Journal of Medical Virology 83:1458–1468 (2011)

Contribution of Common and Recently Described Respiratory Viruses to Annual Hospitalizations in Children in South Africa

Marietjie Venter,[1,2]* Ria Lassaunière,[1] Tina Louise Kresfelder,[1] Yvette Westerberg,[1] and Adele Visser[1,3,4]

[1]*Department of Medical Virology, Faculty of Health Sciences, University of Pretoria, South Africa*
[2]*Respiratory Virus Unit, National Institute for Communicable Diseases, National Health Laboratory Services (NHLS), Sandringham, South Africa*
[3]*Department Clinical Pathology, Faculty of Health Sciences, University of Pretoria, South Africa*
[4]*Tshwane Academic Division, NHLS, Pretoria, South Africa*

The contribution of viruses to lower respiratory tract disease in sub-Saharan Africa where human immunodeficiency virus may exacerbate respiratory infections is not well defined. No data exist on some of these viruses for Southern Africa. Comprehensive molecular screening may define the role of these viruses as single and co-infections in a population with a high HIV-AIDS burden. To address this, children less than 5 years of age with respiratory infections from 3 public sector hospitals, Pretoria South Africa were screened for 14 respiratory viruses, by PCR over 2 years. Healthy control children from the same region were included. Rhinovirus was identified in 33% of patients, RSV (30.1%), PIV-3 (7.8%), hBoV (6.1%), adenovirus (5.7%), hMPV (4.8%), influenza A (3.4%), coronavirus NL63 (2.1%), and OC43 (1.8%). PIV-1, PIV-2, CoV-229E, -HKU1, and influenza B occurred in <1.5% of patients. Most cases with adenovirus, influenza A, hMPV, hBoV, coronaviruses, and WU virus occurred as co-infections while RSV, PIV-3, and rhinovirus were identified most frequently as the only respiratory pathogen. Rhinovirus but not RSV or PIV-3 was also frequently identified in healthy controls. A higher HIV sero-prevalence was noticed in patients with co-infections although co-infections were not associated with more severe disease. RSV, hPMV, PIV-3, and influenza viruses had defined seasons while rhinovirus, adenovirus, and coronavirus infections occurred year round in this temporal region of sub-Saharan Africa. ***J. Med. Virol. 83:1458–1468, 2011.***

KEY WORDS: respiratory virus infection; epidemiology; disease severity; viral load; co-infection; HIV

INTRODUCTION

Acute lower respiratory tract infections are major causes of pediatric morbidity and mortality worldwide, annually accounting for ~1.9 million deaths in children <5 years of age, of which up to 90% occur in the developing world [Williams et al., 2002; Black et al., 2003]. In the past respiratory syncytial virus (RSV; *Paramyxoviridae*), influenza viruses A and B (*Orthomyxoviridae*), parainfluenza viruses (PIV) 1–3 (*Paramyxoviridae*), and adenoviruses (*Adenoviridae*) were viewed to be the leading viral causes of acute lower respiratory tract infections [Law et al., 2002; Crowe and Williams, 2003]. Since 2001, several novel respiratory viruses have been described including human metapneumovirus (hMPV) [van den Hoogen et al., 2001] (*Paramyxoviridae*), human coronaviruses (hCoV) NL63 [van der Hoek et al., 2004] and HKU1 [Woo et al., 2005] (*Coronaviridae*), human bocavirus (hBoV) [Allander et al., 2005] (*Parvoviridae*), and two new polyomaviruses (WU virus (WUV) and KI virus (KIV)) [Allander et al., 2007; Bialasiewicz et al., 2007; Gaynor et al., 2007] (*Polyomaviridae*). Furthermore, improved sensitivity of diagnostic tests has lead to the increased detection of viruses previously only considered as upper respiratory tract infections in cases

Grant sponsor: Poliomyelitis Research Foundation; Grant sponsor: University of Pretoria.

Institute where the work was done: Department of Medical Virology, University of Pretoria/NHLS Tshwane Academic Division, Pretoria, South Africa.

Marietjie Venter's present address is Respiratory Virus Unit, NICD, Private bag x 4, Sandringham 2131, South Africa.

*Correspondence to: Marietjie Venter, Respiratory Virus Unit, NICD, Private bag x 4, Sandringham 2131, South Africa. E-mail: marietjiev@nicd.ac.za

Accepted 30 March 2011

DOI 10.1002/jmv.22120
Published online in Wiley Online Library (wileyonlinelibrary.com).

of acute lower respiratory tract disease, including hCoV-OC43, hCoV-229E, and rhinovirus (family *Picornaviridae*) [Pene et al., 2003; Vabret et al., 2003; Jartti et al., 2004; Hayden, 2006; Briese et al., 2008].

Respiratory viruses are important causes of morbidity and mortality in immunocompromised individuals, including patients with acquired immunodeficiency syndrome (www.unaids.org) [UNAIDS]. Increased disease burden due to RSV [Madhi et al., 2000, 2001] and hMPV [Madhi et al., 2003] infection have been demonstrated in children infected with HIV. As yet comparisons of a comprehensive range of newly implicated viruses as well as other well defined respiratory viruses by sensitive molecular techniques are lacking in sub-Saharan African countries where >65% of individuals infected with HIV reside [UNAIDS, 2007]. A recent study in Kenya indicated that RSV accounts for the majority of respiratory infections in young children [Berkley et al., 2010]. No data are yet available on the role of rhinovirus or most of the other new viruses in hospitalized patients from the African continent or on the seasonal distribution of the individual viruses in temperate regions in South Africa. The contribution of these viruses to hospitalizations as single and co-infections and disease severity in hospitalized children in populations with a high HIV disease burden is also not yet defined.

To investigate the contribution of new and common respiratory viruses to hospitalizations in children <5 years of age in South Africa PCR assays for 14 different respiratory viruses were used to screen specimens from patients seeking medical attention or requiring hospitalization at public sector hospitals serving the Pretoria area in South Africa over a period of 2 years and compared to a healthy control group of the same age in the same region. Seasonal trends, age distribution, co-infection rate, and association of single and mixed infections in disease severity were assessed in HIV sero-positive and negative children from this region.

