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Molecular Viral Epidemiology and Clinical Characterization of Acute Febrile Respiratory Infections in Hospitalized Children in Taiwan

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Acute respiratory infection (ARI) is a leading cause of morbidity and hospitalization in children. To profile the viruses causing ARI in children admitted to a community-based hospital in central Taiwan, a cross-sectional study was conducted on children under 14 years of age that were hospitalized with febrile ARI. Viral etiology was determined using conventional cell culture and a commercial respiratory virus panel fast assay (xTAG RVP), capable of detecting 19 different respiratory viruses and subtype targets. Demographic, clinical, and laboratory data were recorded and analyzed. The RVP fast assay identified at least one respiratory virus in 130 of the 216 specimens examined (60.2%) and rose to 137 (63.4%) by combining the results of cell culture and RVP fast assay. In order of frequency, the etiological agents identified were, rhinovirus/enterovirus (24.6%), respiratory syncytial virus (13.8%), adenovirus (11.5%), parainfluenza virus (9.2%), influenza B (8.4%), influenza A (5.4%), human metapneumovirus (4.6%), human coronavirus (2%), and human bocavirus (2%). Co-infection did not result in an increase in clinical severity. The RVP assay detected more positive specimens, but failed to detect 6 viruses identified by culture. The viral detection rate for the RVP assay was affected by how many days after admission the samples were taken ($P = 0.03$). In conclusion, Rhinovirus/enterovirus, respiratory syncytial virus, and adenovirus were prevalent in this study by adopting RVP assay. The viral detection rate is influenced by sampling time, especially if the tests are performed during the first three days of hospitalization. ***J. Med. Virol. 87:1860–1866, 2015***.

KEY WORDS: acute respiratory infection; children; epidemiology; respiratory viruses; xTAG RVP fast assay

INTRODUCTION

Acute respiratory tract infection (ARTI) is a long-standing public health issue and the most common reason for doctor visits and hospitalization of young children, and is a major contributor to morbidity and mortality in that age group of the population [Aramburo et al., 2011; Bezerra et al., 2011; Sung et al., 2011; Garcia-Garcia et al., 2012]. Viruses are one of the major causes of ARTIs, and accurate and timely identification of the responsible viral pathogens would delineate their epidemiology and clinical significance and reduce unnecessary antimicrobial use [Garcia-Garcia et al., 2012].

In the past, detection of respiratory viruses relied on virus isolation by cell culture and antigen testing. However, in addition to influenza virus (FLU), respiratory syncitial virus (RSV), adenovirus (ADV), and

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parainflenza virus (PIV), an increasing number of newly discovered viruses, such as human metapneumovirus (HMPV), human coronaviruses (HCoV) NL63, HKU1, and human bocavirus (HBoV), have been recognized as possible viral pathogens in ARTI. Because these viruses are not routinely cultivated in clinical laboratories, molecular detection utilizing high throughput multiplex PCR assays has been developed and widely applied in the past decade [Freymuth et al., 2006; Lee et al., 2007; Khamis et al., 2012]. Using molecular detection technologies, simultaneously detect a broad spectrum of respiratory viruses is feasible but awaits further validation to better understand their roles in diagnosing respiratory infections.

This study aimed (1) to profile the viruses causing acute respiratory infections in children admitted to a community-based hospital in central Taiwan using both conventional virus isolation and the xTAG respiratory virus panel (RVP) fast assay; (2) to investigate the significance of viral co-infections

MATERIALS AND METHODS

Patients

This study was conducted prospectively at Chang Bing Show Chwan Memorial Hospital in Taiwan between May 2010 and July 2011. All children below 14 years of age admitted to the pediatric department ward with fever and ARI symptoms were eligible for enrollment. The inclusion criteria were (1) within 7 days after hospitalization, (2) not receiving rapid influenza (flu) test or negative for flu teat was enrolled (A rapid test for influenza A and B antigens was frequently applied in our routine clinical practice.), and (3) fever $\geqq 38^{\circ}C$ on the sampling day. Patients with malignancy, metabolic or genetic diseases, immunodeficiency were excluded. This protocol was approved by the Institutional Review Board of Chang Bing Show Chwan Memorial Hospital (No. IRB-1000505).

