not found as the sole microbe in any patient with suspected lower respiratory tract infection.

DISCUSSION

PCR was employed to investigate intensively a predominantly paediatric population with suspected respiratory tract infection for the presence of characterized and newly identified viruses. The study produced a 4.4-fold improvement in the number of microbial diagnoses from 15.2% to 67.0% of specimens. Following the study, viruses accounted for 89.9% of all microbial detections and at least one virus was detected in 59.7% of specimens mostly collected from infants between 6 and 12 months of age.

Detailed clinical reviews were not performed during this epidemiological study, nonetheless clinical notes made at consultation provided a guide to the impact of the acute respiratory tract infection. More than one microbe was detected in 17.8% of the Brisbane study specimens however the synergistic impact of combined viral infection on illness is unclear from these data. It is possible that each infection occurred in series, rather than in parallel and it may even be possible for one infection to prime the host for subsequent infections perhaps by temporarily exhausting the innate immune response. It is also possible that some of the infections detected in these patients were acquired during their hospital attendance however a more detailed medical chart review will be required to determine this.

HRVs were the most common viruses to be detected among the Brisbane virus study population, occurring in 44.4% of specimens collected from most age groups, a similar value to that reported by others [Hyypiä et al., 1998]. Sequencing studies using Arola et al.'s HRV/HEV primers led us to assign our HRV-A strains into two genetic sublineages we termed HRV-A1 and HRV-A2. Further studies using additional gene targets will be required to test the validity of this proposal. In contrast to Ledford et al. [2004] and Savolainen et al. [2004] who described sublineages of HRV-A, no previously characterized sublineages of HRV fell into our proposed HRV-A2 clade. In agreement with Monto [2002] and Druce et al. [2005], HRV detections were made in every season, however HRV-B strains were only detected during winter and most HRV-A2 detections occurred during spring. In the primary population, almost half of the summer specimens were HRV-positive whereas a third of specimens in the continuous summer population were positive, indicating intra-seasonal variation of HRV prevalence. An HRV was detected in almost half of all specimens from subjects with suspected lower respiratory tract infection and as the sole microbe among more than a third of all HRV-positive specimens. The dominant HRV sublineage detected overall and

from subjects with suspected lower respiratory tract infection was HRV-A1.It was also found that two-thirds of HRV-positive specimens contained no other microbe and the remainder contained up to three additional microbes. We recommend that HRVs be considered routinely in the differential laboratory diagnosis of suspected acute respiratory tract infection.

HAdVs were the second most frequently detected viruses, occurring as sole agent in over half of all HAdV detections. Typically, HAdV type C has been associated with less severe respiratory illnesses while HAdV type E has been linked to viral pneumonia [Horwitz, 2001; Heim et al., 2003]. The type C HAdVs were sequenced most commonly in this study, present alongside other viruses in three patients with suspected bronchiolitis. One patient with suspected pneumonia was positive for HAdV type E in the absence of any other microbe. HAdV type C was also the predominant lineage not detected by the original PCR method, demonstrating the clinical inadequacy of that assay.

HBoV, a newly described member of the family *Parvoviridae*, was the fourth most frequently detected microbe after HMPV. We found 67% of HBoV detections coincided with another virus, similar to a previous report [Sloots et al., 2006]. No HBoV detections were made from subjects with suspected LRTI therefore it remains difficult to elucidate the full spectrum of illnesses resulting from infection with HBoV until controlled community and hospital-based studies are performed.

HHV-6 infection alone was not linked with serious respiratory illness in our population and most instances of HHV-6 detection coincided with another microbe. Our screening did not detect HCoV-HKU1, Mimivirus, Mossman virus, HPIV-4, IFCV nor human Parvovirus B19. The existence of HCoV-HKU1 in Brisbane was described previously among specimens collected during the winter of 2004 at a rate of 3.1% [Sloots et al., 2006]. Its absence in the present study population may reflect that HCoV-HKU1 has an outbreak pattern of incidence missed by our specimen sampling, as has been reported for HCoV-OC43 [Vabret et al., 2003]. Similarly, the low rate of IFAV and IFBV can be explained by a paucity of specimens from some months of the winter season, particularly August, a peak period of influenza virus activity. A study of more specimens would address this issue. Overall, DNA viruses were more frequently co-detected with another microbe than were RNA viruses.

The Brisbane respiratory virus research study did not seek to develop optimal assays for the detection of each target, instead it aimed to identify those assays capable of improving upon existing tests or to develop assays where none existed. We considered the inclusion of an endogenous internal control important to monitor the PCR components of testing. In contrast, other recent studies have used an exogenous RNA target [Druce et al., 2005] or a DNA target for RT-PCR assays [Syrmis et al., 2004]. A supplemented control does not test template integrity although it does monitor the extraction process, while a DNA template cannot monitor the most inefficient step of the RT-PCR process, namely reverse transcription [Kolls et al., 1993]. Both endogenous internal control and viral target failed to amplify in four of tested specimens but in three other specimens for which the internal control failed, viral sequences were still detected. The latter result suggests that human DNA may sometimes be absent in NPA specimens.

