Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Journal of Virological Methods 269 (2019) 1–6

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Diversity of respiratory viruses detected among hospitalized children with acute lower respiratory tract infections at Hospital Serdang, Malaysia

Mohammad Reza Etemadi[a,b], Farid Azizi jalilian[c,*], Norlijah Othman[d], Munn-Sann Lye[e], Sara Ansari[f], Putri Yubbu[d], Zamberi Sekawi[b]

[a] Department of Biology, Faculty of Basic Sciences, Islamic Azad University of Arak, Arak, Iran
[b] Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[c] Department of Medical Virology, Faculty of Medicine, Hamadan University of Medical Sciences, Hamadan, Iran
[d] Department of Pediatrics, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[e] Department of Community Health, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia
[f] Department of Anatomy, Faculty of Medicine and Health Sciences, University Putra Malaysia, 43400 UPM, Serdang, Selangor, Malaysia

ARTICLE INFO

Keywords:
Respiratory virus
Hospitalized
Malaysia

ABSTRACT

Background: The role of respiratory viruses as the major cause of acute lower respiratory tract infections (ALRTIs) in children is becoming increasingly evident due to the use of sensitive molecular detection methods. The aim of this study was to use conventional and molecular detection methods to assess the epidemiology of respiratory viral infections in children less than five years of age that were hospitalized with ALRTIs.

Methods: The cross-sectional study was designed to investigate the occurrence of respiratory viruses including respiratory syncytisl virus (RSV), human metapneumovirus (HMPV), influenza virus A and B (IFV-A and B), parainfluenzavirus 1, 2, 3 and 4 (PIV 1, 2, 3 and 4), human rhinoviruses (HRV), human enterovirus (HEV), human coronaviruses (HCoV) 229E and OC43, human bocavirus (HBoV) and human adenovirus (HAdV) in hospitalized children with ALRTIs, at Hospital Serdang, Malaysia, from June 16 to December 21, 2009. The study was also designed in part to assess the performance of the conventional methods against molecular methods.

Results: Viral pathogens were detected in 158 (95.8%) of the patients. Single virus infections were detected in 114 (67.9%) patients; 46 (27.9%) were co-infected with different viruses including double-virus infections in 37 (22.4%) and triple-virus infections in 9 (5.5%) cases. Approximately 70% of samples were found to be positive using conventional methods compared with 96% using molecular methods. A wide range of respiratory viruses were detected in the study. There was a high prevalence of RSV (50.3%) infections, particularly group B viruses. Other etiological agents including HAdV, HMPV, IFV-A, PIV 1–3, HBoV, HCoV-OC43 and HEV were detected in 14.5, 9.6, 9.1, 4.8, 3.6, 2.4 and 1.8 percent of the samples, respectively.

Conclusion: Our results demonstrated the increased sensitivity of molecular detection methods compared with conventional methods for the diagnosis of ARTIs in hospitalized children. This is the first report of HMPV infections in Malaysia.

1. Background

Most respiratory tract infections are caused by viruses or bacteria with viruses causing the highest proportion of infections. The major causes of acute respiratory infections (ARI) in children are respiratory syncytial virus (RSV), parainfluenzavirus (PIV), influenza virus (IFV), adenovirus (AdV), and human rhinoviruses (HRV). Human metapneumovirus (HMPV), human coronaviruses (HCoV) HKU1, NL63, 229E and OC43, PIV4, human enterovirus (HEV) (Casas et al., 2005), human bocavirus (HBoV), parvovirus type 4 and 5, and mimivirus have recently been implicated as etiological agents of acute lower respiratory tract infections (ALRTIs) albeit at a lower frequency (Arden et al., 2006; Kesson, 2007; Mahony, 2008). Laboratory confirmation is required to identify aetiological agents of respiratory virus infections since viral agents cannot be differentiated on clinical features alone (Kesson, 2007). Sensitive and rapid diagnosis of respiratory infections in

* Corresponding author.
E-mail addresses: mr_etemadi@yahoo.com (M.R. Etemadi), azizifarid@gmail.com (F.A. jalilian), nor@upm.edu.my (N. Othman), mslye@upm.edu.my (M.-S. Lye), sara.ansari1986@gmail.com (S. Ansari), drputri@upm.edu.my (P. Yubbu), zamberi@upm.edu.my (Z. Sekawi).

https://doi.org/10.1016/j.jviromet.2019.03.013
Received 8 December 2018; Received in revised form 21 March 2019; Accepted 21 March 2019
Available online 22 March 2019
0166-0934/

hospitalized children is cost-effective. It is pivotal in directing active treatment early in the course of the illness following detection, reducing unnecessary antibiotic prescription, and limiting nosocomial transmission to high-risk patients (Adcock et al., 1997; Lanata et al., 2004; Osiowy, 1998; Woo et al., 1997). In addition, assessment of the morbidity of specific etiological agents of ALRTIs identified using sensitive detection methods in hospitalized patients is important to determine agent-specific interventions such as vaccination against RSV (Lanata et al., 2004). Extensive detection of infections will also expand our knowledge of the etiology of pneumonia and assist in determining which etiological agents should be considered for vaccine development (Rudan et al., 2008). Recent developments in molecular diagnosis of respiratory viruses and the discovery of new viruses have renewed the interest in respiratory virus epidemiology. However, there is still a considerable deficiency in the diagnosis of viruses which cause ALRTI (Ieven, 2007). Far too little attention has been paid to the epidemiology of respiratory viral infections in Malaysia. Therefore, in this study, the goal was to detect a panel of classical and newly discovered respiratory viruses including IFV, RSV, PIV, AdV, HMPV, HRV, HEV, HCoV, and HBoV which cause ALRTI in children below 5 years of age at Hospital Serdang. Both conventional methods including direct immunofluorescence assay, cell culture and shell vial culture and molecular diagnostic techniques including multiplex PCR and sequencing were used.

