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Identification of Viral and Atypical Bacterial Pathogens in Children Hospitalized With Acute Respiratory Infections in Hong Kong by Multiplex PCR Assays

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Acute respiratory tract infection is a leading cause of hospital admission of children. This study used a broad capture, rapid and sensitive method (multiplex PCR assay) to detect 20 different respiratory pathogens including influenza A subtypes H1, H3, and H5; influenza B; parainfluenza types 1, 2, 3, and 4; respiratory syncytial virus (RSV) groups A and B; adenoviruses; human rhinoviruses; enteroviruses; human metapneumoviruses; human coronaviruses OC43, 229E, and SARS-CoV; *Chlamydophila pneumoniae*; *Legionella pneumophila*; and *Mycoplasma pneumoniae*; from respiratory specimens of 475 children hospitalized over a 12-month period for acute respiratory tract infections. The overall positive rate (47%) was about twice higher than previous reports based on conventional methods. Influenza A, parainfluenza and RSV accounted for 51%, and non-cultivable viruses accounted for 30% of positive cases. Influenza A peaked at March and June. Influenza B was detected in January, February, and April. Parainfluenza was prevalent throughout the year except from April to June. Most RSV infections were found between February and September. Adenovirus had multiple peaks, whereas rhinovirus and coronavirus OC43 were detected mainly in winter and early spring. RSV infection was associated with bronchiolitis, and parainfluenza was associated with croup; otherwise the clinical manifestations were largely nonspecific. In general, children infected with influenza A, adenovirus and mixed viruses had higher temperatures. In view of the increasing concern about unexpected outbreaks of severe viral infections, a rapid multiplex PCR assay is a valuable tool to enhance the management of hospitalized patients, and for the surveillance for viral infections circulating in the community. ***J. Med. Virol. 81:153–159, 2009.***

KEY WORDS: respiratory tract infection; seasonality; nasopharyngeal aspirate; nasal swab; multiplex PCR; atypical bacteria; Hong Kong

INTRODUCTION

Acute respiratory infection is the most common cause of hospitalization of children in Hong Kong. A review of the standardised discharge data for all Hong Kong government hospitals from 1997 to 1999 showed that acute respiratory infections accounted for 38% of all paediatric admissions and 63% of those age under 5 years [Nelson et al., 2007]. A large spectrum of viruses can cause respiratory tract infection. The routine rapid diagnostic method used currently in Government hospitals in Hong Kong is immunofluorescence test on nasopharyngeal aspirate. This method can cover upto

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seven common respiratory viruses (influenza A and B; parainfluenzae types 1, 2, and 3; respiratory syncytial virus and adenovirus). By this method alone, an aetiological diagnosis was made in less than 20% of cases [Lam et al., 2007]. Viral culture, although being carried out routinely in referent laboratories, is less helpful in patient management because of the long turnaround time, and inability to detect fastidious viruses. The lack of a highly sensitive and rapid test to confirm the etiology of acute respiratory infection has caused greater concern since the outbreaks of avian influenza A H5N1 and severe acute respiratory syndrome in Hong Kong in the past. These outbreaks have posed special challenges for paediatric practice in hospitals as suspected cases had to be confined in isolation rooms before the suspected pathogens were exlcuded [Leung et al., 2004]. This aggravated the shortfall of isolation cubicles and also caused great distress to children and their family. Urgent PCR tests for coronavirus and infleunza A H5N1 have since become available for pneumonia cases with severe respiratory distress, and for avian infuenza cases suspected on the ground of history of travelling to endemic areas or contact with birds; but this policy of selective testing is inadequate to cover all the clinical needs.