An informed consent form signed by parents or guardians was obtained for each enrolled patient, and two sequential throat swabs were taken by a pediatrician or physician's assistant and placed in liquid transport medium (COPAN, Brescia, Italy). One sample was subjected to molecular viral detection, and the other to conventional viral culture. All samples for molecular detection were stored at $-70^{\circ}C$ before batch analysis. Demographic data, clinical features, and laboratory results for all the enrolled patients were collected and analyzed by medical chart review.

Nucleic Acid Extraction

Nucleic acids were extracted from stock clinical samples using QiaAmp Viral RNA/DNA Mini Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions. The extracted DNA (or RNA) was eluted with 140 μl of elution buffer and stored at $-70^{\circ}C$ until further analysed.

Respiratory Virus Detection

A commercial kit, xTAG RVP Fast Assay, Version 2.0 (Luminex Molecular Diagnostics Inc., Toronto, Canada), was used for respiratory viral detection. This kit allows simultaneous screening for 19 common respiratory viral and subtype targets, including influenza A virus (Flu A), with additional subtyping of positive specimens into subtypes H1, H3, and 2009 H1N1v, influenza B virus (Flu B), respiratory syncytial virus (RSV) types A and B, human coronaviruses (HCoVs, NL63, 229E, OC43, and HKU1), parainfluenza viruses (PIV) types 1–4, human metapneumovirus (HMPV), picornaviruses (including rhinoviruses and enteroviruses, RV/EV), human bocavirus (HBoV), and adenovirus (ADV). The assay includes RNA bacteriophage MS2 as an internal extraction/inhibition control, and DNA bacteriophage lambda as an amplification and assay performance control. It was carried out using the xMAP 100 IS instrument (Luminex Molecular Diagnostics Inc.), and analyzed with TDAS RVP FAST software (version 2.0, Abbott Molecular).

Statistical Analysis

Continuous variables were analyzed using the independent *t*-test and one way ANOVA, and categorical variables by the χ^2 test. All comparisons were two-tailed, and *P*-values less than 0.05 were considered statistically significant. Odd ratios and 95% confidence interval (CI) of antibiotic usage and intensive care during hospitalization were calculated by multivariate logistic regression. All statistical analyses were carried out with the SPSS software package (PAWS Statistics 18.0.0; IBM Corporation, Somers, NY)

RESULTS

Overview of Demographic and Clinical Characteristics

Two hundred and sixteen febrile children hospitalized with acute respiratory infections were enrolled from May 2010 to July 2011 after obtaining informed consent from their parents or guardians. Overall demographic data, laboratory and clinical findings, and final etiological pathogens as determined by RVP are shown in Table I and Figure 1. The mean age was 3.5 years, and the sex ratio (male–female, M:F) was 125:91. The mean age was significantly lower in the acute bronchiolitis group (1.9 ± 1.6 years, $P < 0.001$). The clinical diagnoses included 76 (35.2%) of upper respiratory tract infection, 46 (22.2%) of acute tonsillitis, 46 (21.3%) of acute bronchiolitis, and 48 (22.2%) of lower respiratory tract infection (LRTI). The most common clinical presentations were, in

TABLE I. Demographic Data, Laboratory Results, Clinical Symptoms, and Viral Etiology for Each Category of Respiratory Infections

Diagnosis	URI	Acute tonsillitis	Acute bronchiolitis	LRI	Total
No.	76	46	46	48	216
Age (year; mean ± SD)	4.1 ± 3.3	4.4 ± 2.8	1.9 ± 1.6*	3.4 ± 2.2	3.5 ± 2.8
M:F	49:27	28:18	26:20	22:26	125:91
Laboratory results and clinical symptoms					
WBC (mean ± SD)	12,366 ± 12,892.4	13,178 ± 5,879.2	11,904.4 ± 6,886.1	100,018.8 ± 5,399.2	11,917.6 ± 9,095.4
Neutrophil count (mean ± SD)	7,770.2 ± 7,695.3	8,592.9 ± 4,758.1	6,621.0 ± 5,459.9	5,433.4 ± 3,295.6	7,182.9 ± 5,954.7
CRP (mg/dl)	2.7 ± 4.3	5.0 ± 4.0*	1.9 ± 2.1	2.5 ± 3.8	3.0 ± 3.9
Rhinorrhea	61.8%	54.3%	78.2%	79.2%	67.6%
Cough	65.8%	67.4%	97.8%	97.9%	80.1%
Wheezing	0%	2.2%	79.3%*	33.3%	23.6%
Dyspnea	1.3%	0%	34.8%	27.1%	13.9%
GI symptoms	26.3%	19.6%	8.7%	22.9%	20.4%
Conjunctivitis	9.2%	4.3%	2.2%	8.3%	6.5%
Skin rashes	10.5%	4.3%	6.5%	4.2%	6.9%
Antibiotic use During hospitalization	30.3%	34.8%	47.8%	68.8%	43.5%

