Use of a Multiplex PCR/RT-PCR Approach to Assess the Viral Causes of Influenza-Like Illnesses in Cambodia During Three Consecutive Dry Seasons

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Acute respiratory infections are a major cause of mortality and morbidity worldwide. Using multiplex PCR/RT-PCR methods for the detection of 18 respiratory viruses, the circulation of those viruses during 3 consecutive dry seasons in Cambodia was described. Among 234 patients who presented with influenza-like illness, 35.5% were positive for at least one virus. Rhinoviruses (43.4%), parainfluenza (31.3%) viruses and coronaviruses (21.7%) were the most frequently detected viruses. Influenza A virus, parainfluenza virus 4 and SARS-coronavirus were not detected during the study period. Ninety apparently healthy individuals were included as controls and 10% of these samples tested positive for one or more respiratory viruses. No significant differences were observed in frequency and in virus copy numbers for rhinovirus detection between symptomatic and asymptomatic groups. This study raises questions about the significance of the detection of some respiratory viruses, especially using highly sensitive methods, given their presence in apparently healthy individuals. The link between the presence of the virus and the origin of the illness is therefore unclear. *J. Med. Virol. 82:1762–1772, 2010.*

INTRODUCTION

Acute respiratory infections are the leading cause of acute illnesses and a major cause of morbidity and mortality worldwide, accounting for approximately two million deaths each year in infants [The World Health Report, 2004; Kieny and Girard, 2005; Mizgerd, 2006]. Acute respiratory infections are also associated with high mortality in the elderly and individuals with compromised cardiac, pulmonary, or immune systems. Clinical manifestations in respiratory infections range from self-limited upper respiratory tract infections to more serious lower respiratory tract infections. Determining etiological diagnoses for patients with respiratory symptoms remains a clinical and laboratory challenge. Most laboratory-confirmed viral infections are attributed to respiratory syncytial viruses (RSV), parainfluenza viruses (PIV), influenza viruses A and B (IAV, IBV), human rhinoviruses (HRhV), and adenoviruses (AdV). Clinical virology laboratories have used historically traditional methods such as direct fluorescent-antibody assay (DFA) for the diagnosis of viral respiratory tract infections, however, these methods fail to detect a large proportion of infections.

Over the past 10 years, nucleic acid amplification techniques have been developed for several respiratory viruses. Polymerase chain reaction (PCR) and reverse transcription-PCR (RT-PCR) assays provide rapid results with equivalent or greater sensitivity than conventional methods [Erdman et al., 2003; Weinberg et al., 2004]. Furthermore, multiplex PCR assays have been used to detect the presence of two or more respiratory viruses in a single reaction tube [Fan et al., 1998; Coiras et al., 2004; Bellau-Pujol et al., 2005].

Recent advances in molecular technology have enabled the detection of several new viral agents in specimens collected from human respiratory tracts, including the human metapneumovirus (HMPV) in 2001 [van den Hoogen et al., 2001], the severe acute respiratory syndrome-associated coronavirus (SARS-CoV) in 2003 [Ksiazek et al., 2003], the human

coronavirus NL63 (HCoV-NL63) in 2004 [van der Hoek et al., 2004], the human coronavirus HKU1 (HCoV-HKU1) in 2005 [Woo et al., 2005], as well as the human bocavirus (HBoV) in 2005 [Allander et al., 2005]. However, data regarding the prevalence of respiratory viruses in the community are still lacking in many parts of the world.

In order to assess the distribution of the major viral agents responsible for acute respiratory infections in Cambodia, respiratory specimens were tested retrospectively for 18 respiratory viruses using multiplex PCR assays. In addition, asymptomatic individuals were also tested in order to evaluate the importance of asymptomatic carriers of respiratory viruses and the significance of the detection of some viruses in the human respiratory tract.

MATERIALS AND METHODS

Respiratory Specimens

For this study, 324 nasopharyngeal swab specimens were collected in children and adults between February and May (the dry season in Cambodia) over a 3-year period (2005–2007). Nasopharyngeal swabs was collected in 1 mL of viral transport medium and stored at $-80°C$ prior to testing.

Two hundred thirty four symptomatic patients were recruited in five different provincial hospitals. When sufficient specimens were available in an age group, they were randomly chosen. Otherwise, all samples were tested (Fig. 1).