MATERIALS AND METHODS

Patients and Specimens

During 2006 and 2007, a total of 737 and 965 respiratory specimens were, respectively, submitted and tested by routine virus diagnosis in the department Medical Virology, University of Pretoria/NHLS Tshwane Academic Division, which serves three public sector hospitals in the Pretoria region, viz. Steve Biko Academic Hospital, Kalafong Secondary Hospital, and 1-Military Hospital. Nasopharyngeal aspirates were collected on the day of admission. Of the total specimen group, 525/737 (71.2%) patients in 2006 and 561/965 (58.1%) patients in 2007 required hospitalization (Table I). Of all specimens received, 93.1% and 97.3% were from children less than 5 year of age. For this reason only specimens from children <5 was included in this study. Throughout each year, 244 (2006) and 240 (2007) specimens that previously tested negative, together with 75 (2006) and 66 (2007) specimens that tested positive by routine antigen detection assays, were randomly selected for retrospective screening with RT-PCR assays representing approximately one-third of the total specimens received during each year. Table I lists the demographics of the study group. Clinical diseases severity was retrospectively recorded from patients' hospital files and broadly categorized as outpatients on the basis of patients presenting to the emergency room but not requiring admission; hospitalized patients that were ill enough to be admitted to the pediatric wards and patients needing ICU treatment. Control specimens were nasopharyngeal aspirates collected, following written informed consent, from 46 healthy children <2 years of age with a similar epidemiological background, attending a vaccine clinic in the same region. Prior to specimen collection, parents at the vaccine clinic were asked to complete a questionnaire regarding the occurrence of any symptoms associated with respiratory disease within the previous month. Only healthy children without recent respiratory disease were included in the healthy control group.

Viral Diagnosis

Antigen detection assays. Routine diagnosis was performed with direct immunofluorescence assays using the Light Diagnostics™ Respiratory Panel 1 Viral Screening and Identification Kit (Millipore, Billerica, MA) for detection of RSV A and B, PIV-1, -2, and -3, influenza A and B, and adenovirus, and the Light Diagnostics™ Cytomegalovirus (CMV) Immunofluorescence Assay (Millipore, Billerica, MA). Rapid RSV antigen detection ELISA with the BD Directigen™ RSV test (BD, Franklin Lakes, NJ) was performed on request by the attending clinician.

TABLE I. Demographic Characteristics of Patients Suffering From Acute Respiratory Tract Infections (ARI) Diagnosed at the NHLS Laboratory in 2006 and 2007

	All ARI patients[a]	Patients screened by multiplex PCR[b]
Total	n = 1,702	n = 627 (36.8%)
Hospitalized	1,086 (63.8%)	399 (63.6%)
Outpatients	616 (36.2%)	228 (36.4%)
Age		
Mean	1 year 7 months	1 year 2 months
Median	4 months	3 months
Range	0 days to 77 years	0–77 years
<5 years old (%)	93.1%	97.3%
Sex (% males)	53.0%	52.8%
HIV Status		
HIV negative	145 (8.5%)	80 (12.8%)
HIV sero-positive[c]	548 (32.2%)	383 (61.1%)
Unknown	1,009 (59.3%)	164 (26.2%)

[a]Include all patients suffering from acute respiratory tract infections of which respiratory samples were submitted for routine diagnosis of respiratory viruses by antigen detection methods.
[b]Include patients suffering from acute respiratory tract infections of which respiratory samples were randomly selected throughout each year to screen with the multiplex real-time RT-PCR assay.
[c]Both HIV sero-positive (exposed) and DNA PCR positive patients (infected) are included.

Nucleic acid extraction. Nucleic acids were extracted with the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics, Mannheim, Germany).

cDNA synthesis. cDNA was synthesized using random hexamer primers and Expand Reverse Transcriptase (Roche Diagnostics), according to the manufacturer's instructions.

Multiplex real time RT-PCR. A two-step multiplex real-time reverse-transcriptase (RT) PCR for the detection and quantitation of 13 respiratory viruses was performed as previously described [Lassauniere et al., 2010]. In brief, four multiplex PCR assays targeting RSV, hMPV, and hBoV in multiplex 1; PIV-1, -2, -3, and hCoV-NL63 in multiplex 2; influenza A, B, and adenovirus in multiplex 3; and hCoV-OC43, hCoV-HKU1, and hCoV-229E in multiplex 4 was run on a Roche light cycler 2 (Roche Diagnostics).

Rhinovirus RT-PCR. Rhinovirus was detected by conventional nested RT-PCR. First round RT-PCR was performed with the Titan One-Tube RT-PCR System (Roche Diagnostics), using primers 1-EV/RV and 2-EV/RV [Coiras et al., 2004]. In brief, 10 μl of RNA was added to 10 μl of 5× reaction buffer, 5 mM DTT solution, 40 pmol of each primer, 10 mM of each dNTP, 1 μl of Titan^{TM} enzyme mix, 10 U of Protector RNase Inhibitor (Roche Diagnostics), and 5 μl of 5× Q Solution (Qiagen, Hilden, Germany) to a final volume of 50 μl. Reactions were performed according to the following program: 50°C for 30 min, 94°C for 2 min, (94°C for 30 sec, 56°C for 1 min, 68°C for 45 sec) for 35 cycles, and 68°C for 7 min. Nested PCR was performed with the Expand High Fidelity^{PLUS} PCR system (Roche Diagnostics), using primers hRV 01.3 [Arden et al., 2006] and RV2n (kindly provided by Drs. Thomas Briese and W. Ian Lipkin, Columbia University, New York). The nested PCR was conducted in a 50 μl reaction using 2 μl of the RT-PCR product, mixed with 10 μl of 5× reaction buffer, 10 mM of each dNTP, 20 pmol of each primer, and 2.5 U of Expand High Fidelity^{PLUS} enzyme mix, according to the following program: 94°C for 2 min (94°C for 30 sec, 57°C for 30 sec, 72°C for 45 sec) for 35 cycles, and 72°C for 7 min. Cycling was performed on the Palm-Cycler^{TM} version 2.2 (Corbett Research, Sydney, Australia). PCR products were assessed by agarose gel electrophoreses.

Data analysis. To determine if viruses described recently accounted for undiagnosed respiratory cases, the majority of specimens in the sample group for retrospective screening by PCR assays were selected from IFA negative specimens. In order to determine the accurate prevalence rates for the viruses already included in the IFA assay the prevalence as determined by both IFA and PCR assays and extrapolated as follows: Conventional respiratory viruses = ((respective virus percentage identified by RT-PCR in IFA-negative sample group × total IFA-negative samples) + number of virus present in IFA-positive group) ÷ total number of specimens in the study group. This calculation gave similar results as adding the % positive in the total specimen group to the % PCR positive in the negative sample group.

The Fisher's exact test was used to compare categorical data including demographic data, co-infection rates, and quantitative data associated with the different respiratory viruses. The confidence level of mean quantitative data analyzed was determined with the Student's *t*-test with two-tailed distribution. Statistical analyses were performed using GraphPad InStat software, version 3.10 (GraphPad Software, San Diego, CA).