SD, standard deviation; Gastrointestinal (GI) symptoms, the presence of vomiting and/or diarrhea.
*$P < 0.001$.

descending order, cough (80.1%), rhinorrhea (67.6%), wheezing (23.6%), and gastrointestinal symptoms (20.4%). The mean duration of hospital stay was 6.5 days. The laboratory findings for the different clinical categories were generally similar, with the overall mean white blood cell count (WBC) of 11,918/μl, neutrophil count of 7,183/μl, and serum C-reactive protein (CRP) level of 3.0 mg/dl. But the CRP levels were significantly higher in the acute tonsillitis group ($P < 0.001$).

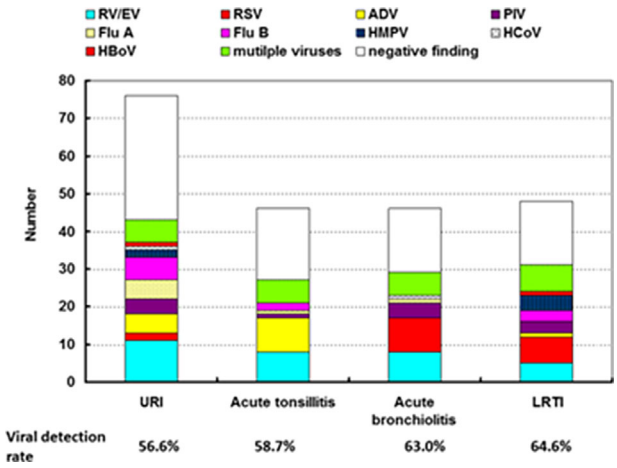

Fig. 1. Viral etiology distribution of each one clinical diagnosis category detected by xTAG RVP fast assay. The viral etiology distribution and detection rate using xTAG RVP fast assay were shown in each clinical diagnosis category. ADV, human adenovirus; HBoV, human bocavirus; HCoV, human coronavirus 229E/NL63/OC43; FLU A, influenza A virus; FLU B, influenza B virus; HMPV, human metapneumovirus; PIV, human parainfluenza virus 1 2, 3, 4; RV/EV, human rhinovirus/enterovirus; RSV, human respiratory syncytial virus.

Overall percentage of antibiotic administration during hospital stay was 43.5% (94/216), but it varied for each clinical group (Table I). Non-respiratory infection was identified in 4 of 94 cases using antibiotics (one of Salmonella bacteremia and three of urinary tract infection), and the four cases were excluded in the calculation of risk factors of antibiotic usage. Potential risk factors related to a higher rate of antibiotic use including intensive care, clinical diagnoses, laboratory analysis of white cell count and CRP level, and viral co-infection were analyzed by logistic regression. The significant factors were shown as in order: (1) CRP ≧ 4.0 ($P < 0.001$; odds ratio = 3.99; 95%CI = 1.9–8.4; (2) LRTI group including acute bronchiolitis ($P < 0.001$; odds ratio = 3.3; 95%CI = 1.8–6.5); (3) viral co-infection group ($P = 0.04$; odds ratio = 2.7; 95%CI = 1.0–7.2), and (4) fever persisting more than 4 days after admission ($P = 0.04$; odds ratio = 2.6; 95%CI = 1.0–6.8)