Along with rapid advances in molecular diagnostic techniques, an increasing number of multiplex PCR assays for respiratory pathogens have been developed by different research groups for early and more comprehensive assessment of viral and bacterial etiologies [Gröndahl et al., 1999; Coiras et al., 2004; Oosterheert et al., 2005; Freymuth et al., 2006; Mahony et al., 2007; Nolte et al., 2007]. The current study employed the multiplex nested PCR assays established recently for the detection of 20 respiratory viruses and atypical bacteria: influenza A (subytpes H1, H3, and H5); influenza B; parainfluenza types 1, 2, 3, and 4; respiratory syncytial virus groups A and B; adenoviruses; human rhinoviruses; enteroviruses; human metapneumoviruses; human coronaviruses OC43 and 229 E and SARS-CoV; *Chlamydophila pneumoniae*; *Legionella pneumophila*; and *Mycoplasma pneumoniae* [Lam et al., 2007]. This study aimed at delineating the spectrum of respiratory viruses and atypical bacteria that accounted for hospitalization of children with acute respiratory tract illnesses, and examined the seasonality and clinical manifestations of these infections.

MATERIALS AND METHODS

Setting and Patients

This prospective study examined children under 5 years old who were admitted to a university-affiliated general hospital (Prince of Wales Hospital) with acute respiratory infections during November, 2005 to October, 2006. The Prince of Wales Hospital is an acute hospital situated at the East New Territories of Hong Kong serving approximate a population of 1.5 million with 15% are under 15 years old. The first 10 children admitted on Monday (or Tuesday if Monday was a public holiday) for suspected acute respiratory tract infections and whose parent provided a written consent were included in this study. The selection of Monday was arbitary. The diagnostic criteria for acute respiratory infection were sudden onset (<36 hr) of one or more of the following symptoms and signs: rhinorrhoea, cough, sore throat, earache, hoarseness, stridor, wheeze, dyspnoea with or without fever.

Sample Collection

Paired nasopharyngeal aspirate and nasal swab samples were taken under strict infection control guidance in a dedicated area by trained nurses. For nasopharyngeal aspirate, a catheter was inserted into one nostril to a depth of 5–7 cm and drawn back while applying gentle suction with an electric suction device [Heikkinen et al., 2002]. For a nasal swab, a Dacron swab with a plastic shaft (Copan, Italy) was placed 1–1.5 cm into the opposite nostril and rotated three times against the surface of the nasal cavity. Both specimens were collected into viral transport medium and kept at 4–10°C until processing further. All specimens were subjected to respiratory virus detection using immunofluorescence, conventional virus culture and multiplex nested PCR.

Immunofluorescence

All specimens were processed within the same of day of collection. Specimens were tested for influenza A, influenza B, parainfluenza (types 1, 2, and 3), respiratory syncytial virus and adenovirus by direct immunofluorescence using specific antibodies (Chemicon, Temecula, CA).

Virus Culture

Virus culture was performed by inoculating approximately 200 μl of specimens onto HEp-2, MDCK, and LLC-MK_2 cell monolayers. After adsorption for 1 hr, the medium was replaced with corresponding maintenance medium, and the tubes were then incubated in a roller drum, at 37°C for HEp-2 cells or 33°C for MDCK and LLC-MK_2 cells. HEp-2 tubes were incubated for 14 days and examined daily for cytopathic effects, while haemadsorption was performed on Day 7 for MDCK and LLC-MK_2 monolayers. The growth of viruses was confirmed by immunofluorescence using virus-specific antibodies.

Viral Nucleic Acid Extraction and cDNA Synthesis

Viral RNA and DNA present in the specimens were extracted by a commercial kit according to the manufacturer's protocol (QIAamp MinElute Virus Spin Kit, Qiagen, Valencia, CA). The RNA template was mixed with 1 μl of random primer (2.5 ng/μl) and 1 μl of dNTPs (0.5 mM each) in a final volume of 10 μl, and heated at 65°C for 5 min. The solution was equilibrated at 4°C and

mixed with 2 U of RNase OUT, 4 μl of 5× First-Strand buffer, 0.5 mM DTT and 10 U M-MLV Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), in a final volume of 20 μl. Reverse transcription was performed at 50°C for 50 min, and then stopped by heating for 15 min at 70°C. cDNAs were stored at −20°C until further analysis.