The inclusion criteria followed the World Health Organization (WHO) case definition for influenza-like illness: sudden onset of a fever over $38°C$ and cough or sore throat and absence of other diagnoses [WHO, 2008].

The negative control group included 90 specimens collected from apparently healthy individuals, who were sampled only because they had contact with H5N1-infected patients (such as family members and health-care workers) between March and April 2005, 2006, and 2007 (Fig. 1). None of them declared having fever or any upper or lower respiratory symptom at the time of sampling. All the specimens were anonymized and not linked to the identity of any person.

RNA/DNA Preparation

RNA was extracted from 140 µl of nasopharyngeal secretion collected in viral transport medium, using the QIAamp Viral RNA Mini kit (QIAGEN®, Hilden, Germany). For DNA viruses (HBoV, AdV), nucleic acid was extracted from 200 µl of specimen using the MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostic®, Mannheim, Germany) on a MagNA Pure Compact instrument (Roche Diagnostic®).

Multiplex PCR/RT-PCR

Five multiplex PCR/RT-PCR methods, targeting 18 respiratory viruses, were used. IAV, IBV, HMPV (A and B), and RSV (A and B) were detected by the multiplex RT-PCR 1; PIV-1, -2, -3, and -4 by the multiplex RT-PCR 2; HRhV, enterovirus (EnV), influenza C virus (ICV) and SARS-CoV by the multiplex RT-PCR 3; HCoV-OC43, -229E, -NL63 and -HKU1 by the multiplex RT-PCR 4; AdV and HBoV by the multiplex PCR 5 (Table I).

Multiplex RT-PCR 1, 2, 3 are a single-step combined PCR amplification performed using the One-step RT-PCR kit from Qiagen, as described previously
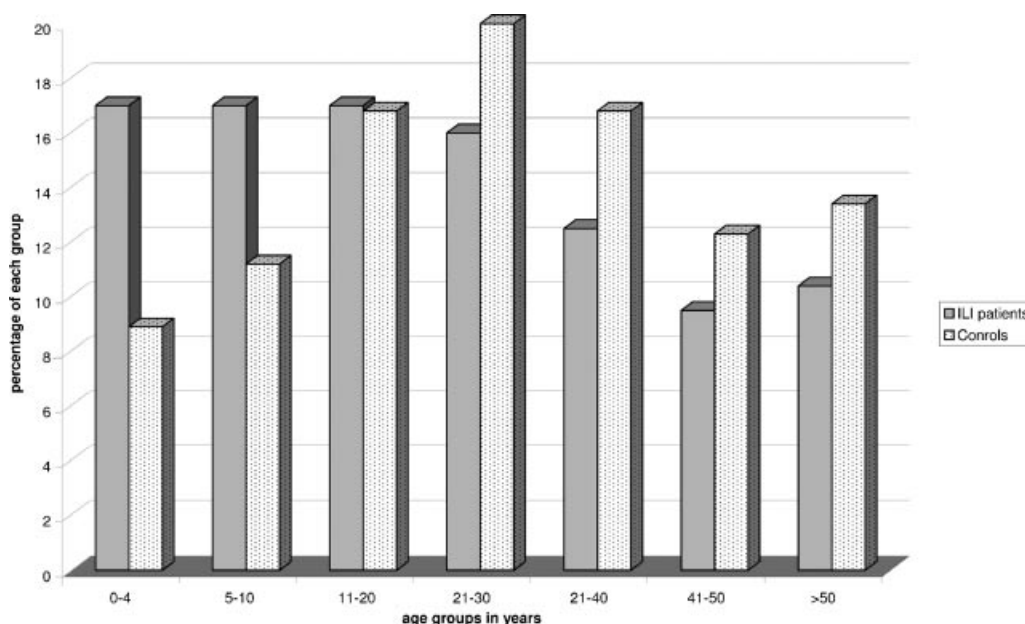

Fig. 1. Sample distribution by age group of influenza-like illness patients and control individuals.

