

# Viruses Associated With Influenza-Like-Illnesses in Papua New Guinea, 2010

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Influenza-like-illness can be caused by a wide range of respiratory viruses. The etiology of influenza-like-illness in developing countries such as Papua New Guinea is poorly understood. The etiological agents associated with influenza-like-illness were investigated retrospectively for 300 nasopharyngeal swabs received by the Papua New Guinea National Influenza Centre in 2010. Real-time PCR/RT-PCR methods were used for the detection of 13 respiratory viruses. Patients with influenza-like-illness were identified according to the World Health Organization case definition: sudden onset of fever ( $>38^{\circ}\text{C}$ ), with cough and/or sore throat, in the absence of other diagnoses. At least one viral respiratory pathogen was detected in 66.3% of the samples tested. Rhinoviruses (17.0%), influenza A (16.7%), and influenza B (12.7%) were the pathogens detected most frequently. Children  $<5$  years of age presented with a significantly higher rate of at least one viral pathogen and a significantly higher rate of co-infections with multiple viruses, when compared to all other patients  $>5$  years of age. Influenza B, adenovirus, and respiratory syncytial virus were all detected at significantly higher rates in children  $<5$  years of age. This study confirmed that multiple respiratory viruses are circulating and contributing to the presentation of influenza-like-illness in Papua New Guinea. **J. Med. Virol.** 86:899–904, 2014 © 2013 Wiley Periodicals, Inc.

**KEY WORDS:** respiratory; influenza; virus; Papua New Guinea

## INTRODUCTION

Influenza-like-illnesses are a major cause of morbidity and mortality worldwide, particularly in developing countries where the majority of deaths due to respiratory illnesses occur [Williams et al., 2002]. Influenza viruses are the predominant pathogens

involved with the presentation of influenza-like-illness with the World Health Organization (WHO) estimating that 5–10% of the world's population are infected with influenza each year, resulting in 250,000–500,000 deaths annually [WHO, 1999]. However, the clinical symptoms of an influenza infection are difficult to distinguish from infections with other respiratory viruses such as respiratory syncytial virus (HRSV), parainfluenza viruses 1–3 (HPIV-1, HPIV-2, and HPIV-3), adenovirus (HAdV), coronaviruses (HCoV-229E, HCoV-OC43, HCoV-NL63, and HCoV-HKU1), metapneumovirus (HMPV), and rhinovirus (HRV). Nevertheless, it is important to distinguish these infections to monitor influenza epidemiology and vaccine efficacy.

Children in particular are greatly impacted by influenza-like-illness. Acute respiratory infections are the leading cause of infectious-related deaths in children worldwide. However, the majority of these deaths occur in children in developing countries [Williams et al., 2002]. The differing etiologies of respiratory illnesses in young children and adults is poorly understood, although it is well known that some viruses such as HRSV are much more likely to cause severe disease in children  $<5$  years [Ogra, 2004]. In Papua New Guinea acute respiratory infections have a high burden, with pneumonia being the most common cause of hospitalization and death in children  $<5$  years of age [National Health Plan, 2010]. The high carriage rate of *Streptococcus pneumoniae* in this population [Francis et al., 2009] and the absence of vaccination against seasonal influenza or *S. pneumoniae* undoubtedly contribute

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to the high morbidity and mortality due to acute respiratory infections.

Due to the poor diagnostic facilities in developing nations such as Papua New Guinea, disease classification is generally based upon clinical diagnoses. Consequently, the etiology and epidemiology of respiratory illnesses is poorly characterized in these settings. The advent and continued improvement in molecular diagnostic methods have revolutionized the detection and characterization of viral respiratory pathogens. The aim of this study was to retrospectively investigate alternative viral causes of influenza-like-illness in samples received by the Papua New Guinea National Influenza Centre during 2010.