Multiplex PCR

Five separate multiplex nested PCR assays were used for detection of 20 respiratory viruses and atypical bacteria. Multiplex PCR Group 1 comprised of influenza A and influenza B group-specific, and subtype H1, H3, H5-specific primers. Group 2 comprised of parainfluenza viruses type 1, 2, 3, and 4. Group 3 comprised of respiratory syncytial virus groups A and B, rhinoviruses, and enteroviruses. Group 4 was comprised of human coronaviruses OC43, 229E, SARS-CoV, and human metapneumovirus. Group 5 included *Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, *Legionella pneumophila*, and adenovirus. The details of the assay procedures and performance have been described previously [Lam et al., 2007]. The nested PCR assays were run in a "fast" thermal cycler (Applied Biosystem fast PCR machine, Foster City, CA), which allow the completion of a 35-cycle PCR assay within 35 min, compared to the ∼180 min of ordinary cyclers.

Clinical Information

A standard form for demographic data and detailed history was completed for each patient as soon as they were recruited for the study. Additional laboratory results, information on treatment, clinical course and final diagnosis were retrieved from the medical records. The routine immunofluorescence results for seven common respiratory viruses were available to clinicians within one working day. The multiplex PCR results were retained by the virology laboratory until the end of the study.

RESULTS

Paired samples were collected from 475 children under 5 years old (253 boys, 222 girls) with a mean age of 23.8 months (SD, 17 m). The study population represented 20% of children under 5 years old admitted for acute respiratory infections during the study period. The results of comparing the diagnostic yield between nasopharyngeal aspirates and nasal swabs with respect to different testing methods (immunofluorescence, isolation, multiplex PCR) for influenza A and B, respiratory syncytial virus, parainfluenza 1, 2, and 3, and adenovirus have been described previously [Sung et al., in press]. The following are analyses of the results of the multiplex PCR assays covering all the 20 respiratory pathogens.

Altogether, 47% (225/475) of cases had at least one virus or atypical bacteria detected by the multiplex PCR assays. The positive rate for nasopharyngeal aspirate specimens (43%) was higher than nasal swab specimens (39%), but the difference was not significant ($P > 0.05$ by Chi squared test). A single pathogen was detected in 206 children and two pathogens were detected in 19 children. Influenza A, parainfluenza, and respiratory syncytial virus were the most frequently identified pathogens which accounted for 51% of all positive cases (Table I). Nineteen patients had coinfection with two pathogens. These included three cases with metapneumovirus plus mycoplasma, two cases with adenovirus plus chlamydophila; and the remaining 14 cases had other combinations. Parainfluenza virus, *mycoplasma pneumoniae*, rhinovirus and metapneumovirus were the pathogens found most commonly among coinfections. SARS-CoV and influenza A H5N1 was not detected during the study period. Figure 1 shows the monthly distribution of the number of positive specimen for each pathogen. Influenza A infection peaked in March and June. Influenza B was detected in January, February, and April. Parainfluenza virus was prevalent throughout the year except from April to June. Respiratory syncytial virus was also prevalent throughout the year but was more common from February to September. Adenovirus showed multiple peaks, and was more active from November to April. Both rhinovirus and coronavirus OC43 were detected more often in winter and early spring. *Mycoplasma pneumoniae* peaked at March.

The clinical manifestations and discharge diagnoses of the 225 children with respiratory pathogens identified are shown in Table II. More than one third of the cases had a principal diagnosis of upper respiratory tract infection on discharge, four cases had croup, and 44 (20%) cases had lower respiratory tract infections (14 cases with pneumonia, 30 with bronchiolitis). Twenty-six (12%) cases had acute gastrointestinal symptoms as the main feature of their illness. Thirteen cases (6%) had febrile convulsions and eight cases (4%) had asthma exacerbation in association with the acute respiratory infection. None of the patients required intensive care or ventilatory support. The duration of fever before admission was 3.4 ± 3.2 days, the temperature on admission was 38.7 ± 0.9°C. The total duration of fever was 4.74 ± 2.54 days. In general, children infected with adenovirus, influenza A and mixed viruses had higher temperatures; whereas those infected with respiratory syncytial virus had a lower degree of fever.