Distribution of Respiratory Viruses With Known Etiology

Using the xTAG RVP fast assay, at least one viral pathogen was identified in 130 of the 216 patients (60.2%). The distribution of single viral etiology was, in descending order, RV/EV (32, 24.6%), RSV (18, 13.8%), ADV (15, 11.5%), PIV (12, 9.2%), Flu B (11, 8.4%), Flu A (7, 5.4%), HMV (6, 4.6%), HCoV (2, 1.5%), and HBoV (2, 1.5%). The percentage of each viral etiology of each clinical category was shown in Figure 1. Picornavirus (RV/EV) was the predominant virus in the URTI group (14.4%), and ranked the second in the tonsillitis (17.4%) and bronchiolitis groups (17.4%). Adenovirus predominated in the tonsillitis group (19.5%), and RSV was the virus most frequently detected in the bronchiolitis group

(19.5%). In contrast, 71 of 216 specimens (32.9%) showed positive results by conventional viral culture, whereas at least one viral pathogen could be found in 63.4% of specimens by combining cell culture and RVP fast assay data.

Comparison of Cell Culture and RVP Fast Assays

The performance of the two assay methods is summarized in Table II. There was good agreement in the detection ADV, enterovirus, and Flu A. In general, the RVP fast assay detected more pathogens than conventional viral isolation (60.2% vs. 32.9%), but occasionally failed to detect Flu B (5 of 12; sensitivity, 58.3%; specificity, 98.1%), some RSV (1 of 5; sensitivity, 80%; specificity, 91%) and PIV clinical strains (1 of 11; sensitivity, 90.9%; specificity, 96.6%). The viral detection rate by conventional viral isolation was significantly higher for URTI than LRTI (38.5% vs. 25.5%; $P = 0.04$), but not for RVP fast assay. To assess the influence of sampling time, for the reduction in viral load after 3 days of hospitalization has been reported [Franz et al., 2010], the detection rate of RVP fast assay or conventional cell culture was analyzed. The viral detection rate for RVP fast assay, but not for conventional cell culture, was statically different between time of sampling less than 3 days of hospital admission and greater than 3 days ($P = 0.03$, Fig. 2). On the other hand, other factors, including age, gender, and body temperature on the sampling day, did not affect the viral detection rate for either method ($P > 0.05$).

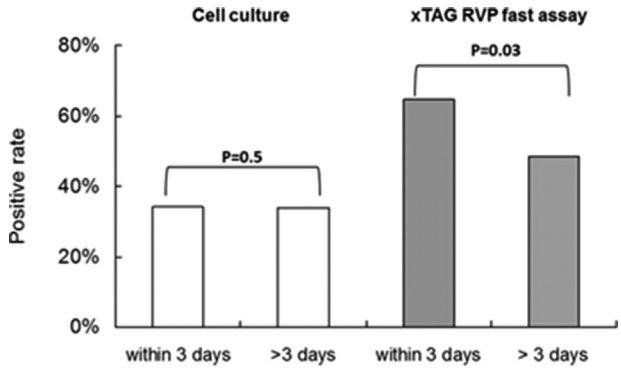

Fig. 2. Viral detection rate by xTAG RVP fast assay was influenced by sampling day after admission. Influence of sampling day after admission on viral identification rate. The viral positive identification rate by xTAG RVP fast assay was significantly influenced by the sampling day after admission (64.6% vs. 48.3%, $P = 0.03$), but not by cell culture (34.2% vs. 33.9%, $P = 0.5$). Three days after admission was served as a cut-off.

Incidence of Viral Co-Infections

More than one virus was detected in 19.2% cases (25/130). The most frequently detected viruses in co-infections were picornavirus (RV/EV) (15; 60%), ADV (11; 44%), and HMPV (5; 20%), and the most common viral pairs were ADV/picornavirus (n = 4) and PIV-1/picornavirus (n = 3). There were no significant differences in terms of clinical symptoms, severity, and laboratory findings between patients with single or multiple viral infections (Table III), except for the percentage with rhinorrhea (61.3% vs. 87.5%, $P = 0.016$) and the percentage receiving antibiotics (37.7% vs. 62.5%, $P = 0.039$). Although there was no significant difference of intensive care between single or multiple viral infection groups ($P = 0.46$), the risk factors contributing the intensive care were: age $\leqq 2$ years ($P = 0.032$, odds ratio = 6.1; 95%CI = 1.1–32.3) and presence of dyspnea ($P < 0.001$, odds ratio = 22.4; 95%CI = 4.9–103.3), respectively.