## MATERIALS AND METHODS

### Sample Collection and Inclusion Criteria

Retrospective analysis was conducted on 300 samples collected in 2010 from two major hospitals in Papua New Guinea during routine influenza surveillance by the PNG National Influenza Centre at the PNG Institute of Medical Research. The two hospitals where influenza-like-illness surveillance was conducted were Goroka General Hospital in the highland region of the country and Vanimo General Hospital on the north-west coast. Patients with influenza-like-illness were identified according to the WHO case definition: sudden onset of fever ( $>38^{\circ}\text{C}$ ), with cough and/or sore throat, in the absence of other diagnoses [WHO, 2009]. Nasopharyngeal swabs and detailed patient data were collected from enrolled participants according to standard methods and stored at  $-80^{\circ}\text{C}$  until required for testing. A maximum of five samples were collected from each site per day. The swabs were sent in 3 ml of universal transport media (Copan Italia, Brescia, Italy) within 48 hr of collection and then aliquoted and stored at  $-80^{\circ}\text{C}$ .

This study was approved by the Papua New Guinea Institute of Medical Research Institutional Review Board and the Papua New Guinea Medical Research Advisory Council. Nasopharyngeal samples were collected from eligible children following informed consent from parents or guardians.

### Nucleic Acid Extraction and Real-Time PCR/RT-PCR Detection

Nucleic acids were extracted from nasopharyngeal samples using the QIAamp Viral RNA Minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Eluates were stored at  $-80^{\circ}\text{C}$  until required for testing.

The samples were screened for a large range of respiratory viruses which have previously been associated with influenza-like-illness (Table I). All viruses were detected using individual Taqman real-time PCR assays. RNA viruses (influenza A, influenza B, HRSV, HPIV-1, HPIV-2, HPIV-3, HCoV-229E, HCoV-OC43, HCoV-NL63, HCoV-HKU1, HMPV, and HRV) were

detected using the QuantiTect Multiplex RT-PCR Master Mix (Qiagen) and the DNA viruses (HAdV) were detected using the QuantiTect Multiplex PCR Master Mix (Qiagen). All reactions and cycling conditions were conducted according to the mastermix manufacturer's instructions. Viral positive controls, and negative controls of nuclease-free water, were included with each assay to ensure consistency of the assays.

### Statistical Analysis

Patients were divided into two groups based upon age: children  $<5$  years of age and all other patients  $>5$  years of age. The Fisher's exact test was used to compare the infection rates between the two different age groups. A  $P$ -value of  $<0.05$  was considered to be statistically significant.

## RESULTS

### Patients

The patients (161 females and 139 males) enrolled in the study were 1 month to  $>60$  years of age. The median age could not be determined due to the inaccurate recording of birth dates in this population, particularly in adults. The participant samples were split into two groups for analysis: children  $<5$  years of age ( $n = 167$ ) and all other patients  $>5$  years of age ( $n = 133$ ).

### Virus Detection

Among the 300 patients presenting with an influenza-like-illness, a viral respiratory pathogen was detected in 66.3% ( $n = 199$ ) of samples. HRV (17.0%,  $n = 51$ ), influenza A (16.7%,  $n = 50$ ), and influenza B (12.7%,  $n = 38$ ) were the viruses most commonly detected in the nasopharyngeal swabs (Table II). All other viruses targeted during this study were detected at varying rates below 10%: HRSV in 8.7% ( $n = 26$ ) of patients; HAdV in 6.7% ( $n = 20$ ) of patients; HPIV-3 in 6.3% ( $n = 19$ ) of patients; HCoV-OC43 and HPIV-1 in 2.7% ( $n = 8$ ) of patients; HPIV-2 in 1.3% ( $n = 4$ ) of patients; HMPV in 1.0% ( $n = 3$ ) of patients; HCoV-HKU1, HCoV-NL63 in 0.7%, and HCoV-229E in 0.7% ( $n = 2$ ) of patients. Mixed infections were detected in 10.0% of patients, and influenza A and HRV were most commonly associated with co-infections with other viruses (Table III).