DISCUSSION

This study used multiplex PCR assays to assess the role of 20 respiratory viruses and atypical bacteria as a cause of hospital admission for children with suspected acute respiratory infections in Hong Kong. These multiplex PCR assays have been shown to provide a much greater sensitivity compared to immunofluorescence and conventional virus isolation [Lam et al., 2007; Sung et al., in press]. This improved sensitivity together with the broad coverage allows a more precise evaluation on the relative importance of these pathogens in Hong Kong.

TABLE I. Detection of Respiratory Viruses and Atypical Bacteria in 475 Paired Nasal Swab and Nasopharyngeal Aspirate Specimens by Multiplex PCR Assays

	Nasopharyngeal aspirate (+), nasal swab (−)	Nasopharyngeal aspirate (−), nasal swab (+)	Nasopharyngeal aspirate (+), nasal swab (+)	Any +ve (% of all cases)
Single infection				
Adenovirus	5	0	18	23 (4.8%)
Influenza A	3	5	26	34 (7.2%)
H1	2	4	24	
H3	1	2	1	
H5	0	0	0	
Influenza B	2	2	12	16 (3.4%)
Parainfluenza (PIV)	7	3	32	42 (8.8%)
PIV1	2	0	17	
PIV2	2	0	4	
PIV3	2	2	10	
PIV4	1	1	1	
Respiratory syncytial virus (RSV)	10	2	28	40 (8.4%)
RSV A	9	2	18	
RSV B	1	0	10	
Rhinovirus	2	0	15	17 (3.6%)
Coronavirus	1	1	16	18 (3.8%)
OC43	1	1	14	
229E	0	0	2	
SARS	0	0	0	
Metapneumovirus	2	3	2	7 (1.5%)
Enterovirus	1	1	0	2 (0.4%)
Legionella	0	0	0	
Mycoplasma	3	2	2	7 (1.5%)
Chlamydophila	0	0	0	
Coinfection	4	2	13	19 (4.0%)

Positive yield for nasal swab: 185/475 = 39%.
Positive yield for nasopharyngeal aspirate: 202/475 = 43%.
Positive yield either for nasal swab or nasopharyngeal aspirate: 225/475 = 47%.

In this study, one or more respiratory pathogen(s) was found in 47% of cases. This positive rate was about twice of those based on conventional methods [Lam et al., 2007]. In concordance with previous reports [Druce et al., 2005; Weigl et al., 2007], respiratory syncytial virus, influenza A, and parainfluenza virus were identified most frequently, and accounted for 51% of positive cases in the current study population. Respiratory syncytial virus and parainfluenza virus were the predominant viruses detected in infants younger than 6 months old, whereas 39% of adenovirus infections occurred in children between 3 and 4 years old. Influenza A were evenly distributed in children between 1 and 5 years old. Other viruses identified more frequently included influenza B, coronavirus OC43 and rhinoviruses; each accounted for 7–8% of positive cases. Mycoplasma and human metapneumovirus each accounted for about 3% of positive cases. Enterovirus, reported to be circulating widely in children [Juvén et al., 2000; Tsai et al., 2001], was identified in three cases only (two single and one coinfection). A previous local study on the viral etiology of acute respiratory infections in children carried out from August 2001 to July 2002 using RT-PCR for the detection of rhinovirus, metapneumovirus and coronavirus; and immunofluorescence and culture for other respiratory viruses showed slightly lower positive rates for adenovirus (5% vs. 3%), influenza B (3% vs. 1%), parainfluenza (9% vs. 6%) and respiratory syncytial virus (8% vs. 7%). The positive rates for influenza A and coronavirus were similar, whereas the positive rate for rhinovirus (4% vs. 35%) was remarkably higher in the previous study [Cheuk et al., 2007]. This discrepancy of