RESULTS

Patients and Specimens

Over the 2-year period, 1,702 specimens were submitted for respiratory virus investigation to the routine diagnostic laboratory. Of these 1,637(93%) were from children <5 years of age. For the purpose of this study 627 (36.8%) were selected randomly out of every month for real-time PCR screening of which 610 (97.3%) were from children <5 years of age and used in further investigations (Table I). Within each year, the patients in the selected study group did not differ significantly from the complete patient group with respect to percentage hospitalized patients, age, and sex. The HIV sero-prevalence of the patient group (32%) is comparable to the HIV sero-prevalence in the Gauteng province of South Africa (~29.3% among antenatal clinic attendees by 2008) (www.doh.gov.za/docs/nasspsf.html, 2009) although 58.5% of patients were of unknown status. The sampled patient group had a higher HIV sero-prevalence (61.45%) but a greater number of patients were of known status due to subsequent blind testing of unknown samples as part of another study for which the data were made available for this study [Kresfelder et al., unpublished data; Venter et al., 2009a] (Table I).

Virus Detection

Over the 2-year period (2006–2007), in children less than 5 years of age rhinovirus accounted for 33.0% of respiratory infections, followed by RSV (30.1%); PIV-3 (7.8%); hBoV (6.1%); adenovirus (5.7%); hMPV (4.8%); influenza A (3.4%); hCoV-NL63 (2.1%); hCoV-OC43 (1.8%); influenza B (1.6%); PIV-2 (1.2%) and PIV-1 (1.0%); hCoV-229E (0.3%) while hCoV-HKU1 was only detected once in 2006 (0.2%) (Table II). WUV was detected in 6.8% and KIV in 1.0% of cases, as described previously [Venter et al., 2009b].

Compared to IFA, PCR assays identified distinctly higher co-infection rates for RSV (5.3% vs. 51.5%), influenza A (12.2% vs. 61.1%), PIV-1 (18.2% vs. 66.6%), PIV-3 (16.7% vs. 37.5%), and adenovirus (24.4% vs. 74.1%) (Table II). With the exception of influenza A and adenovirus, the recently described viruses had a greater number of co-infections with 68.9% for hMPV; 74.3% for hBoV; 92.3% for hCoV-NL63; 75% for hCoV-OC43 (Table II). A lower

TABLE II. Respiratory Virus Frequencies Over a 2-year Period as Identified by Routine Antigen Detection Assays and PCR in Patients < 5 years of Age

	Viruses detected by IFA assays (patients <5 years) (total: 1,637)	Viruses detected by PCR assays (patients <5 years) (total: 610)	Frequencies (patients <5 years)	IFA co-infection frequency	PCR co-infection frequency	Healthy patient group (n = 46)
RSV	353	57	30.1%[a]	5.3%	51.1%	2 (4.2%)
PIV-1	11	2	1.0%[a]	18.2%	66.6%	0
PIV-2	8	5	1.2%[a]	25.0%	16.7%	0
PIV-3	88	16	7.8%[a]	16.7%	37.5%	0
Influenza A	38	7	3.4%[a]	12.2%	61.1%	0
Influenza B	6	8	1.6%[a]	33.3%	30.0%	0
Adv	39	22	5.7%[a]	24.4%	74.1%	1 (2.1%)
CMV	5	Not tested	0.3%[a]	0.0%	Not tested	Not tested
Total tested						
hMPV	N/A	29	4.8%	N/A	68.9%	0
hBoV	N/A	37	6.1%	N/A	74.3%	2 (4.2%)
hCoV-NL63	N/A	13	2.1%	N/A	92.3%	0
hCoV-OC43	N/A	11	1.8%	N/A	75.0%	0
hCoV-229E	N/A	2	0.3%	N/A	50.0%	0
hCoV-HKU1	N/A	1	0.2%	N/A	100.0%	0
Rhinovirus	N/A	201	33.0%	N/A	46.6%	9 (18.8%)
WU[b]	N/A	21	6.8%	N/A	68.2%	0
KI[b]	N/A	3	1.0%	N/A	100.0%	0

[a]The prevalence of each of the conventional viruses in the complete specimen group was adjusted to take into account the reduced sensitivity of the IFA relative to the complete specimen group since RT-PCR was used to screen mainly specimens that already tested negative by IFA: (% IFA positive in total group) + (% RT-PCR positive in negative specimen group). This was equivalent to extrapolation values calculated as follow: Conventional respiratory viruses = ((Respective virus percentage identified by RT-PCR in IFA-negative sample group × total IFA-negative samples) + number of virus present in IFA-positive group) ÷ total number of specimens in the study group.
[b]Data from Venter et al., 2009.

co-infection rate was observed for rhinovirus (46.6%) relative to many of the recently described and some conventional respiratory viruses and was significant relative to hMPV, hBoV, hCoV-NL63, and adenovirus ($P < 0.030$ for each comparison).

In the healthy control patients, rhinovirus was detected most frequently 9/46 (18.8%), followed by RSV and hBoV (2/46 (4.2%), each), and adenovirus 1/46 (2.1%). In this patient group, rhinovirus was detected as a single infection in 8/46 healthy children while RSV, adenovirus and hBoV was each only detected once as a single infection. One patient was co-infected with RSV, hBoV, and rhinovirus.

Age Distribution

The age distribution of the different respiratory viruses in children attending public sector hospitals in Pretoria is shown in Figure 1a,b. The majority of respiratory viruses were identified in children <1-year of age. RSV was predominant in all age groups younger than 1 year, but equivalent to rhinovirus in children 12–24 months. Most RSV and rhinovirus diseases were detected in children <1–3 months of age while most PIV-3, hBoV, and adenovirus infections were in children of 6–12 months. HPMV infections were distributed almost equally in the 1–12 months age groups. Most influenza A infections were identified in the 1–3 and 6–12 months age groups. hCoV-HKU1 was detected in one 4-month old patient and hCoV-229E in two patients of 1.5 and 2 months old.

Seasonal Distribution

The monthly detection of the individual viruses is indicated in Figure 2. The climatic seasons in this region of South Africa are distinguished as follows: Summer (November to February); autumn (March to May); winter (June to August); spring (September–October) with the rainy season being between October and March. RSV peaked in autumn (April–May), while PIV-3 was detected more frequently in spring (August to October). Influenza A peaked from April to September with the majority of cases in June and July in 2006 but had two peaks in 2007 (April–May and August–September). This may be sampling error due to low numbers detected in hospitalized children in this year. Adenovirus was distributed throughout the year. Rhinovirus occurred throughout the year, with peak activity observed in February–May 2006 and April–August 2007. hMPV occurred year-round with peak activity in April 2006 and May–August 2007. hBoV cases were detected throughout 2006 whereas in 2007 peak activity occurred from July to October. hCoV-OC43 was predominantly detected during winter (June–July) and spring (September–October), whereas 91% of hCoV-NL63 cases were detected from mid-summer (January) to mid-autumn (May).