2. Materials and methods

2.1. Study design

The survey was conducted at two 28-bed pediatric wards in Hospital Serdang, a government-funded multi-specialty hospital located in the district of Sepang in the state of Selangor, Malaysia. The participants were children more than one-month-old and less than 5 years of age who were admitted to the hospital between June 16, and December 21, 2009 with the diagnosis of ALRTI. Patients with congenital or acquired immunosuppressive conditions, with conditions that posed a potential hazard in obtaining the nasopharyngeal samples (e.g. bleeding diathesis, severe respiratory compromise) as determined by the clinicians and children with incomplete data or inadequate samples were excluded from the study. All potential subjects (including careers of the patients) were briefed on the study before written informed consent was obtained by the pediatrician in-charge of the case. Approval from the following authorities was obtained prior to the start of the study: the Ministry of Heath Malaysia Research and Ethics Committee (MREC) (NMRR-09-161-638), the Medical Research Ethics Committee, Faculty of Medicine and Health Sciences, UPM (UPM/FPSK/PADS/T7-MJKEtikaPer/F01 (JMPP FEP (9) 32)) and the Research Ethics Board of Hospital Serdang were obtained. Blood samples for bacterial culture were collected by the nurses involved in the study and sent to the Department of Pathology, Hospital Serdang.

2.2. Specimen processing

Nasopharyngeal aspirate (NPA) was taken through both nostrils by inserting a disposable catheter (no. 6 or no. 8) connected to a mucus extractor. NPAs were transported in viral transport medium (VTM) to the laboratory and refrigerated at 4 °C–8 °C until required. In order to avoid repeated freezing and thawing, all NPAs were processed upon receipt. The samples were vortexed vigorously for 15 s and centrifuged at 600 ×g for 7 min. The supernatant was collected and set aside for virus isolation and genome extraction. The cell pellet was used in a direct Immunofluorescence Assay (DFA) after washing several times to remove mucus to avoid nonspecific fluorescence. Most of the samples were detected with DFA at the same day of samples received followed by cell culture and genome extraction and cDNA synthesis. The PCR reaction was performed when a bunch of samples were available. Detection of the viruses was performed concurrently during the six months of the study period.

2.3. Immunological assays

The D3 Ultra 8 Direct Immunofluorescence Assay Respiratory Virus Screening & Identification kit (Diagnostic Hybrids Inc. (DHI), USA) which contains a blend of murine fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (MAbs) was used as the first step to detect eight common viruses including RSV, HMPV, IFV type A and B, PIV 1-3, HAdV. DFA negative samples were inoculated onto shell vial culture (SVC). R-Mix TM Ready cells (DHI, USA), ready-to-use mixed cell monolayers comprising mink lung cells (Mv1Lu) and human Adenocarcinoma cells (A549), were used according to the manufacturer's recommendations.

2.4. Genome extraction and reverse transcription

Viral RNA/DNA was extracted from the filtered supernatant of NPA using MagMAX Viral RNA Isolation Kit (Applied Biosystems, Ambion, USA) according to the manufacturer's instructions. The concentration and purity of the extracts (A260/A280 nm and A260/A230 nm) were measured using a NanoDrop (ThermoFisher Scientific, USA). The first strand cDNA synthesis was carried out on RNA extracts in a final volume of 20 µl by random hexamer primer using RevertAid™ H Minus First Strand cDNA Synthesis kit (Fermentas, USA) following the manufacturer's instructions. The samples were incubated first for 5 min at 25 °C followed by 60 min at 42 °C. The reaction was terminated by heating at 70 °C for 5 min.

2.5. Polymerase chain reaction

Three multiplex RT-PCR (MP/RT-PCR1–3) and subsequently two hemi-nested multiplex PCR assays (HNMP/PCR 1–2) were carried out for the molecular detection of RNA viruses (Bellau-Pujol et al., 2005). MP/RT-PCR1 targeted influenza viruses A and B, RSV (types A and B), HMPV (A and B). MP/RT-PCR2 detected parainfluenza virus types 1, 2, 3 and 4 (A and B) (PIV 1–4). The MP/RT-PCR 3 contained primers for the detection of HRV, HEV, HCoV OC43 and 229E. An internal control consisting of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included in MP/RT-PCR2. HBoV and HAdV were detected in the samples by singleplex PCR and nested PCR respectively (Allander et al., 2005a; Lu and Erdman, 2006). Primer set HAdVhexF1/AdhexR1and nested primer set AdhexF2/AdhexR2 were used to detect HAdV hexon gene hyper-variable regions 1–6 (HVR1 − 6). The PCR products were separated by electrophoresis in a 2.5% agarose gel and visualized using ethidium bromide under UV light. The multiplex PCR was validated using pCR®2.1-TOPO® plasmid vector [TOPO TA cloning® kit (Invitrogen, USA)].