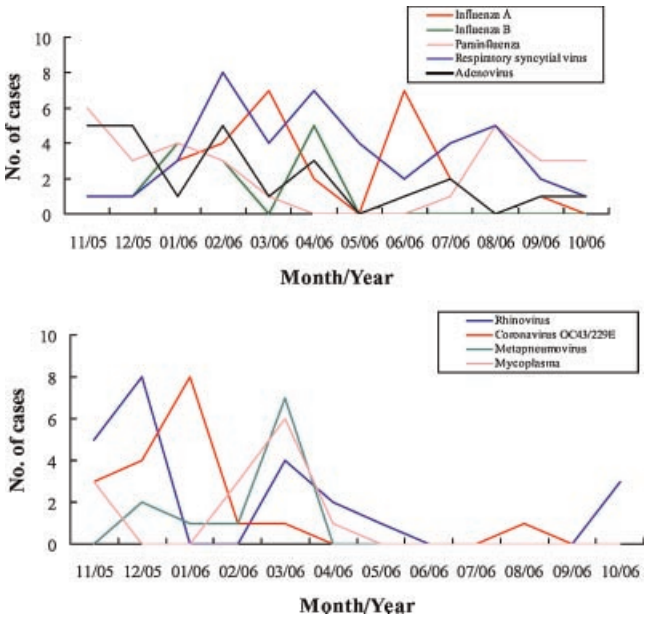

Fig. 1. Seasonal distribution of hospitalization for acute respiratory infections caused by different viruses and atypical bacteria.

TABLE II. Comparison on Clinical Manifestations of 225 Children With Single or Coinfection With Respiratory Viruses and/or Atypical Bacteria

Characteristics	Adeno-virus (n = 23)	Influenza A (n = 34)	Influenza B (n = 16)	Para-influenza (n = 42)	Respiratory syncytial virus (n = 40)	Rhinovirus (n = 17)	Corona-virus (n = 18)	Metapneumo-virus (n = 7)	Mycoplasma (n = 7)	Enterovirus (n = 2)	Co-infection (n = 19)
Age											
<6 m	0	0	1	7	12	0	0	0	0	1	1
6–11.99 m	2	1	1	9	4	3	2	2	2	0	4
1–1.99 y	2	8	3	11	7	4	8	1	2	1	4
2–2.99 y	2	6	0	8	11	4	6	2	1	0	5
3–3.99 y	9	5	2	3	4	2	1	1	2	0	2
4–4.99 y	5	7	5	4	1	3	0	1	0	0	1
5–5.99 y	3	7	4	0	1	1	1	0	0	0	2
Principle discharge diagnosis											
Upper respiratory tract infection	17	21	10	23	15	8	11	3	3	0	9
Croup	0	1	0	3	0	0	0	0	0	0	0
Pneumonia	1	2	1	2	3	1	1	0	1	0	2
Bronchiolitis	1	1	0	3	18	2	2	1	0	1	1
Viral infection	0	2	1	0	0	0	0	0	0	0	0
Gastroenteritis	1	4	2	4	2	3	4	1	1	1	3
Asthma	1	1	1	2	0	2	0	0	1	0	0
Febrile convulsions	2	2	1	3	2	1	0	0	1	0	1
Fever	0	0	0	2	0	0	0	2	0	0	3
Others	0	0	0	0	0	0	0	0	0	0	0
Highest temperature (°C)											
<38	0	3	1	2	12	5	5	2	2	0	1
38–39	0	3	4	10	10	3	3	1	0	2	3
>39	23	28	11	30	18	9	10	4	5	0	15
Total duration of fever	6.5 ± 3.9	5.0 ± 3.0	5.2 ± 2.5	4.8 ± 2.7	4.3 ± 1.9	4.8 ± 1.9	3.1 ± 1.0	4.2 ± 1.7	4.6 ± 2.0	1.5 ± 0.7	4.4 ± 1.5
Antibiotic given before admission	4	4	3	8	5	1	3	2	1	1	3
Antibiotic given during hospitalization	4	2	2	10	7	3	1	2	2	1	2

m, month; y, year.

rhinovirus detection rates might be due to differences in the sensitivity of PCR tests used. Nevertheless, both studies continued for only 1 year and the seasonal pattern of respiratory viral activities might vary in different years. Further prospective studies of longer duration are necessary to elucidate the epidemiology and disease burden associated with rhinovirus in Hong Kong.