Disease Severity in Single Infections

When assessing conventional viruses, of the patients with single infections, 45/60 (75.0%) RSV, 17/

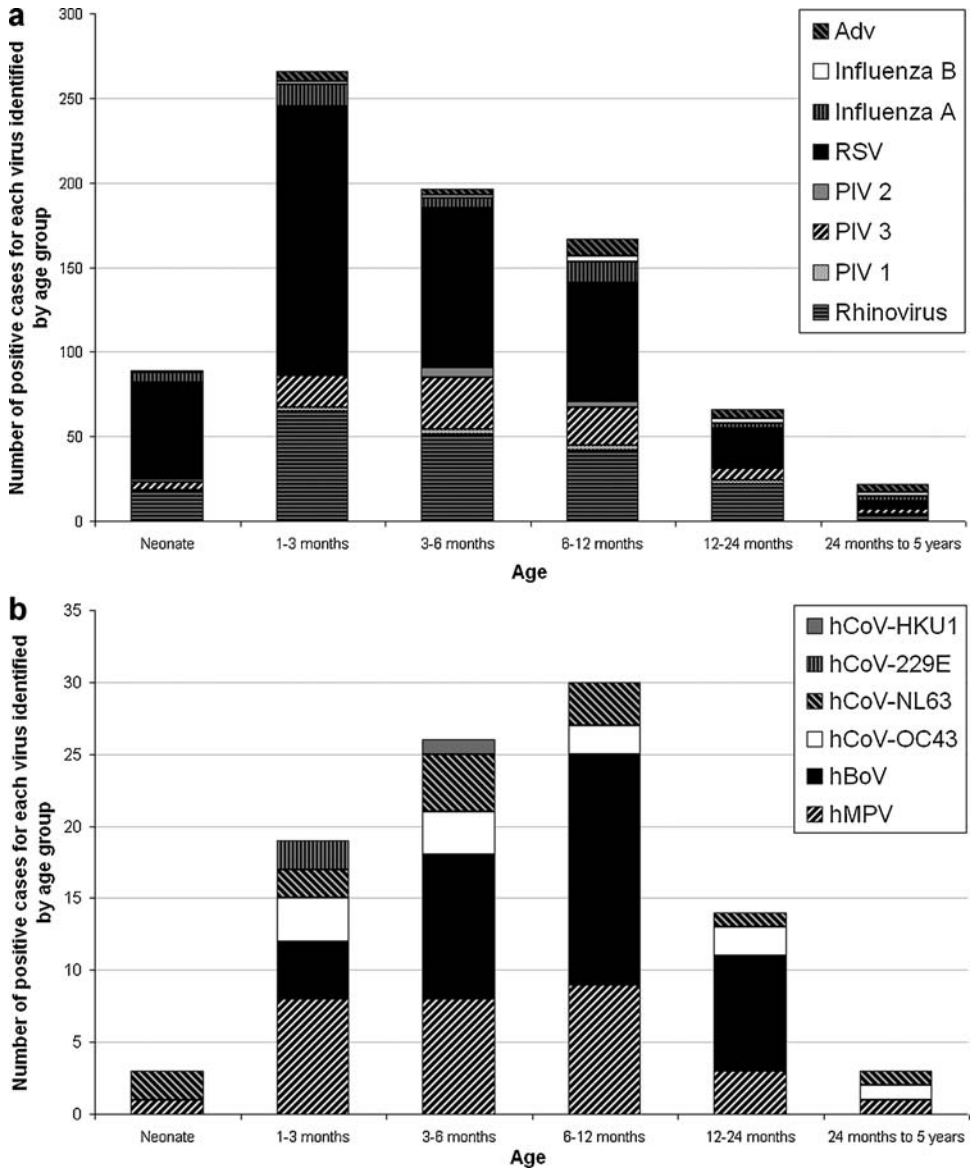

Fig. 1. **a**: Age distribution associated with well-defined viruses (RSV, influenza A and B, PIV-1–3, adenovirus, and rhinovirus). **b**: Age distribution associated with recently described viruses (hMPV, hBoV, and coronaviruses).

25 (68.0%) PIV-3, 4/7 (57.1%) influenza A, and 5/7 (71.4%) adenovirus required hospitalization (Table III). Of the patients with recently described viruses, 5/9 (55.6%) of single hMPV, 5/9 (55.6%) hBoV, 88/100 (80.7%) rhinovirus, 1/1 hCoV-NL63, 3/3 hCoV-OC43, and 1/1 hCoV-229E infections required hospitalization (Table III). Rhinovirus and RSV accounted for most single infections resulting in hospitalization. The association of RSV infections (single- and co-infections) with acute lower respiratory tract infections and hospitalization was highly significant compared to the healthy control group ($P < 0.001$). While the hospitalization rate of RSV and rhinovirus infected patients was comparable, significantly more RSV single infected patients required intensive care treatment than patients infected rhinovirus ($P < 0.020$). As a single infection, rhinovirus prevalence was neither significantly higher in all patients with acute respiratory infection (109/624 (17.6%)) nor in hospitalized patients (88/399 (22.1%)) compared to single infections in the healthy control group (8/46 (17.4%)) ($P = 1.000$ and 0.572, respectively) (Table III). However collectively, the frequency of detection for all rhinovirus infections (single- and co-infections) was notably higher in all patients with acute respiratory infection (204/627 (32.5%),