2.6. Virus culture

HEp-2 (ATCC CCL-23, USA), MRC-5 (ATCC CCL-171, USA), Vero (ATCC (CCL-81, USA) and HeLa cells (ATCC, USA) were purchased from American Type Culture Collection (ATCC) and cultured according to their guidelines. All RSV positive samples were cultured on Vero and HEp-2 cells. HAdV positive samples were inoculated onto HeLa and HEp-2 cells. MRC-5 and HeLa cells were used for samples which were positive for HRV. Cell cultures with characteristic CPE for RSV and HAdV were harvested and confirmatory testing was performed with DFA. The tube cultures showing CPE for HRV were harvested and confirmed by PCR. A second blind passage was performed after a week for cultures without characteristic CPE.

2.7. Statistical analysis

Data were analyzed using SPSS version 16.0. All *p*-values were two-tailed and *p*-values of < 0.05 were considered statistically significant. Comparisons between the results obtained by molecular methods and conventional methods were evaluated by McNemar's test and paired-samples t-test.

3. Results

3.1. Conventional methods

A total of 165 children less than five years of age who fulfilled the inclusion criteria as outlined above and were hospitalized with ALRTIs during a 20-week period between June 16, and December 21, 2009 were enrolled in the study. DFA was the first conventional method of detection of common respiratory viruses used to identify eight viruses including IFV A & B, PIVs 1–3, RSV, HMPV and HAdV. HRV, HEV, HCoVs, PIV4 and HBoV were excluded from immunological detection because of a lack of specific monoclonal antibodies. For each virus the pattern of immunofluorescent staining was specific and was used for confirmatory purposes. Eighty eight (53.9%) of the 165 NPA samples were DFA positive as follows: 67 (40.6%) for RSV, 9 (5.5%) for HMPV, 4 (2.4%) for HAdVs, 3 (1.8%) for IFV A and 6 (3.61%) for PIVs 1-3. Detection of RSV, HMPV, PIV1–3, IFV-A & B, HAdV in shell vial culture was attempted as the second stage of the conventional method on the remaining 76 DFA negative NPA samples. Seven additional viruses were detected and a total of 95 (57.6%) samples were virus positive by DFA followed by shell-vial culture. Therefore, this method was able to detect some viruses which were missed by DFA: 4 RSV and one case each for HAdV, HMPV and PIV2. For RSV, CPE was detected in 31 of 83 specimens representing a 37% recovery. Twenty-four samples positive for HAdVs were inoculated into HeLa and HEp-2 cell lines. Five of 24 samples (21%) showed characteristic CPE. Semi-confluent monolayers of HeLa and MRC-5 cell lines were used to isolate 26 of the 54 HRV positive samples detected using RT-PCR.

3.2. Molecular methods

The ability of the multiplex method to specifically detect multiple viruses in the same reaction tube was evaluated by testing a mixture of cloned plasmids of targeted viruses. Analysis of the PCR products showed that each multiplex method simultaneously detected all three control viruses included in each reaction as well as the internal control with the expected band sizes. In the presence of all primer sets in the multiplex reaction, no mispriming was observed in the positive and negative control tubes. The specificity of the MP/RT-PCR products was confirmed by nucleotide sequence analysis. Three multiplex RT-PCR (MP/RT-PCR 1–3) and subsequently 2 hemi-nested multiplex PCR (HNMP/PCR 1–2) were carried out on nucleic acids extracted from 165 clinical specimens. The specific products were clearly separated and identified on a 2.5% Seakem agarose gel, both for virus control and for clinical specimens. In total, 154 samples (93.3%) from the panel of 165 were virus positive and 11 (6.7%) specimens were virus negative using this method. Almost all viruses (97%) were detected in the first stage of the MP/RT-PCR1-3 assay. In the second stage six (6/183, 3%) additional viruses were detected by HNMP/PCR1-2. Using normal and nested PCR, of the 165 samples tested, 6 were found to be positive for HBoV and 24 (14.5%) for adenovirus. Of these positive samples, single infections were documented in one HBoV and three cases of HAdV infection. In total, 158 samples (95.8%) were positive for respiratory viruses using the molecular method while 7 (4.2%) were negative. GAPDH was successfully amplified from all of the NPA samples tested by PCR indicating that there were no PCR inhibitors in the reactions. Therefore, false negative results were excluded using this internal control.

Table 1
Single and Multiple Viral Infections by Viral Etiology (n = 165).

Pathogens	Total	Single-infection	Double-infection	Triple-infection
RSV	83 (50.3)[a]	49 (29.7)	29(17.6)	5(3.0)
HRV	54 (32.7)	36 (21.8)	14(8.5)	4(2.4)
HAdV	24 (14.5)	3 (1.8)	14 (8.5)	7(4.2)
HMPV	16 (9.6)	8 (4.8)	5(3.0)	3(1.8)
IFV-A	15 (9.1)	10 (6.1)	3 (1.8)	2(1.2)
PIVs	8 (4.8)	3 (1.8)	2(1.2)	3(1.8)
PIV-1	1 (0.6)	0	0	1 (0.6)
PIV-2	3 (1.8)	1 (0.6)	1(0.6)	1(0.6)
PIV-3	4 (2.4)	2 (1.2)	1(0.6)	1(0.6)
HBoV	6 (3.6)	1 (0.6)	3(1.8)	2(1.2)
HCoV-OC43	4 (2.4)	0	3(1.6)	1(0.6)
HEV	3 (1.8)	2 (1.2)	1(0.6)	0

[a] Numbers in parentheses, percentages.