We propose that future research studies deploy new formulations of molecular diagnostic tests when studying paediatric populations with suspected ARTI. One such formulation includes HRV, HAdV, HMPV, HBoV, HRSV, HCoV-OC43, HCoV-NL63, HPIV-2, HPIV-3, and IFAV targets, which together encompassed 81.1% of all microbial detections in our study. While some of these viruses do not appear to be the sole agent of suspected acute respiratory tract infection in our population, future studies are needed to better define their roles and further refine the composition of diagnostic panels for the benefit of routine diagnostic laboratories. Whilst the implication of an expanded laboratory test panel is increased cost this is not the case on a per test basis [Hyypiä et al., 1998]. Furthermore, any additional expenditure should be measured against increased laboratory diagnoses which can better direct expensive patient therapies, reduce overall hospital stay and its associated costs, and minimize the spread of nosocomial infection. The number of specimens lacking a laboratory diagnosis (33.0%; $n = 104$) fell within the range reported by other epidemiological studies which describe 12% to 66.2% of negative specimens [Jartti et al., 2004; Syrmis et al., 2004].

This seasonal epidemiology study provided an important insight into the scope of respiratory viral infections burdening a pediatric hospital-based population. Additionally, the PCR methods employed in this study permitted the detection of many viruses whose routine isolation by culture was impossible without expending considerable time and effort and for which no molecular testing was currently employed on our campus. The recent discoveries of HMPV, HCoV-NL63, HBoV HCoV-HKU1, and an increasing body of literature implicating HRV in lower respiratory tract infection make it clear that the importance of any respiratory virus should not be underestimated. It is essential to keep an open mind about the potential for newly identified viruses to cause illness and about the possible requirement to redefine associations between characterized viruses and respiratory illnesses as molecular diagnostic techniques improve our understanding of the dynamic nature of respiratory tract infections.

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REFERENCES

Arola A, Santti J, Ruuskanen O, Halonen P, Hyypiä T. 1996. Identification of enteroviruses in clinical specimens by competitive PCR followed by genetic typing using sequence analysis. J Clin Microbiol 34:313–318.

Bernet C, Garret M, De Barbeyrac B, Bebear C, Bonnet J. 1989. Detection of *Mycoplasma pneumoniae* by using the polymerase chain reaction. J Clin Microbiol 27:2492–2496.

Carrière C, Boulanger P, Delsert C. 1993. Rapid and sensitive method for the detection of B19 virus DNA using the polymerase chain reaction with nested primers. J Virol Methods 44:221–234.

Coiras MT, Pérez-Breña P, García ML, Casas I. 2003. Simultaneous detection of influenza A, B and C viruses, respiratory syncytial virus, and adenoviruses in clinical samples by multiplex reverse transcriptase nested-PCR assay. J Med Virol 69:132–144.

Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB, Mutton KJ. 2002. Development and evaluation of a 'real-time' RT-PCR for the detection of enterovirus and parechovirus RNA in CSF and throat swab samples. J Med Virol 67:555–562.

Druce J, Tran T, Kelly H, Kaye M, Chibo D, Kostecki R, Amiri A, Catton M, Birch C. 2005. Laboratory diagnosis and surveillance of human respiratory viruses by PCR in Victoria, Australia, 2002-2003. J Med Virol 75:122–129.

Fouchier RAM, Rimmelzwaan GF, Kuiken T, Osterhaus ADME. 2005. Newer respiratory virus infections: Human metapneumovirus, avian influenza virus, and human coronaviruses. Curr Opin Infect Dis 18:141–146.

Hayden FG. 2004. Rhinovirus and the lower respiratory tract. Rev Med Virol 14:17–31.

Heikkinen T, Järvinen A. 2003. The common cold. Lancet 361:51–59.

Heim A, Ebnet C, Harste G, Pring-Åkerblom P. 2003. Rapid and quantitative detection of human adenovirus by real-time PCR. J Med Virol 70:228–239.

Hibbitts S, Rahman A, John R, Westmoreland D, Fox JD. 2003. Development and evaluation of NucliSens® basic kit NASBA for diagnosis of parainfluenza virus infection with 'end-point' and 'real-time' detection. J Virol Methods 108:145–155.

Horwitz MS. 2001. Adenoviruses. In: Knipe DM, Howley PM, editors. Fields virology, Vol. 2. Philadelphia: Lippincott Williams and Wilkins, pp 2301–2326.