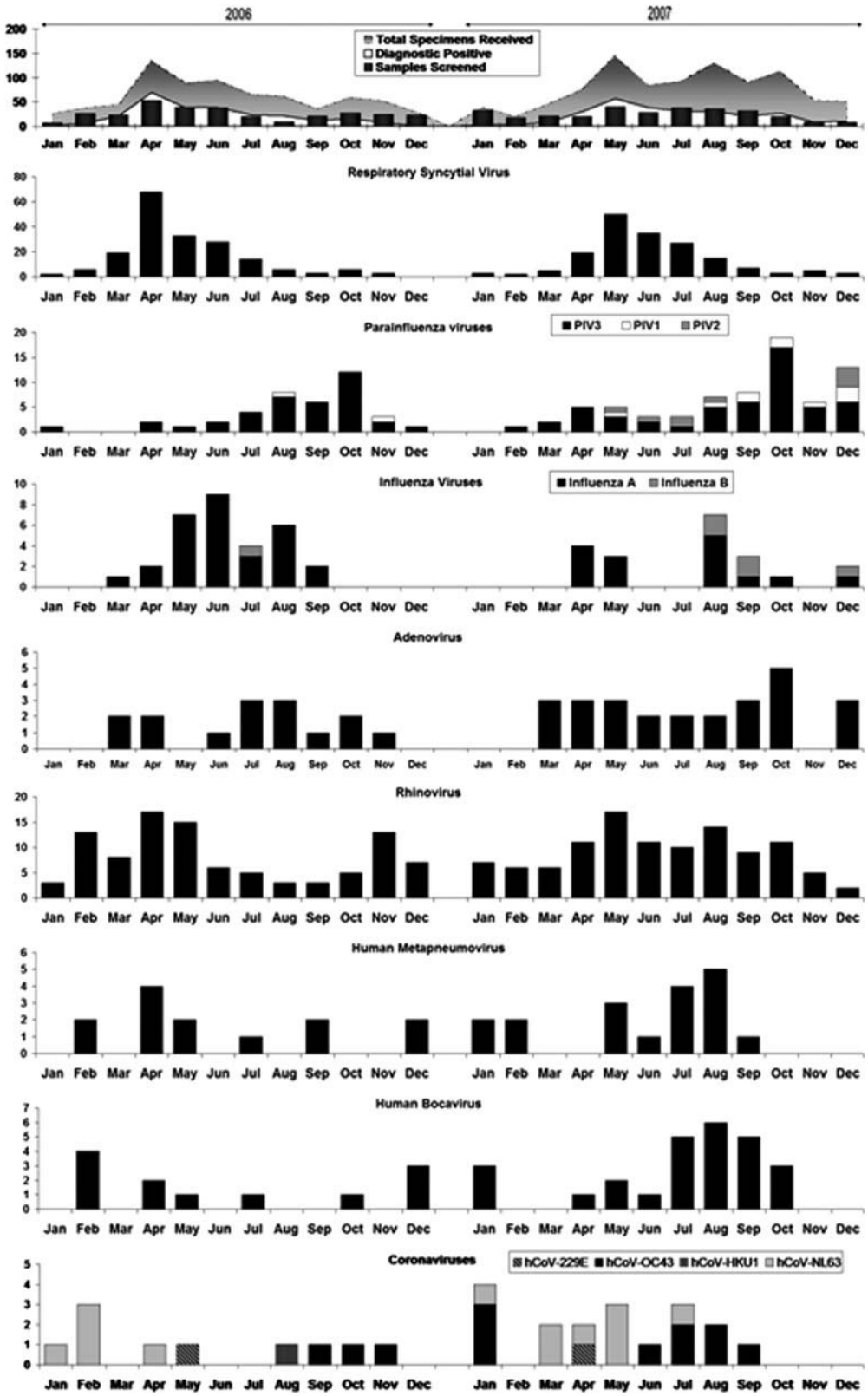

Fig. 2. Seasonal distribution of viruses detected in respiratory specimens from public sector hospitals in Pretoria submitted for viral diagnosis from January 2006 to December 2007. The top graph shows the total number of specimens received in each month, specimens positive by routine diagnostic assays and the sample group that was screened with the multiplex. The number of positive specimens for each virus is shown in the graphs below indicating the traditional viruses detected by the antigen detection tests, and the new viruses detected in the screened sample group and do therefore not reflect the extrapolated frequencies but rather the actual positives in the sample group which was selected randomly out of each month.

TABLE III. Disease Severity in Patients With Single and Co-infections With Conventional (RSV, PIV-3, Influenza A, Adenovirus) and Newly Recognized Viruses (Rhinovirus, hMPV, hBoV, hCoV-NL63, and hCoV-OC43)

	a: Patients with single infections									
	RSV (n = 60)[a]	PIV-3 (n = 25)	Influenza A (n = 7)	Adenovirus (n = 7)	Rhinovirus (n = 109)	hMPV (n = 9)	hBoV (n = 9)	hCoV-NL63 (n = 1)	hCoV-OC43 (n = 3)	hCoV-229E (n = 1)
Hospitalized	45 (75.0%)	17 (68.0%)	4 (57.1%)	5 (71.4%)	88 (80.7%)	5 (55.6%)	5 (55.6%)	1 (100.0%)	3 (100.0%)	1 (100.0%)
ICU admission	21 (35.0%)	6 (24.0%)	0	1 (14.3%)	19 (17.4%)	1 (11.1%)	0	1 (100.0%)	0	1 (100.0%)
Premature	13 (21.7%)	5 (20.0%)	0	0	9 (8.3%)	0	0	1 (100.0%)	1 (33.3%)	0
Died	6 (10.0%)	1 (4.0%)	0	?	8 (7.3%)	1 (11.1%)	1 (11.1%)	0	0	0
HIV status										
HIV negative	9 (15.0%)	2 (8.0%)	4 (57.1%)	1 (14.3%)	12 (11.0%)	1 (11.1%)	1 (11.1%)	0	0	1 (100.0%)
HIV sero-positive	38 (63.3%)	13 (52.0%)	3 (42.9%)	2 (28.6%)	69 (63.3%)	4 (44.4%)	5 (55.6%)	1 (100.0%)	3 (100.0%)	0
Unknown status	13 (21.7%)	10 (40.0%)	0	4 (57.1%)	28 (25.7%)	4 (44.4%)	3 (33.3%)	0	0	0
	b: Patients with co-infections									
	RSV (n = 70)	PIV-3 (n = 15)	Influenza A (n = 11)	Adenovirus (n = 20)	Rhinovirus (n = 95)	hMPV (n = 20)	hBoV (n = 26)	hCoV-NL63 (n = 12)	hCoV-OC43 (n = 9)	hCoV-229E (n = 1)
Hospitalized	39 (55.7%)	11 (73.3%)	7 (63.6%)	15 (75.0%)	55 (57.9%)	15 (75.0%)	19 (73.1%)	9 (75.0%)	7 (77.8%)	0
ICU admission	12 (17.1%)	2 (13.3%)	1 (9.1%)	1 (5.0%)	1 (9.5%)	1 (5.0%)	2 (7.7%)	3 (25.0%)	0	1 (100.0%)
Premature	3 (4.3%)	0	1 (9.1%)	0	3 (3.2%)	0	0	0	0	0
Died	2 (2.9%)	0	0	1(5%)	5 5.2%	1 (5.0%)	2 (7.7%)	1 (8.3%)	0	0
HIV status										
HIV negative	15 (21.4%)	3 (20.0%)	3 (27.3%)	4 (20.0%)	15 (15.8%)	3 (15.0%)	3 (11.5%)	5 (41.7%)	2 (22.2%)	1 (100.0%)
HIV sero-positive	41 (58.6%)	11 (73.3%)	7 (63.6%)	13 (65.0%)	70 (73.7%)	11 (55.0%)	19 (73.1%)	5 (41.7%)	5 (55.6%)	0
Unknown status	14 (20.0%)	1 (6.7%)	1 (9.1%)	3 (15.0%)	20 (21.1%)	6 (30.0%)	4 (15.4%)	2 (16.6%)	2 (22.2%)	0

Conventional viruses parainfluenza 1, parainfluenza 2, and influenza B are not shown due to low prevalence; ICU, intensive care unit; N/D, not determined.
[a]Additional information for 60/67 (89.6%) of hospitalized and outpatients with RSV single infections as identified by real-time RT-PCR were included in this analysis.

$P = 0.072$) and significantly higher in hospitalized patients (143/399 (35.8%), $P = 0.047$) compared to the healthy control group (9/46 (18.8%)) (Tables I and III). Patients with RSV and rhinovirus had similar HIV sero-prevalence.