TABLE I. Primer Sequences Used in the 5 Multiplex PCR/RT-PCR

Virus	Assay	Primer designation	Primer sequences (5′–3′)	Expected amplicon size (bp)	Refs.
Multiplex 1					
IAV	RT-PCR	A1 (For)	CAG AGA CTT GAA GAT GTC TTT GCT (A)G	212	Adapted from Bellau-Pujol et al. [2005]
		A2 (Rev)	GCT CTG TCC ATG TTA TTT GG(A) ATC		Adapted from Bellau-Pujol et al. [2005]
	Hemi nested	MIA3 (For)	CTC TGA CTA AGG GGA TTT TG	130	Bellau-Pujol et al. [2005]
		A2 (Rev)	GCT CTG TCC ATG TTA TTT GG(A) ATC		Bellau-Pujol et al. [2005]
IBV	RT-PCR	B1 (For)	GAA AAA TTA CAC TGT TGG TTC GGT G	365	Adapted from Bellau-Pujol et al. [2005]
		B2B (Rev)	AGC GTT CCT AGT TTT ACT TGC ATT GA		Adapted from Bellau-Pujol et al. [2005]
	Hemi nested	MIB3 (Rev)	CAT GAA ARC TCA CAC ATC T	260	Bellau-Pujol et al. [2005]
		B1 (For)	GAA AAA TTA CAC TGT TGG TTC GGT G		Bellau-Pujol et al. [2005]
RSV	RT-PCR	Cane P1 (For)	GGA ACA AGT TGT TGA GGT TTA TGA ATA TGC	278	Adapted from Bellau-Pujol et al. [2005]
		Cane P2 (Rev)	CTG CTG TCA AGT CTA GTA CAC TGT AGT		Adapted from Bellau-Pujol et al. [2005]
	Hemi nested	VRSi (Rev)	GGT GTA CCT CTG TAC TCT C	180	Bellau-Pujol et al. [2005]
		Cane P1 (For)	GGA ACA AGT TGT TGA GGT TTA TGA ATA TGC		Bellau-Pujol et al. [2005]
HMPV	RT-PCR	MPV M1a (For)	GGA GTC CTA CCT AGT AGA C	537	Freymuth F [unpublished work]
		MPV M2 (Rev)	GCA GCT TCA ACA GTA GCT G		Bellau-Pujol et al. [2005]
	Hemi nested	MPV M3 (For)	AGG CCA ACA CAC CAC CAG	410	Bellau-Pujol et al. [2005]
		MPV M2 (Rev)	GCA GCT TCA ACA GTA GCT G		Bellau-Pujol et al. [2005]
Multiplex 2					
PIV-1	RT-PCR	PiS1+ (For)	CCG GTA ATT TCT CAT ACC TAT G	317	Echevarria et al. [1998]
		PiS1-(Rev)	CTT TGG AGC GGA GTT GTT AAG		Echevarria et al. [1998]
	Hemi nested	piS1i (For)	AGC TGC AGG AAC AAG GGG	261	Bellau-Pujol et al. [2005]
		PiS1-(Rev)	CTT TGG AGC GGA GTT GTT AAG		Bellau-Pujol et al. [2005]
PIV-2	RT-PCR	PiP2+ (For)	AAC AAT CTG CTG CAG CAT TT	507	Echevarria et al. [1998]
		PiP2- (Rev)	ATG TCA GAC AAT GGG CAA AT		Echevarria et al. [1998]
	Hemi nested	PARA2i (For)	CTA GCT GAA CTG AGA CTT G	340	Bellau-Pujol et al. [2005]
		PiP2- (Rev)	ATG TCA GAC AAT GGG CAA AT		Bellau-Pujol et al. [2005]
PIV-3	RT-PCR	P3-1 (For)	CTC GAG GTT GTC AGG ATA TAG	189	Karron et al. [1994]
		P3-2 (Rev)	CTT TGG GAG TTG AAC ACA GTT		Karron et al. [1994]
	Hemi nested	PARA3i (Rev)	GCT AGA GAA CAT GAC TTC C	145	Bellau-Pujol et al. [2005]
		P3-1 (For)	CTC GAG GTT GTC AGG ATA TAG		Bellau-Pujol et al. [2005]
PIV-4	RT-PCR	Pi4S+ (For)	CTG AAC GGT TGC ATT CAG GT	451	Aguilar et al. [2000]
		Pi4S- (Rev)	TTG CAT CAA GAA TGA GTC CT		Aguilar et al. [2000]
	Hemi nested	Pi4i (Rev)	GTC TGA TCC CAT AAG CAG C	390	Bellau-Pujol et al. [2005]
		Pi4S+ (For)	CTG AAC GGT TGC ATT CAG GT		Bellau-Pujol et al. [2005]
Multiplex 3					
ICV	RT-PCR	CHAA (For)	ACA CTT CCA ACC CAA TTT GG	485	Zhang and Evans [1991]
		CHAD (Rev)	CCT GAC AGC AAC TCC C TC AT		Zhang and Evans [1991]
	Hemi nested	MICi (For)	GAG GAT GTG GCA ACT ACT	391	Bellau-Pujol et al. [2005]
		CHAD (Rev)	CCT GAC AGC AAC TCC C TC AT		Bellau-Pujol et al. [2005]
HRhV	RT-PCR	SRHI 1 (For)	GCA TCI GGY ARY TTC CAC CAC CAN CC	549	Savolainen et al. [2002]
		SRHI 2 (Rev)	GGG ACC AAC TAC TTT GGG TGT CCG TGT		Savolainen et al. [2002]
	Hemi nested	NESTRHI1 (Rev)	ATG GGN GCW CAN GTN TCH ANH CA	450	Bellau-Pujol et al. [2005]
		SRHI 1 (For)	GCA TCI GGY ARY TTC CAC CAC CAN CC		Bellau-Pujol et al. [2005]
SARS-CoV	RT-PCR	SARS-Bin out-AS (For)	CAT AAC CAG TCG GTA CAG CTA	195	Drosten et al. [2003]
		SARS-Bin out-S2 (Rev)	RTG AAT TAC CAA GTC AAT GGT		Drosten et al. [2003]
	Nested	SARS-Bni/in-AS (For)	CTG TAG AAA ATC CTA GCT GGA	110	Drosten et al. [2003]
		SARS-Bni/in-S (Rev)	GAA GCT ATT CGT CAC GTT CG		Drosten et al. [2003]
Multiplex 4					
HCoV-OC43	RT-PCR	MF 1 (For)	GGC TTA TGT GGC CCC TTA CT	334	Vabret et al. [2001]
		MF3 (Rev)	GGC AAA TCT GCC CAA GAA TA		Vabret et al. [2001]
	Hemi nested	MF2i (Rev)	CTC CAA AAA CTT CCA GTT C	170	Bellau-Pujol et al. [2005]
		MF 1 (For)	GGC TTA TGT GGC CCC TTA CT		Bellau-Pujol et al. [2005]