With the exception of the strong association between respiratory syncytial virus infection and bronchiolitis, and between parainfluenza and croup; the clinical manifestations of other viral infections were largely nonspecific with fever and cough as the main symptoms. Among the 225 patients with positive results, 26 (12%) cases had prominent gastrointestinal symptoms, which appeared to be overrepresented (25%) in human coronavirus OC43 infections. This is in agreement with a previous report that gastrointestinal symptoms were common in human coronavirus OC43 infections [Vabret et al., 2001]. Febrile convulsions and asthmatic attacks were observed in 6% and 4% of positive cases respectively, which were in line with previous reports [Grünberg et al., 2001; Chung and Wong, 2007]. Notably, more rhinoviruses were detected between October and December which are peak months of asthma admissions in Hong Kong. In this study, 2 of 17 patients with rhinovirus infection had asthmatic attack as the principal diagnosis of their admission. However, the number of rhinovirus positive asthma cases was too small to allow further evaluation on the association. In line with a previous report [Lin et al., 2007], the current study revealed that adenovirus, influenza A and mixed viral infections were associated with higher fever than other single viral infections.

Rapid identification of the aetiology of acute respiratory infections is essential for better decisions on the usage of antiviral agents and antibiotics, and to implement proper isolation to prevent transmission [Woo et al., 1997; Oosterheert et al., 2005]. In this regard, the rapid PCR system used in this study can reduce the time required for a single round of PCR from 3 hr to 35 min, and therefore the whole testing process can be completed within 1 day. This rapid turnaround time may alleviate the anxiety of the parents, and potentially decrease the duration of hospital stay and the need for isolation cubicles. Nevertheless, it should be noted that this gain in turnaround time can only be achieved when the rapid PCR system is available on site at the hospital or clinic.

While multiplex PCR assays have major advantages, their sensitivities and specificities are in general inferior to the equivalent monoplex PCR assays. This technical hurdle has to be overcome before multiplex PCR can be recommended for routine clinical use. Under certain circumstances for examples, pathogens of great public health concern including SARS-CoV and avian influenza, a positive multiplex PCR result requires further testing with a supplementary assay to ascertain the infection status.

A practical concern about routine use of multiplex PCR assays is the cost which can however be justified in view of the devastating economic and human loss sustained during the outbreaks of H5N1 influenza in 1997 and SARS in 2003, and the increasing concern about repeated unexpected outbreaks of other viral infections in Hong Kong [Yuen et al., 1998; Chan, 2002; Lee et al., 2003]. The importance of establishing a sustainable surveillance system for monitoring circulating respiratory pathogens, disease burden, and economic impact of a wide range of respiratory infections has become increasingly recognized. The results of this study support that multiplex PCR assays with expanded detection spectrum are valuable tools for surveillance which could be performed at one or more sentinel hospitals and government or private clinics.

One limitation of the current study is that bacterial culture of nasopharyngeal aspirate or sputum was not performed systematically except when a bacterial aetiology was suspected clinically. So it is possible that the clinical manifestations in some cases might have been contributed in part by co-existing bacterial infections.

In conclusion, the data generated from this study have expanded the knowledge of the epidemiology of respiratory viral infections in children in Hong Kong. The management of children with severe acute respiratory infections in hospital, and surveillance for respiratory viral infections in the community could be greatly enhanced with the use of multiplex PCR assays.

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