3.3. Multiple viral infections

One hundred and twelve patients (67.9%) were found to be infected with a single virus with the most frequently detected viruses being RSV (30%), HRV (22%), IFV-A (6.1%) and HMPV (4.8%)(Table1). No single infection due to HCoVs was observed. Multiple respiratory viral infections were documented in 46 (28%) samples; consisting of 37 (22.4%) double infections and 9 (5.5%) triple infections. The most frequently detected viruses in these patients were RSV (34, 73.9%), followed by HAdV (21, 45.6%) and HRV (18, 39.1%). Dual infections of RSV with HAdV and HRV were the most prevalent multiple viral infections found in the study (13/46, 28% and 11/46, 24%, respectively). Culture of blood samples from 165 patients revealed bacterial infections in only five (3%) cases including one patient with a single infection (0.6%) with ά-hemolytic *Streptococcus viridans*. Four other samples were also virus positive and were considered to be nosocomial infections (blood cultures results were positive after 48 h) as follows: 2 RSV/*Burkholderia cepacia*, 1 HRV/ *B. cepacia* and 1HRV/ coagulase-negative staphylococcus. *M. pneumonia* infections were not identified in any of the 165 patients.

3.4. Conventional methods versus molecular methods

In total, 158 specimens (95.8%) from the panel of 165 were virus positive by a combination of conventional and molecular methods, and seven (4.2%) specimens were virus negative. The comparison between conventional and molecular methods is depicted in Table 2. A greater number of samples (95.8%) were found to be virus positive using molecular methods compared to conventional methods (69.1%) ($p < 0.001$, McNamara's test). There were 69 (41.9%), 63 (38.2%) and 36 (21.9%) more positive samples compared with DFA, DFA plus SVC and DFA plus SVC plus conventional cell culture methods, respectively. On the other hand molecular assays were able to detect 91 more viruses ($p < 0.001$, Paired-samples t-test).

3.5. Monthly distribution of viral infections

The monthly distribution of cases with respiratory tract viruses is shown in Fig. 1. During the study period, a continuously persisting activity was seen for RSV, HRV, HAdV, and HMPV. Influenza A was detected from July to September, with a peak in August followed by a plateau from September onwards. An increased incidence of HRV and RSV cases was seen after the influenza. A peak from September onwards, peaking in October. For the viruses with low incidence, no distinct pattern was seen.

4. Discussion

Recent developments in molecular diagnostics and the discovery of new viruses have created a renewed interest in the epidemiology of

Table 2
Comparison of Conventional and Molecular Methods.

Variables	Conventional Assays[a]	Molecular Assay	*p value*
No. of detectable viruses	9	14	
No. of NPA tested	165	165	
No. (%) of positive NPA	114 (69.1)	158 (95.8)	< 0.001[d]
No. of virus detected	114	213	< 0.001[e]
No. of extra virus detected	0	92	
No. of samples with co-infections	0	46 (27.9)	
No. of positive NPA by virus types			
1. Conventional viruses	**94 (56.9)**	**146 (88.5)**	
RSV	71 (43.0)	83 (50.3)	< 0.001 [d]
HMPV	11 (6.7)	16 (9.7)	0.062 [d]
IFV-A	3 (1.8)	15 (9.1)	< 0.001 [d]
IFV-B	0	0	–
PIV1	1 (0.6)	1 (0.6)	–
PIV2	2 (1.2)	3 (1.8)	–
PIV3	1 (0.6)	4 (2.4)	–
HAdV	5 (3.0)	24 (14.5)	< 0.001 [d]
2. Unconventional viruses	**27 (16.4)**	**67 (41.0)**	–
PIV4	NA	0	–
HRV	27 (16.4)	54 (32.7)	< 0.001 [d]
HEV	NA	3 (1.8)	–
HCOV OC43	NA	4 (2.4)	–
HCOV 229E	NA	0	–
HBoV	NA	6 (3.6)	–

[a]including DFA and SVC and conventional cell culture; NA: not available; [b] No of positive samples detected by conventional but not by molecular assay; [c]No of positive samples detected by molecular but not immunological assay;[d] McNemar test; [e] Paired-samples t-test.

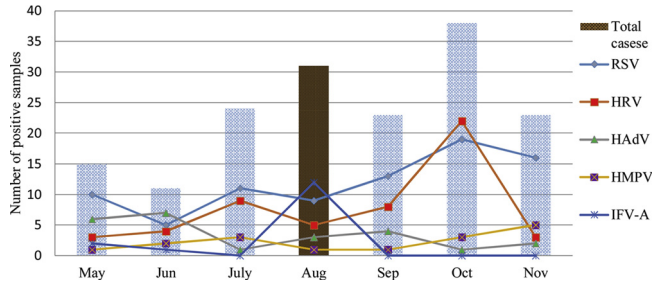

Fig. 1. Monthly Distribution of RSV, HRV, HAdV, HMPV, and IFV-A Infections.

respiratory viruses. Different ensitivities of the diagnostic assays, make evaluation of the exact contribution of each virus in epidemiology difficult (van den Hoogen et al., 2003). Regional determination of the epidemiology of specific viral infections will improve the treatment guidelines for doctors (Irmen and Kelleher, 2000). The main goal of the current study was to detect a broad panel of respiratory viruses associated with hospitalized children with ALRTIs in Malaysia. We report a high prevalence of respiratory viruses in hospitalized children (Allander et al., 2007; Jartti et al., 2004; Jennings et al., 2004; Richard et al., 2008). This was achieved using highly sensitive nested PCR which was applied to a broad spectrum of viruses. NPA samples from 158 (95.8%) patients were positive for single and/or multiple viruses. Single virus infections were detected in approximately two-thirds of the samples compared with almost one-third of samples which were found to contain multiple viruses. The performance of conventional diagnostic methods and molecular methods was also evaluated using the samples in this study. As reported in other studies (Coiras et al., 2004, 2003; Freymuth et al., 2006; LaSala et al., 2007; Weinberg et al., 2004) we established that molecular methods were more sensitive than conventional methods for the detection of respiratory viruses in children hospitalized with ALRTIs.