TABLE II. Comparison of Conventional Cell Culture and xTAG RVP Fast Assay

Cell culture		xTAG RVP fast assay		
Virus	No.	No. of agreement results	Additional co-infected viruses (No.)	Discordance (No.)
Adenovirus	20	19	CoV (3); RV/EV (2); HMPV (1)	Read as PIV 3 (1)
Coxsakievirus B5	1	1	0	0
Echovirus	4	4	PIV 1 (1)	
Enterovirus	4	4	ADV (1)	
Flu A	7	7 (H3: 5)	HBoV (1)	
Flu B	12	7		Not detected (5)
HSV-1	7	0		ADV (2); RV/EV(3)
PIV	11	10	ADV (1); RV/EV (2); Flu A3 (1)	Not detected (1)
RSV	5	4	RV/EV (2)	Not detected (1)
Negative finding	145	75		Extra víruses: ADV (5); RV/EV (31); RSV (19); HBoV(4); HCoV(3); Flu A3(1); Flu B(4); HMPV(10); PIV (6)
Positive rate	32.9%		60.2%	

TABLE III. Comparison of Clinical Characteristics of Single and Multiple Viral Infections

	Single virus infection (n = 105)	Multiple virus infection (n = 25)	*P*-value
White cell count (/μl)	11,810.4 ± 6,770.5	12,304.2 ± 5,541.1	0.740
Neutrophil count (/μl)	7,163.6 ± 5,227.7	7,912.9 ± 4,836.0	0.529
CRP (mg/dl)	2.6 ± 3.5	3.1 ± 2.9	0.544
Duration of hospital stay (day)	6.3 ± 2.6	6.2 ± 1.5	0.809
Rhinorrhea	61.3%	87.5%	0.016*
Cough	80.2%	87.5%	0.564
Wheezing	28.3%	29.2%	1.000
Dyspnea	15.1%	20.8%	0.541
GI symptoms	21.7%	12.5%	0.404
Conjunctivitis	5.6%	16.7%	0.087
Intensive care	11.3%	4.2%	0.46
Antibiotic use during hospitalization	37.7%	62.5%	0.039*

*$P < 0.05$.

DISCUSSION

Acute respiratory infection is a leading cause of hospitalization and morbidity in children. The profile of respiratory viral infection is expanding with advances in molecular diagnosis. In the present study, we conducted a cross section surveillance of viral pathogens in children admitted for ARTI at a community hospital in the rural area of central Taiwan. Of the 216 enrolled cases, at least one viral etiology could be identified in more than half (137, 63.4%) of the cases by combining the results of a commercial multiplex NAT assay and conventional cell culture. Our results are in line with previous studies that identified viral etiology in 34.6–83% of cases [Ren et al., 2009; Niang et al., 2010; Shafik et al., 2012; Marcone et al., 2013; Rhedin et al., 2014]. Of the respiratory viruses analyzed, the three most common pathogens were, in descending order, RV/EV (24.6%), RSV (13.8%), and ADV (11.5%). Viral co-infection was not uncommon (19.2%), with the most common viral pair being ADV and RV/EV (n = 4). Viral detection rates for upper and lower respiratory infections were comparable in this study, whereas Ambrosioni et al. reported a lower rate for lower respiratory infections in a pediatric population [Ambrosioni et al., 2014]. These discrepancies might reflect differences in the profile of respiratory viruses in the study populations and/or the differences in the sensitivity and/or limitations of the viral detection techniques applied.

Although RSV had been reported as the most common virus detected in many investigations, especially in children under 5 years old [Bonzel et al., 2008; Bezerra et al., 2011; Do et al., 2011; Garcia-Garcia et al., 2012], this study, using molecular diagnostics, found RV/EV to be the most common virus, in agreement with other observations, stressing the prevalence and importance of picornavirus in upper and lower respiratory tract infections [Fabbiani et al., 2009; Iwane et al., 2011; Guerrier et al., 2013; Renois et al., 2013]. Furthermore, rhinovirus (belonging to RV/EV) infection had been demonstrated to be associated with the severity of bronchiolitis[Ricart et al., 2013]. For acute tonsillitis our findings were consistent with those of Hsieh et al. in that adenovirus and enterovirus were the two most common viral etiologies, accounting for half of the known pathogens[Hsieh et al., 2011].