TABLE I. (*Continued*)

Virus	Assay	Primer designation	Primer sequences (5′–3′)	Expected amplicon size (bp)	Refs.
HCoV-229E	RT-PCR	MD1 (For)	TGG CCC CAT TAA AAA TGT GT	574	Vabret et al. [2001]
		MD3 (Rev)	CCT GAA CAC CTG AAG CCA AT		Vabret et al. [2001]
	Hemi nested	MD2i (Rev)	CCG TAT CAA CAC TCG TTA TGT GG	230	Bellau-Pujol et al. [2005]
		MD1 (For)	TGG CCC CAT TAA AAA TGT GT		Bellau-Pujol et al. [2005]
HCoV-HKU1	RT-PCR	HKU-N-sens3 (For)	ATC TGA GCG AAA TTA CCA AAC	443	Woo et al. [2005]
		HKU1 antisense (Rev)	CGG AAA CCT AGT AGG GAT AGC TT		Woo et al. [2005]
HCoV-NL63	RT-PCR	NL63 sens (For)	GAT AAC CAG TGG AAG TCA CCT AGT TC	255	Vabret et al. [2005]
		NL63 antisense (Rev)	ATT AGG AAT CAA TTC AGC AAG CTG TG		Vabret et al. [2005]
Multiplex 5					
AdV	PCR	ADHEX1F (For)	CAA CAC CTA YGA STA CAT GAA	270	Avellon et al. [2001]
		ADHEX2R (Rev)	ACA TCC TTB CKG AAG TTC CA		Palacious et al. [2003]
HBoV	PCR	188F (For)	GAS CTC TGT AAG TAC TAT TAC	354	Allander et al. [2005]
		542R (Rev)	CTC TGT GTT GAC TGA ATA CAG		Allander et al. [2005]

[Bellau-Pujol et al., 2005]. However, multiplex 3 was modified by including the detection of SARS-CoV instead of HCoV-NL63 and HCoV-229E. The multiplex 4 was developed for the simultaneous detection of the four HCoVs using primers described by others [Vabret et al., 2001, 2005; Woo et al., 2005].