Many respiratory viruses including RSV-A, and B, IFV-A, PIV1, PIV2, PIV3, HMPV, HRV-A and C, HEV, HAdV, HBoV, and HCoV-OC43 were detected in the patients in this study. RSV was the most prevalent virus detected in 50% of samples. Previous publications have frequently identified RSV as the major viral pathogen associated with LRTI in children (Chan et al., 1999; Grimwood et al., 2008; Hall et al., 2009; Richard et al., 2008; Zamberi et al., 2003). Our results provide further supporting evidence that RSV infection is a frequent cause of hospitalization among children in tropical and developing countries (Weber et al., 1998; WHO, 2009). HRV was the second most prevalent virus and was detected in one-third of patients which is a similar infection rate to that reported in other studies (Chung et al., 2007; Kim and Hodinka, 1998; Lau et al., 2009; Linsuwanon et al., 2009; Miller et al., 2009, 2007; Papadopoulos et al., 2002; Peltola et al., 2009).

RSV and HRV have also been reported as the most common causes of LRTIs in other studies (Calvo et al., 2010; Franz et al., 2010; Gruteke et al., 2004; Jennings et al., 2004; Papadopoulos et al., 2002). The finding of the current study is also consistent with study by Nathan who found RV and HRV as the most detected virus associated with ALRTIs in a prospective study in Malaysia (Nathan et al., 2017). Co-infections with other viruses were found in approximately 33% (18/54) of HRV infections. The high prevalence of HRV in this study suggests that virus testing should be routinely aq

HAdV was the third (14.5%) most common virus detected in this study. Adenoviruses are responsible for 3.6–13% of all LRTIs occurring in infants and children (Chen et al., 2004; Jennings et al., 2004; John et al., 1991; Lee et al., 2010; Rocholl et al., 2004). Our findings seem to be consistent with the detection rate of 14% among hospitalized children with ALRTIs in Argentina (Videla et al., 1999). This high detection rate is especially important in developing countries with high prevalence of measles and malnutrition.

HMPV was detected as the fourth most prevalent virus with an infection rate of 10%. This is consistent with a 5.3–13% detection rate among otherwise healthy children hospitalized with LRTIs in several other studies (Foulongne et al., 2006a; Lee et al., 2007; Williams et al., 2005; Wolf et al., 2006). We demonstrated for the first time that HMPV could be an important cause of LRTI in children in Malaysia. The positivity rate is also comparable with a recent study of children hospitalized with ALRTI in subtropical Brazil (11.4%) (Oliveira et al., 2009). The prevalence of HBoV was between 1.5% (Bastien et al., 2006) to 19% (Allander et al., 2005b) in these children with respiratory infections. In our study, HBoV was detected for the first time in Malaysia in 6 of 165 nasopharyngel aspirates giving a prevalence of 3.6% (Etemadi et al., 2012). The detection rate was comparable to that (3.4%) reported in France (Foulongne et al., 2006b) and Sweden (3.1%) (Allander et al., 2005b) but was lower than that reported (8.0%) in Singapore (Tan et al., 2009).

Sensitive multiplex PCR assays give useful information about the presence of multiple pathogens and their epidemiological and clinical effects (Bellau-Pujol et al., 2005). In the current study, all of the multiple infections were diagnosed exclusively using PCR, which further supports the superiority of molecular methods over conventional methods (Rovida et al., 2005; van de Pol et al., 2006). Evaluation of the relative importance of each coexisting agent may play an important role in understanding the etiopathogenesis of these viruses (Tsolia et al., 2004). Identification of all infectious agents is especially important in high-risk immonocompromised patients for appropriate antiviral therapy (Leland and Ginocchio, 2007). In this study, multiple viral infections were found in 28% of patients and were usually combinations of RSV with HAdV and/or HRV. The co-detection rate is similar to that found by Calvo (2010) (Calvo et al., 2010)(23%), Belau-Pujol (2005) (Bellau-Pujol et al., 2005)(23%), Richard (2008) (Richard et al., 2008) (24.4%) and van de Pol (2006) (van de Pol et al., 2006)(35%). The relatively high coinfection rate in this study may be explained by the broad panel of viruses investigated. This supports the theory that HRV, HAdV, HCoV and HMPV are highly prevalent in multiple infections (van de Pol et al., 2006). It is surprising that a high diversity of virus combinations was found in the samples. This may be due in part to the

continued presence of all of the surveyed viruses during the study period with no distinct seasonality. RSV and HAdV was the most commonly detected co-infection. The high proportion of HAdV co-infections (87.5%) with other viruses in this study is comparable with (89%) HAdV co-infection rates in a study by Calvo (2010)(Calvo et al., 2010). The second most prevalent combination was RSV and HRV (24%). This combination has been reported as the most common co-infection in other studies (Chung et al., 2007; Papadopoulos et al., 2002; Paranhos-Baccalà et al., 2008; Richard et al., 2008). The high incidence of RSV and HRV co-infection can be explained by the substantial overlapping of the monthly distribution observed for these viruses during the study period (Paranhos-Baccalà et al., 2008). Quantification of the viruses in the samples may help to better understand the etiological role of each virus (Rovida et al., 2005). A study of the clinical features is required to clarify the disease severity in mixed infections (Paranhos-Baccalà et al., 2008).