### Age Distribution

The rates of virus detection for most of the viruses included in this study were not significantly different between the two age groups analyzed ( $<5$  and  $>5$  years). However, influenza B ( $P = 0.0081$ ), HAdV ( $P = 0.0001$ ), and HRSV ( $P = 0.0017$ ) were all detected at significantly higher rates in patients  $<5$  years of age with an influenza-like-illness (Table II). There was also a significant difference in the two age

TABLE I. Primer Sequences Used for the Real-Time PCR/RT-PCR Detection of Respiratory Viruses

Virus	Primer/probe	Sequence	Refs.
FluA		Not available	CDC <sup>a</sup>
FluB		Not available	CDC <sup>a</sup>
RSV	RSV-F	GCAAATATGGAAACATACGTGAACA	Brittain-Long et al. [2008]
	RSV-R	GCACCCATATTGTWAGTGATGCA	
	RSV-P	Cy5-CTTCACGAAGGCTCCACATACACAGCWG-BHQ3	
HPIV-1	PF1-F	GCAAAGAGARAATGCRGATCTAG	Chidlow et al. [2009]
	PF1-R	AGCTCCGAGACATGCAGGAT	
	PF1-P	6FAM-TCCATATGTCTGAAGCAAT-MGBNFQ	Chidlow et al. [2009]
HPIV-2	PF2-F	ATTCCAGATGCTCGATCAACTATG	
	PF2-R	TCYTCAGCTAATGCTTCRAARGC	
	PF2-P	VIC-AGCACYTCTCCTCTGG-MGBNFQ	Chidlow et al. [2009]
HPIV-3	PF3-F	CGCGCTCCWTTYATCTGTATC	
	PF3-R	TTGCCTGGTGCGAACTCA	
	PF3-P	VIC-TCAGAGATCCYATACATG-MGBNFQ	Gunson et al. [2005]
HRV	RV-F	TGGACAGGGTGTGAAGAGC	
	RV-R	CAAAGTAGTCGGTCCCATCC	
	RV-P	Cy5-TCCTCCGGCCCCCTGAATG-BHQ3	Heim et al. [2003]
HAdV	AdV-F	GCCACGGTGGGGTTTCTAAACTT	
	AdV-R	GCCCCAGTGGTCTTACATGCACATC	
	AdV-P	Cy5-TGCACCCAGACCCGGCTCAGGTACTCCGA-BHQ2	Chidlow et al. [2009]
HCov229E	229E-F	CTGCCAAGAGTCTTGCTCGTT	
	229E-R	TCTTTTCCACCGTGGCTTTT	
	229E-P	VIC-AGAACAAAAGCATGAAATG-MGBNFQ	Chidlow et al. [2009]
HCoVOC43	OC43-F	GACATGGCTGATCAAATTGCTAGT	
	OC43-R	GCTGAGGTTTtagTGGCATCCTT	
	OC43-P	6FAM-TCTGGCAAAACTTGG-MGBNFQ	Chidlow et al. [2009]
HCoVNL63	NL63-F	AACCTCGTTGGAAGCGTGTT	
	NL63-R	CGAGGACCAAAGCACTGAATAA	
	NL63-P	VIC-ATTTTCTCTCTGGTAG-MGBNFQ	Chidlow et al. [2009]
HCoVHKU1	HKU1-F	CCCGCAAACATGAATTTTGTT	
	HKU1-R	CATTCATTCGCAAGGCGATA	
	HKU1-P	6FAM-AATCTATCACCATGTGAA-MGBNFQ	Klemenc et al. [2012]
HMPV	MPV-F	CATAYAARCATGCTATATTTAAAAGAGTCTC	
	MPV-R	CCTATYTCWGCAGCATATTTGTAATCAG	
	MPV-P	FAM-CAACHGCAGTRACACCYTCATCATRCA-BHQ1	

<sup>a</sup>US Centers for Disease Control and Prevention (CDC) Real-time RT-PCR Influenza A/B Typing Panel.