The four assays were undertaken in 30 μl reaction volume containing 3 μl RNA extract, 6 μl Qiagen One-Step RT-PCR buffer 5×, 1.2 μl 10 mM deoxynucleoside triphosphate (dNTP), 1 μl Qiagen OneStep RT-PCR Enzyme Mix (OneStep RT-PCR kit, Qiagen), 1.44 μl of each primer (concentration 10 μM), 3.6 μl Qiagen One-Step RT-PCR kit Q solution, and RNase-free water to complete the 30 μl final volume. The reaction was carried out in a MyCycler thermo-cycler (Bio-Rad Laboratories, Hercules, CA) with an initial reverse transcription step at 50°C for 30 min, followed by PCR activation at 94°C for 15 min, 8 initial cycles of amplification (30 sec at 94°C, 30 sec at 58°C, 1 min at 72°C), 30 additional cycles of amplification (30 sec at 94°C, 30 sec at 50°C, 1 min at 72°C) and a final extension step at 72°C for 10 min.

The multiplex PCR 5 was designed for the detection of DNA viruses using primers described by others [Allander et al., 2005; Bellau-Pujol et al., 2005; Casas et al., 2005]. Multiplex 5 is a single-step combined PCR amplification. The 25 μl reaction mix consisted of 2.5 μl DNA extract, 2.5 μl 10× PCR buffer (Promega, Madison, WI), 0.5 μl of 10 mM deoxynucleoside triphosphate (dNTP), 0.5 μl of 5 U/μl Taq DNA Polymerase (Promega) 1 μl of each primer (concentration: 10 μM), 2.5 μl of MgCl₂ 25 mM, and RNase-free water to a final volume of 25 μl. The reaction was carried out in a MyCycler thermo-cycler (Bio-Rad Laboratories). After 2 min at 94°C, 40 cycles of amplification (94°C for 30 sec, 58°C for

30 sec, and 72°C for 1 min) were performed, followed by a final extension step at 72°C for 10 min.

Hemi-nested or nested singleplex PCR methods were used to confirm positive results obtained by multiplex RT-PCR. Products were visualized on a 2% agarose gel. Primer sequences used in these protocols are listed in Table I.

In vitro-transcribed RNA was generated from different viruses to use as positive controls and to assess the sensitivity of each multiplex PCR/RT-PCR (Table II). The PCR-amplified fragments of each virus were inserted into the pGEM-T Easy Vector (Promega). The plasmid T7 RNA polymerase transcription initiation site and promoter sequence located upstream of viral cloned sequences were utilized for in vitro synthesis of RNA transcripts, using RiboMAX™ Large Scale RNA Production System-T7 (Promega) following the instructions of the manufacturer. The concentration of each RNA transcript was measured by an ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). To assess sensitivity, RNA was serially diluted (10-fold), ranging from 10^5 to 1 RNA copies/reaction (Table II). The specificity of multiplex 1, 2, and 3 (which did not initially test for SARS-CoV) was assessed by Bellau-Pujol et al. [2005] who did not observe false positives. For multiplex 3, 4, and 5, the analytical specificity was checked by including the following in each RT-PCR/PCR: an appropriate positive control, two negative controls, a strain of *Mycoplasma pneumonia* and a strain of *Chlamydia pneumonia*, two nucleic acids extracted from clinical specimens and confirmed by sequencing or two RNA transcripts for: influenza A/H1N1, H3N2, H5N1 and H1N1 pandemic, influenza B, influenza C, RSV, HMPV, PIV-1, -2, -3, -4, HRhV, EnV, HCoV-OC43, -229E, -NL63, -HKU1, SARS-CoV, AdV,

TABLE II. Sensitivity of Monoplex and Multiplex PCR/RT-PCR

Multiplex	Virus	Expected amplicon size (bp)	Sensitivity of monoplex PCR/RT-PCR (number of copy/μl of VTM[a])	Sensitivity of multiplex PCR/RT-PCR (number of copy/μl of VTM[a])
1	IAV	212	4	4
	IBV	365	4	36
	RSV	278	4	36
	HMPV	537	4	4
2	PIV-1	317	36	3572
	PIV-2	507	358	3572
	PIV-3	189	4	36
	PIV-4	451	4	358
3	ICV	485	4	36
	HRhV	549	358	358
	SARS	195	4	4
4	HCoV-OC43	335	4	4
	HCoV-229E	573	4	4
	HCoV-HKU1	443	4	4
	HCoV-NL63	255	358	358
5	AdV	270	36	36
	HBoV	354	4	4

[a]Viral transport medium.

and HBoV. Non-specific amplifications were not observed (data not shown).