5. Conclusions

A wide range of respiratory viruses were detected in this study facilitated by molecular diagnostic methods. Molecular methods increased the detection rate by up to 27% compared with conventional methods. The high detection rate of HRV confirmed its association with severe LRTI and hospitalization. Our study also demonstrated that HMPV and HBoV can be important causes of hospitalization of Malaysian children. Yearly variations in the incidence of respiratory viruses may influence their association with ALRTIs and therefore our six months study should be considered as a snapshot of viral ALRTIs in pediatric inpatients.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This work was partially supported by grants from the Ministry of Science, Technology and Innovation Malaysia (grant number 5450401). We are grateful to all of the doctors and nurses of the Pediatric Department, Hospital Serdang for providing nasopharyngeal aspirates for the study.

References

Adcock, P.M., Stout, G.G., Hauck, M.A., Marshall, G.S., 1997. Effect of rapid viral diagnosis on the management of children hospitalized with lower respiratory tract infection. Pediatr. Infect. Dis. J. 16, 842.

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

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

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

Arden, K.E., McErlean, P., Nissen, M.D., Sloots, T.P., Mackay, I.M., 2006. Frequent detection of human rhinoviruses, paramyxoviruses, coronaviruses, and bocavirus during acute respiratory tract infections. J. Med. Virol. 78, 1232–1240.

Bastien, N., Brandt, K., Dust, K., Ward, D., Li, Y., 2006. Human bocavirus infection, Canada. Emerging Infect. Diseases 12, 848–850.

Bellau-Pujol, S., Vabret, A., Legrand, L., Dina, J., Gouarin, S., Petitjean-Lecherbonnier, J., Pozzetto, B., Ginevra, C., Freymuth, F., 2005. Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. J. Virol. Methods 126, 53–63.

Calvo, C., Pozo, F., García-García, M.L., Sanchez, M., Lopez-Valero, M., Pérez-Breña, P., Casas, I., 2010. Detection of new respiratory viruses in hospitalized infants with bronchiolitis: a three-year prospective study. Acta Paediatr. 99, 883–887.

Casas, I., Avellon, A., Mosquera, M., Jabado, O., Echevarria, J.E., Campos, R.H., Rewers, M., Perez-Brena, P., Lipkin, W.I., Palacios, G., 2005. Molecular identification of adenoviruses in clinical samples by analyzing a partial hexon genomic region. J. Clin. Microbiol. 43, 6176–6182.

Chan, P., Goh, A., Chua, K.B., Kharullah, N.S., Hooi, P.S., 1999. Viral aetiology of lower respiratory tract infection in young Malaysian children. J. Paediatr. Child Health 35, 287–290.

Chen, H.-L., Chiou, S.-S., Hsiao, H.-P., Ke, G.-M., Lin, Y.-C., Lin, K.-H., Jong, Y.-J., 2004. Respiratory adenoviral infections in children: a study of hospitalized cases in Southern Taiwan in 2001-2002. J. Trop. Pediatr. 50, 279–284.

Chung, J.Y., Han, T.H., Kim, S.W., Kim, C.K., Hwang, E.S., 2007. Detection of viruses identified recently in children with acute wheezing. J. Med. Virol. 79, 1238–1243.

Coiras, M.T., Perez-Brena, P., Garcia, M.L., Casas, I., 2003. Simultaneous detection of influenza A, B, and C viruses, respiratory syncytial virus, and adenoviruses in clinical samples by multiplex reverse transcription nested-PCR assay. J. Med. Virol. 69, 132–144.

Coiras, M.T., Aguilar, J.C., Garcia, M.L., 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.

Etemadi, M., Azizi jalilian, F., Sekawi, Z., 2012. First detected human bocavirus in a malaysian child with pneumonia and pre-existing asthma: a case report. Med. J. Malaysia 76, 429–430.

Foulongne, V., Guyon, G., Rodière, M., Segondy, M., 2006a. Human metapneumovirus infection in young children hospitalized with respiratory tract disease. Pediatr. Infect. Dis. J. 25, 354–359.

Foulongne, V., Rodiere, M., Segondy, M., 2006b. Human bocavirus in children. Emerg Infect. Dis. 12 (862), 863.

Franz, A., Adams, O., Willems, R., Bonzel, L., Neuhausen, N., Schweizer-Krantz, S., Ruggeberg, J.U., Willers, R., Henrich, B., Schroten, H., Tenenbaum, T., 2010. Correlation of viral load of respiratory pathogens and co-infections with disease severity in children hospitalized for lower respiratory tract infection. J. Clin. Virol. 48, 239–245.

Freymuth, F., Vabret, A., Cuvillon-Nimal, D., Simon, S., Dina, J., Legrand, L., Gouarin, S., Petitjean, J., Eckart, P., Brouard, J., 2006. Comparison of multiplex PCR assays and conventional techniques for the diagnostic of respiratory virus infections in children admitted to hospital with an acute respiratory illness. J. Med. Virol. 78, 1498–1504.