Disease Severity in Co-infections

For patients with co-infections, a greater percentage of patients had to be hospitalized relative to those with single infections for PIV-3 (11/15), influenza A (7/11), adenovirus (15/20), hMPV (15/20), and hBoV (19/26) although not statistically significant due to the low number of single infected cases (Table IIIb). For RSV and rhinovirus, a significantly higher percentage of patients with single infections (Table IIIa) had to be hospitalized than with co-infections (Table IIIb) (RSV (45/60) single vs. (39/70) co-infections; rhinovirus (88/100) single vs. 55/95 co-infections) ($P = 0.027$ and <0.001, respectively) and needed ICU treatment ($P = 0.026$ and <0.001, respectively). A greater percentage of patients with co-infections were HIV sero-positive relative to single infected patients with PIV-3, influenza A, adenovirus, rhinovirus, hMPV, and hBoV although not statistically significant due to low numbers.

Clinical Characteristics of the New Viruses

Although most of the new viruses were detected at too low numbers to draw significant conclusion regarding the disease presentation clinical data were investigated for all single infections of RSV and hRV cases that had data available and compared to the single PIV3, influenza A, hPMV, hBoV, hCoV-NL63, and hCoV-OC43 and -229E patients (Table IV). The following observations were made: Single infections of hBoV and hMPV were detected in association with respiratory distress, crepitations, wheezing, and gastrointestinal symptoms in several cases. Lower respiratory tract infections were diagnosed in 9/9 (100.0%) hMPV and 7/9 (77.9%) hBoV patients with single infections, with many of these patients receiving a diagnosis of bronchiolitis, bronchopneumonia, or pneumonia. hBoV was also detected in two patients with upper respiratory tract infections. Single infections of rhinovirus were associated with a wide variety of diagnoses, ranging from moderate upper respiratory tract infections to severe pneumonia and bronchiolitis. hCoV-NL63 was associated with severe pneumonia and croup in a premature, patient exposed to HIV admitted to ICU, while hCoV-OC43 was detected in patients suffering from apnea attacks and lower respiratory tract infections (2/3 patients) and bronchiolitis (1/3). hCoV-229E was only detected once as the single infection in a patient admitted to ICU due to lower respiratory tract infection and apnea attacks. hCoV-HKU1 was detected once as a co-infection with PIV-3 in a patient suffering from bronchopneumonia.

DISCUSSION

The contribution of 14 respiratory viruses to emergency room visits and hospitalizations in children under 5 was investigated over a 2-year period, in the Pretoria region, Gauteng, South Africa. Data are lacking on the role of a comprehensive range of respiratory viruses in acute lower respiratory tract infections in Southern Africa as well as their role in HIV infected patients, in particular the new viruses that are not routinely included in routine diagnostic tests. A significant increase in sensitivity of real-time RT-PCR relative to routine antigenic detection assays was demonstrated through increased detection rate of conventional viruses, new viruses not included in routine tests as well as co-infections in the current study.

In the sampled patient group rhinovirus was the most frequently detected virus followed by RSV, hBoV and PIV3, hMPV, adenovirus, hCoV-NL63, and influenza A while the other viruses were all detected in <3% of cases (Table II). WUV was also detected in 8% of cases in 2006 and warrants further investigation [Venter et al., 2009b]. The overall prevalence of hMPV (4.8%) and hBoV (6.1%) were comparable to influenza A (3.5%) in this pre-pandemic season as well as adenovirus (5.7%). Co-infection frequencies were particularly high in patients infected with the recently described respiratory viruses (68.9% to 92.3%), although high co-infection rates were also observed for influenza A and adenovirus (61.1% and 74.1%, respectively). Collectively most patients that tested positive were <1-year of age and were HIV positive, reflecting the demographics of the patient group for who respiratory specimens are submitted to the routine diagnostic laboratory. hBoV was infrequently detected in patients less than 6 months of age suggesting a possible protective role for maternal antibodies.

Worldwide, pediatric acute lower respiratory tract infections associated hospitalization is most frequently associated with RSV, followed by PIV-3 and influenza A [Forster et al., 2004; Iwane et al., 2004] although some studies have suggested that hMPV associated hospitalization rates may exceed those of the latter two viruses [Vicente et al., 2007]. The most cases of hospitalization associated with single infections in children in this study was associated with rhinovirus, RSV, and PIV-3 (80.7%, 75%, and 68%, of cases respectively), while 56% of hMPV and hBoV single and 43% for influenza A and adenovirus cases were hospitalized. Single infections that resulted in death were the highest for RSV (10%), followed by Rhinovirus (7.3%) while 1 death was identified for PIV-3, hPMV, and hBoV each. Further investigations with larger patients groups will clarify the associated hospitalization rates of the newly described viruses in a population with a high HIV burden. Co-infections with these viruses were not associated with an increased death rate in this study (Table III). Rhinovirus was the virus detected most frequently detected

TABLE IV. Clinical Characteristics Recorded From Patient Files for Those With Single-Infection for RSV, PIV-3, Influenza A, hMPV, hBoV, hCoV-NL63, and hoV-OC43, and Rhinovirus, respectively