Hyypiä T, Puhakka T, Ruuskanen O, Mäkelä M, Arola A, Arstila P. 1998. Molecular diagnosis of human rhinovirus infections: Comparison with virus isolation. J Clin Microbiol 36:2081–2083.

Ireland DC, Kent J, Nicholson KG. 1993. Improved detection of rhinoviruses in nasal and throat swabs by seminested RT-PCR. J Med Virol 40:96–101.

Jartti T, Lehtinen P, Vuorinen T, Österback R, van den Hoogen B, Osterhaus ADME, Ruuskanen O. 2004. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute expiratory wheezing in children. Emerg Infect Dis 10:1095–1101.

Klig JE, Shah NB. 2005. Office pediatrics: Current issues in lower respiratory infections in children. Curr Opin Pediatr 17:111–118.

Kolls J, Deininger P, Cohen C, Larson J. 1993. cDNA equalization for reverse transcription-polymerase chain reaction quantitation. Analyt Biochem 208:264–269.

Kosters K, Reischl U, Schmetz J, Riffelmann M, Wirsing von Konig CH. 2002. Real-time LightCycler for detection and discrimination of *Bordetella pertussis* and *Bordetella parapertussis*. J Clin Microbiol 40:1719–1722.

La Scola B, Marrie TJ, Auffray J-P, Raoult D. 2005. Mimivirus in pneumonia patients. Emerg Infect Dis 11:449–452.

Ledford RM, Patel NR, Demenczuk TM, Watanyar A, Herbertz T, Collett MS, Pevear DC. 2004. VP1 sequencing of all human rhinovirus serotypes: Insights into genus phylogeny and susceptibility to antiviral capsid-binding compounds. J Virol 78:3663–3674.

Mackay IM, Gardam T, Arden KE, McHardy S, Whiley DM, Crisante E, Sloots TP. 2003. Co-detection and discrimination of six human herpesviruses by multiplex PCR-ELAHA. J Clin Virol 28:291–302.

Maertzdorf J, Wang CK, Brown JB, Quinto JD, Chu M, de Graaf M, van den Hoogen BG, Spaete R, Osterhaus ADME, Fouchier RAM. 2004. Real-time reverse transcriptase PCR assay for detection of human metapneumoviruses from all known genetic lineages. J Clin Microbiol 42:981–986.

Monto AS. 2002. The seasonality of rhinovirus infection and its implications for clinical recognition. Clin Therapeut 24:1987–1997.

Nicholson KG, Kent J, Hammersley V, Cancio E. 1997. Acute viral infections of upper respiratory tract in elderly people living in the community: Comparative, prospective, population based study of disease burden. Brit Med J 315:1060–1064.

Radonic A, Thulke S, Mackay IM, Landt O, Siegert W, Nitsche A. 2004. Guideline to reference gene selection for quantitative real-time PCR. Biochem Biophys Res Commun 313:856–862.

Savolainen C, Laine P, Mulders MN, Hovi T. 2004. Sequence analysis of human rhinoviruses in the RNA-dependent RNA polymerase coding region reveals within-species variation. J Gen Virol 85:2271–2277.

Sloots TP, McErlean P, Speicher DJ, Arden KE, Nissen MD, Mackay IM. 2006. Evidence of human coronavirus HKU1 and human bocavirus in Australian children. J Clin Virol 35:99–102.

Syrmis MW, Whiley DM, Thomas M, Mackay IM, Williamson J, Siebert DJ, Nissen MD, Sloots TP. 2004. A sensitive, specific and cost-effective multiplex reverse-transcriptase-PCR assay for the detection of seven common respiratory viruses in respiratory samples. J Mol Diagn 6:125–131.

Vabret A, Mourez T, Gouarin S, Petitjean J, Freymuth F. 2003. An outbreak of coronavirus OC43 respiratory infection in Normandy, France. Clin Infect Dis 36:985–989.

van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJM, Wolthers KC, Wertheim-van DIllen PME, Kaandorp J, Spaargaren J, Berkhout B. 2004. Identification of a new human coronavirus. Nat Med.

van der Hoek L, Sure K, Ihorst G, Stang A, Pyrc K, Jebbink MF, Petersen G, Forster J, Berkhout B, Überla K. 2005. Croup is associated with the novel coronavirus NL63. PloS Med 2:e240.

van Elden LJR, van Loon AM, van Alphen F, Hendriksen KAW, Hoepelman AIM, van Kraaij MGJ, Oosterheert J-J, Schipper P, Schuurman R, Nijhuis M. 2004. Frequent detection of human coronaviruses in clinical specimens from patients with respiratory tract infection by use of a novel real-time reverse-transcription polymerase chain reaction. J Infect Dis 189:652–657.