Grimwood, K., Cohet, C., Rich, F.J., Cheng, S., Wood, C., Redshaw, N., Cunningham, C.W., Pearce, N., Kirman, J.R., 2008. Risk Factors for Respiratory Syncytial Virus Bronchiolitis Hospital Admission in New Zealand 136. pp. 1333–1341.

Gruteke, P., Glas, A.S., Dierdorp, M., Vreede, W.B., Pilon, J.W., Bruisten, S.M., 2004. Practical implementation of a multiplex PCR for acute respiratory tract infections in children. J. Clin. Microbiol. 42, 5596–5603.

Hall, C.B., Weinberg, G.A., Iwane, M.K., Blumkin, A.K., Edwards, K.M., Staat, M.A., Auinger, P., Griffin, M.R., Poehling, K.A., Erdman, D., Grijalva, C.G., Zhu, Y., Szilagyi, P., 2009. The burden of respiratory syncytial virus infection in young children. N. Engl. J. Med. 360, 588–598.

Ieven, M., 2007. Currently used nucleic acid amplification tests for the detection of viruses and atypicals in acute respiratory infections. J. Clin. Virol. 40, 259–276.

Irmen, K.E., Kelleher, J.J., 2000. Use of monoclonal antibodies for rapid diagnosis of respiratory viruses in a community hospital. Clin. Vaccine Immunol. 7, 396–403.

Jartti, T., Lehtinen, P., Vuorinen, T., 2004. Respiratory picornaviruses and respiratory syncytial virus as causative agents of acute expiratory wheezing in children. Emerging Infect. Dis. 10, 1095–1101.

Jennings, L.C., Anderson, T.P., Werno, A.M., Beynon, K.A., Murdoch, D.R., 2004. Viral etiology of acute respiratory tract infections in children presenting to hospital: role of polymerase chain reaction and demonstration of multiple infections. Pediatr. Infect. Dis. J. 23, 1003–1007.

John, T.J., Cherian, T., Steinhoff, M.C., Simoes, E.A.F., John, M., 1991. Etiology of acute respiratory infections in children in tropical southern India. Rev. Infect. Dis. 13, 463–469.

Kesson, A.M., 2007. Respiratory virus infections. Paediatr. Respir. Rev. 8, 240–248.

Kim, J.O., Hodinka, R.L., 1998. Serious respiratory illness associated with rhinovirus infection in a pediatric population. Clin. Diagn. Virol. 10, 57–65.

Lanata, C.F., Rudan, I., Boschi-Pinto, C., Tomaskovic, L., Cherian, T., Weber, M., Campbell, H., 2004. Methodological and quality issues in epidemiological studies of acute lower respiratory infections in children in developing countries. Int. J. Epidemiol. 33, 1362–1372.

LaSala, P.R., Bufton, K.K., Ismail, N., Smith, M.B., 2007. Prospective comparison of R-mix (TM) shell vial system with direct antigen tests and conventional cell culture for respiratory virus detection. J. Clin. Virol. 38, 210–216.

Lau, S.K.P., Yip, C.C.Y., Lin, A.W.C., Lee, R.A., So, L.Y., Lau, Y.L., Chan, K.H., Woo, P.C.Y., Yuen, K.Y., 2009. Clinical and molecular epidemiology of human rhinovirus C in children and adults in Hong Kong reveals a possible distinct human rhinovirus C subgroup. J. Infect. Dis. 200, 1096–1103.

Lee, W.-M., Kiesner, C., Pappas, T., Lee, I., Grindle, K., Jartti, T., Jakiela, B., Lemanske Jr., R.F., Shult, P.A., Gern, J.E., 2007. A diverse group of previously unrecognized human rhinoviruses are common causes of respiratory illnesses in infants. PLoS One 2, e966.

Lee, J., Choi, E.H., Lee, H.J., 2010. Clinical severity of respiratory adenoviral infection by serotypes in Korean children over 17 consecutive years (1991-2007). J. Clin. Virol. 49, 115–120.

Leland, D.S., Ginocchio, C.C., 2007. Role of cell culture for virus detection in the age of technology. Clin. Microbiol. Rev. 20, 49–58.

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.

Lu, X., Erdman, D.D., 2006. Molecular typing of human adenoviruses by PCR and sequencing of a partial region of the hexon gene. Arch. Virol. 151, 1587–1602.

Mahony, J.B., 2008. Detection of respiratory viruses by molecular methods. Clin. Microbiol. Rev. 21, 716–747.

Miller, E.K. Lu, X., Erdman, DeanÏD., Poehling, KatherineÏA., Zhu, Y., Griffin, MarieÏR., Hartert, TinaÏV., Anderson, LarryÏJ., Weinberg, GeoffreyÏA., Hall, CarolineÏB., Iwane, MarikaÏK., Edwards, KathrynÏM., Rhinovirus-Associated Hospitalizations in Young Children. 2007.

Miller, E.K., Khuri-Bulos, N., Williams, J.V., Shehabi, A.A., Faouri, S., Al Jundi, I., Chen, Q., Heil, L., Mohamed, Y., Morin, L.-L., Ali, A., Halasa, N.B., 2009. Human rhinovirus C associated with wheezing in hospitalised children in the Middle East. J. Clin. Virol. 46, 85–89.