	RSV[a] (n = 60)	PIV-3 (n = 25)	Influenza A (n = 7)	hMPV (n = 9)	hBoV (n = 9)	hCoV-NL63 (n = 1)	hCoV-OC43 (n = 3)[b]	hCoV-229E (n = 1)	hRV (n = 109)
Demographics									
Mean age (months) ± SD	3.6 ± 2.5	5.9 ± 4.6	4.0 ± 3.5	6.5 ± 5.0	10.8 ± 7.9	3 months	3.1 ± 1.9	1.6 months	7.3 ± 10.5
Sex (males %)	61.7%	64.0%	42.9%	66.7%	66.7%	0%	33.3%	100.0%	60.5%
O_2 supplementation	38 (63.3%)	14 (56.0%)	3 (42.9%)	6 (66.7%)	6 (66.7%)	1 (100%)	2 (66.7%)	1 (100%)	58 (53.2%)
Malnourished (wasted)	8 (13.3%)	4 (16.0%)	1 (14.3%)	0	2 (22.2%)	0	1 (33.3%)	0	0
Undernourished (stunted)	4 (6.7%)	0	0	1 (11.1%)	2 (22.2%)	0	0	0	0
Unknown nutritional status	17 (28.3%)	2 (8.0%)	0	2 (22.2%)	2 (22.2%)	1 (100%)	1 (33.3%)	1 (100%)	3 (2.7%)
Clinical characteristics									
Respiratory distress	38 (63.3%)	14 (56.0%)	3 (42.9%)	6 (66.7%)	6 (66.7%)	1 (100%)	2 (66.7%)	1 (100%)	58 (53.2%)
Crepitations	29 (48.3%)	16 (64.0%)	3 (42.9%)	6 (66.7%)	1 (11.1%)	0	1 (33.3%)	1 (100%)	43 (39.4%)
Wheezing	13 (21.7%)	9 (36.0%)	1 (14.3%)	3 (33.3%)	3 (33.3%)	1 (100%)	1 (33.3%)	0	28 (25.7%)
Lobar consolidation	2 (3.3%)	2 (8.0%)	0	0	1 (11.1%)	0	0	0	1 (0.1%)
GIT symptoms	9 (15.0%)	3 (12.0%)	1 (14.3%)	2 (22.2%)	3 (33.3%)	0	1 (33.3%)	1 (100%)	1 (0.1%)
Clinical diagnosis									
Upper respiratory tract infection	1 (1.6%)	0	1 (14.3%)	0	2 (22.2%)	0	0	0	5 (4.6%)
Lower respiratory tract infection	47 (78.3%)	25 (100%)	5 (71.4%)	8 (88.9%)	7 (77.8%)	1 (100%)	2 (66.7%)	1 (100%)	79 (72.5%)
Bronchiolitis	15 (25.0%)	6 (24.0%)	2 (28.6%)	2 (22.2%)	1 (11.1%)	0	1 (33.3%)	0	16 (14.7%)
Bronchopneumonia	14 (23.3%)	5 (20.0%)	2 (28.6%)	1 (11.1%)	3 (33.3%)	0	0	0	21 (19.3%)
Pneumonia	17 (28.3%)	14 (56.0%)	1 (14.3%)	2 (22.2%)	1 (11.1%)	1 (100%)	0	0	30 (27.5%)
Bronchitis	1 (1.6%)	0	0	0	1 (11.1%)	0	0	0	2 (1.8%)
LRTI not otherwise specified	6 (10.0%)	0	0	3 (33.3%)	1 (11.1%)	0	1 (33.3%)	1 (100%)	10 (9.2%)
Croup	1 (1.6%)	2 (8.0%)	0	0	0	1 (100%)	0	0	0
Asthma exacerbation	2 (3.3%)	0	0	0	0	0	0	0	0
Apnea attacks	7 (11.7%)	3 (12.0%)	0	0	0	0	1 (33.3%)	1 (100%)	5 (4.6%)

[a]Additional information for 60/67 of hospitalized and outpatients with RSV single infections as identified by real-time RT-PCR were included in this analysis.
[b]Additional information could only be obtained for two hCoV-OC43 infected patients.

virus in healthy individuals (18.8%) which may signify prolonged shedding in healthy patients. However, in concordance with recent findings [Trento et al., 2010], rhinovirus had a significantly higher detection rate in children with respiratory disease requiring medical attention compared to healthy individuals (31.3% vs. 18.8%, $P = 0.006$). If 18.8% of these cases are due to shedding it would suggest that rhinovirus may still be associated with hospitalization in 12.5% of cases. Rhinovirus was also associated with severe disease with 48.5% of single infections requiring oxygen supplementation (Table IV) but appear to be less severe than RSV-associated acute lower respiratory tract infections (as reported by similar studies) [Linsuwanon et al., 2009] with only 17.3% of rhinovirus infected patients required intensive care treatment compared to 35% for RSV patients. Prolonged shedding periods and asymptomatic infections may account for the high detection rate in healthy children and warrants further investigation especially in patients with HIV co-infections [Kaiser et al., 2006; Peltola et al., 2008].

One of the limitations of this study is that not all patients had consented to be tested for HIV and associations with disease severity could therefore not be drawn although the present study does indicate that these viruses are associated frequently with hospitalization in HIV sero-positive patients and that co-infections are observed frequently in this group. Further case–control studies in a HIV cohort may determine the impact of these viruses on hospitalization rates in HIV sero-positive patients compared to HIV-uninfected patients.

This study also demonstrates the contribution of a wide range of respiratory viruses to acute respiratory infections over the different seasons in Southern Africa, a region for which these data were lacking for many of these viruses. The majority of cases in autumn and early winter (March to July) were attributed to RSV, while influenza A occurred mostly in winter and PIV-3 in spring. hBoV (peaking in summer and spring) and hMPV (in autumn and winter) showed variability over the 2 years, while the observed seasonal distribution of hCoV-NL63 appeared to occur mostly in late summer and autumn. Contrary to other temperate regions where hCoV-NL63 is mostly observed during winter [Arden et al., 2005; Bastien et al., 2005; van der Hoek et al., 2005], the present study appears to resemble that of subtropical regions [Lau et al., 2006], despite the small numbers detected. Rhinovirus was found year round.

In conclusion, several respiratory viruses contribute to acute lower respiratory tract infections and hospitalizations over different seasons in children in South Africa and were comparable to those of countries with a significantly lower HIV-AIDS burden. Using molecular screening techniques co-infections were identified much more frequently although this was not associated with more severe disease. RSV was demonstrated to contribute significantly more to severe acute lower respiratory infections than previously appreciated and remains the most important viral cause of acute lower respiratory tract infections in hospitalized children in South Africa.

ACKNOWLEDGMENTS

We thank Stephanie Smit and the diagnostic laboratory at NHLS Tshwane for technical assistance. Ethical approval was obtained by the University of Pretoria ethics committee (Protocol 25/2006). Written informed consent was obtained from all healthy control patients. Specimens from acute lower respiratory tract infections patients submitted for diagnostic purposes were treated anonymously entered in a unique database and all identifiers removed.

REFERENCES

Allander T, Tammi MT, Eriksson M, Bjerkner A, Tiveljung-Lindell A, Andersson B. 2005. Cloning of a human parvovirus by molecular screening of respiratory tract samples. Proc Natl Acad Sci USA 102:12891–12896.

Allander T, Andreasson K, Gupta S, Bjerkner A, Bogdanovic G, Persson MA, Dalianis T, Ramqvist T, Andersson B. 2007. Identification of a third human polyomavirus. J Virol 81:4130–4136.

Arden KE, Nissen MD, Sloots TP, Mackay IM. 2005. New human coronavirus, hCoV-NL63, associated with severe lower respiratory tract disease in Australia. J Med Virol 75:455–462.

Arden KE, McErlean P, Nissen MD, Sloots TP, Mackay IM. 2006. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J Med Virol 78:1232–1240.

Bastien N, Anderson K, Hart L, Van Caeseele P, Brandt K, Milley D, Hatchette T, Weiss EC, Li Y. 2005. Human coronavirus NL63 infection in Canada. J Infect Dis 191:503–506.

Berkley JA, Munywoki P, Ngama M, Kazungu S, Abwao J, Bett A, Lassauniere R, Kresfelder T, Cane PA, Venter M, Scott JAG, Nokes DJ. 2010. Viral etiology of severe pneumonia among Kenyan infants and children. J Am Med Assoc 303:2051–2057.

Bialasiewicz S, Whiley DM, Lambert SB, Wang D, Nissen MD, Sloots TP. 2007. A newly reported human polyomavirus, KI virus, is present in the respiratory tract of Australian children. J Clin Virol 40:15–18.