TABLE II. The Frequency of Viral Detection in Patients With Influenza-Like-Illness

Characteristics	No. of cases (%)			P-value <sup>a</sup>
	Total (n = 300)	Age distribution of viruses		
		<5 years (n = 167)	>5 years (n = 133)	
Tested positive for a virus	199 (66.3)	132 (79.0)	67 (50.4)	<b>0.0001</b>
Multiple viruses	30 (10.0)	25 (15.0)	5 (3.8)	<b>0.0015</b>
Influenza A	50 (16.7)	26 (15.6)	24 (18.0)	0.6405
Influenza B	38 (12.7)	29 (17.4)	9 (6.8)	<b>0.0081</b>
Rhinovirus	51 (17.0)	33 (19.8)	18 (13.5)	0.1667
Respiratory syncytial virus	26 (8.7)	22 (13.2)	4 (3.0)	<b>0.0017</b>
Adenovirus	20 (6.7)	19 (11.4)	1 (0.8)	<b>0.0001</b>
Parainfluenza virus 1	8 (2.7)	4 (2.4)	4 (3.0)	0.7363
Parainfluenza virus 2	4 (1.3)	4 (2.4)	0 (0)	0.1321
Parainfluenza virus 3	19 (6.3)	13 (7.8)	6 (4.5)	0.3408
Metapneumovirus	3 (1.0)	0 (0)	3 (2.3)	0.0860
Coronavirus 229E	2 (0.7)	0 (0)	2 (1.5)	0.1957
Coronavirus OC43	8 (2.7)	5 (3.0)	3 (2.3)	0.7363
Coronavirus NL63	2 (0.7)	0 (0)	2 (1.5)	0.1957
Coronavirus HKU1	2 (0.7)	2 (1.2)	0 (0)	0.5048

<sup>a</sup>P-value—Fisher’s exact test (significant values in bold).

TABLE III. Co-infections of Respiratory Viruses in Patients With influenza-Like-Illness

Co-infections	Positive cases among age groups	
	<5 (n = 167)	>5 (n = 133)
Flu A, HRV	2	2
Flu A, AdV	3	0
HRV, RSV	3	0
Flu A, RSV	2	0
Flu A, PIV-3	2	0
HRV, AdV	2	0
HRV, Flu B	2	0
Flu A, 229E	0	1
Flu A, OC43	1	1
AdV, RSV	0	1
Flu A, PIV-1	1	0
AdV, PIV-1	1	0
AdV, OC43	1	0
AdV, Flu B	1	0
HRV, PIV-1	1	0
AdV, HKU1	1	0
Flu B, PIV-3	1	0
Flu A, RSV, PIV-3	1	0
Total	25 (15.0%)	5 (3.8%)

groups for the detection of at least one viral pathogen in a patient ( $P=0.0001$ ) and the detection of viral co-infections ( $P=0.0015$ ).

## DISCUSSION

In this study, a viral respiratory pathogen was detected in 66.3% of nasopharyngeal swabs collected from patients with influenza-like-illness, which is a higher rate of detection compared to previous studies in some other locations [Puzelli et al., 2009; Buecher et al., 2010]. However, this figure is comparable to the detection rate of respiratory viruses in patients with influenza-like-illness in studies from China [Huo et al., 2012] and Madagascar [Razanajatavo et al., 2011]. These differences may be attributed to the lower socio-economic status of people in these countries, or environmental and other more complex factors. All of the viruses which were targeted in this study were detected in at least two patients. In patients where no etiological agent was detected the respiratory illness may have been caused by bacteria, chlamydia, mycoplasma or other viral respiratory pathogens for which testing was not done.

HRV was the most commonly detected viral pathogen in this study (17%). These results are consistent with many studies that have found HRV to be one of the most commonly detected viral pathogen associated with acute respiratory infections [Bellei et al., 2008; Tokarz et al., 2011; Venter et al., 2011; Thiberville et al., 2012]. The detection of HRV in 17% of samples may be an underestimate as more than 100 serotypes exist [Kennedy et al., 2012] and a single primer set may not be sufficient to amplify all of the existing variations. However, the frequent detection of this virus in the respiratory tracts of

healthy people make it difficult to determine the etiological role it plays in influenza-like-illness and lower respiratory tract infections as many studies that have compared the detection rate of HRV between patients with influenza-like-illness and healthy controls have not found a significant difference between the two groups [Buecher et al., 2010; Chidlow et al., 2012]. Nevertheless, the upper respiratory carriage of HRV may contribute to a greater susceptibility to influenza-like-illness and acute lower respiratory tract infections [Papadopoulos, 2004].