Nathan, A.M., Qiao, Y.L., Jafar, F.L., Chan, Y.-F., Eg, K.P., Thavagnanam, S., Bakar, S.A., Sam, I.-C., Anne deBruyne, J., 2017. Viruses and hospitalization for childhood lower respiratory tract infection in Malaysia: a prospective study. Pediatr. Respirol. Crit. Care Med. 1, 46.

Oliveira, D.B.L., Durigon, E.L., Carvalho, A.C.L., Leal, A.L., Souza, T.S., Thomazelli, L.M., Moraes, C.T.P., Vieira, S.E., Gilio, A.E., Stewien, K.E., 2009. Epidemiology and genetic variability of human metapneumovirus during a 4-year-long study in Southeastern Brazil. J. Med. Virol. 81, 915–921.

Osiowy, C., 1998. Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. J. Clin. Microbiol. 36, 3149.

Papadopoulos, N.G., Moustaki, M., Tsolia, M., Bossios, A., Astra, E., Prezerakou, A., Gourgiotis, D., Kafetzis, D., 2002. Association of rhinovirus infection with increased disease severity in acute bronchiolitis. Am. J. Respir. Crit. Care Med. 165, 1285–1289.

Paranhos-Baccalà, G., Komurian-Pradel, F., Richard, N., Vernet, G., Lina, B., Floret, D., 2008. Mixed respiratory virus infections. J. Clin. Virol. 43, 407–410.

Peltola, V., Jartti, T., Putto-Laurila, A., 2009. Rhinovirus infections in children: a retrospective and prospective hospital-based study. J. Med. Virol. 81 (1831), 1838.

Richard, N., Komurian-Pradel, F., Javouhey, E., Perret, M., Rajoharison, A., Bagnaud, A., Billaud, G., Vernet, G., Lina, B., Floret, D., 2008. The impact of dual viral infection in infants admitted to a pediatric intensive care unit associated with severe bronchiolitis. Pediatr. Infect. Dis. J. 27, 213–217.

Rocholl, C., Gerber, K., Daly, J., Pavia, A.T., Byington, C.L., 2004. Adenoviral infections in children: the impact of rapid diagnosis. Pediatrics 113, e51–56.

Rovida, F., Percivalle, E., Zavattoni, M., Torsellini, M., Sarasini, A., Campanini, G., Paolucci, S., Baldanti, F., Revello, M.G., Gerna, G., 2005. Monoclonal antibodies versus reverse transcription PCR for detection of respiratory viruses in a patient population with respiratory tract infections admitted to hospital. J. Med. Virol. 75, 336–347.

Rudan, I., Boschi-Pinto, C., Biloglav, Z., Mulholland, K., Campbell, H., 2008. Epidemiology and etiology of childhood pneumonia. Bull. World Health Organ. 86, 408–416.

Tan, B.H., Loo, L.H., Lim, E.A.S., Seah, S.L.K., Lin, R.T.P., Tee, N.W.S., Sugrue, R.J., 2009. Human rhinovirus group C in hospitalized children, Singapore. Emerging Infectious Diseases 15, 1318.

Tsolia, M.N., Psarras, S., Bossios, A., Audi, H., Paldanius, M., 2004. Etiology of community-acquired pneumonia in hospitalized school-age children: evidence for high prevalence of viral infections. Clin. Infect. Dis. 39, 681–686.

van de Pol, A., Wolfs, T., Jansen, N., van Loon, A., Rossen, J., 2006. Diagnostic value of real-time polymerase chain reaction to detect viruses in young children admitted to the paediatric intensive care unit with lower respiratory tract infection. Crit. Care 10, R61.

van den Hoogen, B.G., van Doornum, Gjj., Fockens, J.C., 2003. Prevalence and clinical symptoms of human metapneumovirus infection in hospitalized patients. J. Infect. Dis. 188, 1571–1577.

Videla, C., Carballal, G., Kajon, A., 1999. Genomic analysis of adenovirus isolated from Argentinian children with acute lower respiratory infections. J. Clin. Virol. 14, 67–71.

Weber, M., Hartley, J., Ashworth, M., Malone, M., Sebire, N., 1998. Virological investigations in sudden unexpected deaths in infancy (SUDI). Forensic Sci. Med. Pathol. 6, 261–267.

Weinberg, G.A., Erdman, D.D., Edwards, K.M., Hall, C.B., Walker, F.J., Griffin, M.R., Schwartz, B., 2004. Superiority of reverse transcription polymerase chain reaction to conventional viral culture in the diagnosis of acute respiratory tract infections in children. J. Infect. Dis. 189, 706–710.

WHO, 2009. Acute Respiratory Infections: Influenza (Update September 2009). pp. 1–7.

Williams, J.V., Tollefson, S.J., Heymann, P.W., 2005. Human metapneumovirus infection in children hospitalized for wheezing. J. Allergy Clin. Immunol. 115, 1311–1312.

Wolf, D.G., Greenberg, D., Kalkstein, D., 2006. Comparison of human metapneumovirus, respiratory syncytial virus and influenza a virus lower respiratory tract infections in hospitalized young children. Pediatr. Infect. Dis. J. 25.

Woo, P.C., Chiu, S.S., Seto, W.H., Peiris, M., 1997. Cost-effectiveness of rapid diagnosis of viral respiratory tract infections in pediatric patients. J. Clin. Microbiol. 35, 1579.

Zamberi, S., Zulkifli, I., Ilina, I., 2003. Respiratory viruses detected in hospitalised paediatric patients with respiratory infections. Med. J. Malaysia 58, 681.