Black RE, Morris SS, Bryce J. 2003. Where and why are 10 million children dying every year? Lancet 361:2226–2234.

Briese T, Renwick N, Venter M, Jarman RG, Ghosh D, Kondgen S, Shrestha SK, Hoegh AM, Casas I, Adjogoua EV, Akoua-Koffi C, Myint KS, Williams DT, Chidlow G, van den Berg R, Calvo C, Koch O, Palacios G, Kapoor V, Villari J, Dominguez SR, Holmes KV, Harnett G, Smith D, Mackenzie JS, Ellerbrok H, Schweiger B, Schonning K, Chadha MS, Leendertz FH, Mishra AC, Gibbons RV, Holmes EC, Lipkin WI. 2008. Global distribution of novel rhinovirus genotype. Emerg Infect Dis 14:944–947.

Coiras MT, Aguilar JC, Garcia ML, Casas I, Perez-Brena P. 2004. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. J Med Virol 72:484–495.

Crowe JE Jr, Williams JV. 2003. Immunology of viral respiratory tract infection in infancy. Paediatr Respir Rev 4:112–119.

Forster J, Ihorst G, Rieger CH, Stephan V, Frank HD, Gurth H, Berner R, Rohwedder A, Werchau H, Schumacher M, Tsai T, Petersen G. 2004. Prospective population-based study of viral lower respiratory tract infections in children under 3 years of age (the PRIDE study). Eur J Pediatr 163:709–716.

Gaynor AM, Nissen MD, Whiley DM, Mackay IM, Lambert SB, Wu G, Brennan DC, Storch GA, Sloots TP, Wang D. 2007. Identification of a novel polyomavirus from patients with acute respiratory tract infections. PLoS Pathog 3:e64.

Hayden FG. 2006. Respiratory viral threats. Curr Opin Infect Dis 19:169–178.

Iwane MK, Edwards KM, Szilagyi PG, Walker FJ, Griffin MR, Weinberg GA, Coulen C, Poehling KA, Shone LP, Balter S, Hall CB, Erdman DD, Wooten K, Schwartz B. 2004. Population-based surveillance for hospitalizations associated with respiratory syncytial virus, influenza virus, and parainfluenza viruses among young children. Pediatrics 113:1758–1764.

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

Kaiser L, Aubert JD, Pache JC, Deffernez C, Rochat T, Garbino J, Wunderli W, Meylan P, Yerly S, Perrin L, Letovanec I, Nicod L, Tapparel C, Soccal PM. 2006. Chronic rhinoviral infection in lung transplant recipients. Am J Respir Crit Care Med 174: 1392–1399.

Lassauniere R, Kresfelder T, Venter M. 2010. A novel multiplex real-time RT-PCR assay with FRET hybridization probes for the detection and quantitation of 13 respiratory viruses. J Virol Methods 165:254–260.

Lau SK, Woo PC, Yip CC, Tse H, Tsoi HW, Cheng VC, Lee P, Tang BS, Cheung CH, Lee RA, So LY, Lau YL, Chan KH, Yuen KY. 2006. Coronavirus HKU1 and other coronavirus infections in Hong Kong. J Clin Microbiol 44:2063–2071.

Law BJ, Carbonell-Estrany X, Simoes EA. 2002. An update on respiratory syncytial virus epidemiology: A developed country perspective. Respir Med 96:S1–S7.

Linsuwanon P, Payungporn S, Samransamruajkit R, Posuwan N, Makkoch J, Theanboonlers A, Poovorawan Y. 2009. High prevalence of human rhinovirus C infection in Thai children with acute lower respiratory tract disease. J Infect 59:115–121.

Madhi SA, Schoub B, Simmank K, Blackburn N, Klugman KP. 2000. Increased burden of respiratory viral associated severe lower respiratory tract infections in children infected with human immunodeficiency virus type-1. J Pediatr 137:78–84.

Madhi SA, Venter M, Madhi A, Petersen MK, Klugman KP. 2001. Differing manifestations of respiratory syncytial virus-associated severe lower respiratory tract infections in human immunodeficiency virus type 1-infected and uninfected children. Pediatr Infect Dis J 20:164–170.

Madhi SA, Venter M, Alexandra R, Lewis H, Kara Y, Karshagen WF, Greef M, Lassen C. 2003. Respiratory syncytial virus associated illness in high-risk children and national characterisation of the circulating virus genotype in South Africa. J Clin Virol 27:180–189.

Peltola V, Waris M, Osterback R, Susi P, Hyypia T, Ruuskanen O. 2008. Clinical effects of rhinovirus infections. J Clin Virol 43: 411–414.

Pene F, Merlat A, Vabret A, Rozenberg F, Buzyn A, Dreyfus F, Cariou A, Freymuth F, Lebon P. 2003. Coronavirus 229E-related pneumonia in immunocompromised patients. Clin Infect Dis 37: 929–932.

Trento A, Casas I, Calderon A, Garcia-Garcia ML, Calvo C, Perez-Brena P, Melero JA. 2010. Ten years of global evolution of the human respiratory syncytial virus BA genotype with a 60-nucleotide duplication in the G protein gene. J Virol 84:7500–7512.

UNAIDS. 2007. AIDS epidemic update: December 2007. Geneva, Switzerland: Joint United Nations Programme on HIV/AIDS (UNAIDS) and World Health Organization (WHO).

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 den Hoogen BG, de Jong JC, Groen J, Kuiken T, de Groot R, Fouchier RA, Osterhaus AD. 2001. A newly discovered human pneumovirus isolated from young children with respiratory tract disease. Nat Med 7:719–724.

van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJ, Wolthers KC, Wertheim-van Dillen PM, Kaandorp J, Spaargaren J, Berkhout B. 2004. Identification of a new human coronavirus. Nat Med 10:368–373.

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

Venter M, Visser A, Lassauniere R. 2009a. Human polyomaviruses, WU and KI in HIV exposed children with acute lower respiratory tract infections in hospitals in South Africa. J Clin Virol 44:230–234.

Venter M, Visser A, Lassauniere R. 2009b. Human polyomaviruses, WU and KI in HIV exposed children with acute lower respiratory tract infections in hospitals in South Africa. J Clin Virol 44:230–234.

Vicente D, Cilla G, Montes M, Perez-Yarza EG, Perez-Trallero E. 2007. Human bocavirus, a respiratory and enteric virus. Emerg Infect Dis 13:636–637.

Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C. 2002. Estimates of world-wide distribution of child deaths from acute respiratory infections. Lancet Infect Dis 2:25–32.

Woo PC, Lau SK, Chu CM, Chan KH, Tsoi HW, Huang Y, Wong BH, Poon RW, Cai JJ, Luk WK, Poon LL, Wong SS, Guan Y, Peiris JS, Yuen KY. 2005. Characterization and complete genome sequence of a novel coronavirus, coronavirus HKU1, from patients with pneumonia. J Virol 79:884–895.