Viral co-infection is frequently encountered in childhood ARTIs (18–42.5% of the study populations), but the predominant co-infecting viral pathogens vary from different studies [Bharaj et al., 2009; Do et al., 2011; Kouni et al., 2013] and the clinical significance of co-infections remains controversial. In the present study, the differences between infections by single or multiple pathogens are not significant in terms of clinical presentation, severity, and laboratory results, except for the higher rate of rhinorrhea and the use of antibiotics. Although some studies found that multiple viral infections were associated with higher fever, higher rate of hospitalization, more severe disease, and more frequent use of antibiotics [Calvo et al., 2008; Kouni et al., 2013], our findings are more consistent with previous studies that the presence of multiple pathogens showed no association with the severity of clinical manifestations [Martin et al., 2012; Rhedin et al., 2014].

Because conventional cell culture is generally not able to allow timely management of respiratory tract infections, multi-target nucleic acid amplification platforms have became preferred tools for the detection of respiratory pathogens, with the advantages of swiftness, high diagnostic frequency, and specificity. Several multiplex tests are currently available, including the xTAG RVP fast assay used in this study. For most respiratory viruses, the performance of xTAG RVP fast assay was comparable or superior to real time PCR and conventional methods [Gadsby et al., 2010; Gharabaghi et al., 2011]. In the present study of 216 inpatients, we not only identified 105 (48.6%) cases with a single viral pathogen by the xTAG RVP fast assay, but an

additional 25 (11.6%) co-infections. However, reduced sensitivity in the detection of ADV, Flu B, PIV and HCoV by the xTAG RVP fast assay, especially at low viral concentrations, requires careful interpretation, which accords with other studies[Gharabaghi et al., 2011; Pillet et al., 2013].

To optimize the sampling conditions, we demonstrated that, unlike cell culture, the viral detection rate for the xTAG RVP fast assay was influenced by the sampling date after the patient admission. The reduction in viral load after 3 days of hospitalization may be due to clinical improvement, and this may contribute to the reduced rate of viral detection by the xTAG RVP fast assay when sampling is delayed [Franz et al., 2010]. For optimal viral detection, it is recommended that samples be collected within 3–5 days of symptom onset.

This study has some limitations: (1) the major limitation of this study was the selection bias: patients positive in flu antigen tests were excluded. Therefore, the real rate of influenza infection would be underestimated. But we still detected 5.4% of flu A and 8.4% of flu B infection in this study, mostly among URI group. (2) The sampling time varied from the 1st to the 7th day following admission. The positive viral detection rate was then influenced by the progressive decline in viral load. This was ameliorated by testing the samples by both cell culture and molecular detection methods; (3) the period of study was not long enough to investigate seasonal variations, and the enrolled sample size was moderate to determine the impact of individual types of respiratory virus on clinical severity; (4) this study was conducted in a rural community-based hospital, where the viral prevalence pattern has been shown to be different from that in urban pediatric populations [Gern et al., 2012]. Our finding of the viral distribution profile in LRTI group was similar to southern Taiwan, but different from northern Taiwan [Sung et al., 2011; Chou et al., 2014]. With respective of LRTI, RSV and EV/RV have the equal role in this study. In addition, this study population did not enroll critical or immunocompromised patients

In conclusion, this study provides a background viral epidemiology for children hospitalized with febrile ARI in a community-based hospital. Picornaviruses, RSV, and ADV were the most frequently detected viruses. Viral co-infection was frequently observed, but was not associated with clinical disease severity. The introduction of molecular detection methods, if conducted early after admission, is beneficial for the early identification of the causative viral pathogens, even with co-infections. Molecular respiratory viral diagnostic techniques have broadened the understanding of the etiological ARTI profile. Further study is warranted, focusing on the impact of introducing molecular diagnoses in reducing hospital stays and/or unnecessary antimicrobial use.

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