Influenza A and B were detected in a high proportion of influenza-like-illness cases, with the viruses detected in 16.7% and 12.7% of patients, respectively. Seasonal influenza vaccination is very rare in Papua New Guinea, so this is unlikely to have any bearing on the detection rate of these viruses in this population. Outbreaks of influenza have had a major impact on Papua New Guinea and other Pacific island nations in the past. The isolation of many communities and the contribution of poverty, high bacterial carriage and poor access to health services have led to significant mortalities in indigenous populations [Kelso and Reading, 2010]. The samples analyzed in this study were collected closely following the 2009 H1N1 pandemic. All of the influenza A strains that were further subtyped ( $n=25$ ) were confirmed as A/H1N1pdm09 by the WHO Collaborating Centre for Influenza Surveillance and Research, Melbourne Australia (data not shown). The majority of the PNG population most likely had very low immunity to this virus and therefore the rates of influenza A may have been higher during this period than at other times.

Influenza B, HAdV, and HRSV were all detected at significantly higher rates in children <5 years of age than people >5 years. The higher detection rates of HAdV [Langley, 2005] and HRSV [Hombrouck et al., 2012; Huo et al., 2012] in children <5 years of age has been reported previously. In particular, HRSV is accepted as the most important cause of viral lower respiratory tract disease in young children [Leung et al., 2005]. Nair et al. [2010] estimated that HRSV caused 66,000–199,000 deaths in children under 5 years of age in 2005, and 99% of these deaths were in developing countries. HAdV can be associated with respiratory illnesses throughout all age groups, but most commonly in young infants or school aged children. By age 5 almost 75% of children have serological evidence of adenovirus infection [Langley, 2005]. The detection of influenza B at significantly higher rates in young children has not been reported extensively [Peltola et al., 2003]. Previous studies have suggested that influenza B may cause less severe illness in adults due to partial immunity elicited to previously circulating strains of influenza B virus [Glezen et al., 1980; Newland et al., 2006].

Infection with at least one respiratory virus was detected at a significantly higher rate in children <5

years of age than the rest of the population. The majority of the morbidity and mortality due to respiratory infections worldwide is in children <5 years of age. The lower immunity of young children to respiratory viruses undoubtedly plays an important role as these viruses can lead to disruption of the innate immunity of the respiratory tract and thus lead to secondary bacterial pneumonia and other acute lower respiratory infections [Vareille et al., 2011].

This study also found a significantly higher rate of co-infections in young children when compared to the rest of the population. Numerous studies have found that co-infections with respiratory viruses may lead to more severe respiratory illnesses [Cilla et al., 2008; Esposito et al., 2008; Richard et al., 2008]; conversely other studies have observed no such effect [Peng et al., 2009; Suryadevara et al., 2011]. In this study, no data were collected on the severity of influenza-like-illness and therefore we cannot comment on this matter. Influenza A and HRV were the viruses that were most commonly associated with co-infections with other respiratory viruses. This is probably not surprising considering they were the two viruses which were detected most commonly in the study.

Our study has some limitations. Indicators of the severity of disease and the outcome of patient's illnesses were not collected. This information may have provided insights into the relative importance of each of the viruses and the significance of co-infections between viruses. All samples collected were submitted to the Papua New Guinea National Influenza Centre for routine analysis of influenza-like-illnesses. Therefore, testing of control population samples were not conducted during this study. However, a recent study in Papua New Guinea looking at children <5 years of age found that influenza A, HRSV, and HAdV were detected at significantly higher rates in children with acute lower respiratory infections than a healthy control population [Chidlow et al., 2012].

This study demonstrated that multiple respiratory viruses are circulating in Papua New Guinea which may contribute to the high burden of acute respiratory infections in this population. The comparison between children <5 years of age and other patients highlights the differing etiologies of respiratory disease in young children and provides important information for clinicians when treating patients presenting with an acute respiratory illness.

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