Statistical Analysis

All statistical analysis was performed using the Stata/SE version 9.0 (StataCorp, College Station, TX). Significance was assigned at $P < 0.05$ for all parameters. Uncertainty was expressed at 95% confidence intervals. The t student, Mann–Whitney and Kruskal–Wallis rank tests were used for continuous variables and χ^2 and Fisher's exact tests for categorical variables.

RESULTS

A total of 324 nasopharyngeal swabs were tested including 234 (72%) among patients who presented with influenza-like illness and 90 (28%) in controls. Patients with influenza-like illness were significantly younger than apparently healthy control individuals (mean age 22.5 vs. 28.5 years, $P = 0.001$).

Among the 234 influenza-like illness patients tested by multiplex PCR/RT-PCR, 83 (35.5%) were positive for at least one of the respiratory viruses tested (Tables IIIa–IIIc). Of these, HRhV (43.4%), PIVs 1–3 (31.3%) and various HCoVs (21.7%) accounted for most of the etiologies of influenza-like illness. Other viruses associated with influenza-like illness are highlighted in Figure 2. Of note, PIV-4, IAV, and SARS-CoV were not detected during the study period.

Of 36 (15.4%) HRhVs detected in influenza-like illness patients, seven (19.4%) were co-infections with other viruses including 4 PIV-3, 2 HCoV-NL63, 1 IBV, 1 RSV and 1 PIV-3 + CoV-229E (Table IIIc). PIVs of different types were detected in 26 (11.1%) patients: PIV-1 (23%), PIV-2 (11.5%), and PIV-3 (65.4%). Of these, 10 (38.4%) were co-infected with other viruses including HRhV, RSV, AdV, and HCoV.

Of 18 (7.7%) influenza-like illness patients with HCoVs, HCoV-OC43 accounted for 55.5%, followed by

HCoV-NL63 (22.2%), HCoV-HKU1 (11.1%), and HCoV-229E (11.1%). Four (22.2%) HCoV-infected patients were also infected with other viruses (HRhV and PIV-2, -3).

Viruses were also detected in 9 (10%) of 90 control samples using multiplex (Tables IIIa and IIIc). HRhV was the most frequently detected (n = 8, 89%), and HBoV was identified in one case (11%). No co-infections were observed in the control group (Table IIIc).

Among the 83 positive specimens collected from influenza-like illness patients, 47 (57.3%) were obtained in children under 10 years old, 18 (21.7%) in the 11- to 20-year age group, 10 (12.2%) in the 21- to 30-year age group, 2 (2.4%) in the 31- to 40-year age group, 3 (3.7%) in the 41- to 50-year age group and 3 (3.7%) in adults aged over 50 years.

In 59% of cases, the three viruses detected most frequently (HRhV, PIVs, and HCoVs) affected children ≤10 years of age. Sixty-one percent of HRhV infections were diagnosed in children ≤10 years of age (median age 7 years, mean 12.9, range from 3 months to 70 years) as well 61% of HCoV infections (median age 7.5 years, mean 10.7, range 3 months to 31 years) and also 61% of PIV infections (median age 7 years, mean age 9.8, range 3 months to 41 years). When excluding co-infections, no significant differences in age were observed between the three groups of viruses ($P > 0.05$).

The frequency of HRhV detection in the patient group was not statistically different to the control group ($P = 0.37$). Within the patient group, no significant difference in frequency of HRhV infections between each age group was demonstrated (data not shown).

When considering the total number of positive cases in young children ≤10 years and in influenza-like illness patients ≥11 years, the frequency of viruses detection was significantly higher in influenza-like illness patients than in asymptomatic individuals (Table IV).

To compare the relative quantity of HRhV and HBoV RNA and DNA in the samples from the patient group to

TABLE IIIa. Number of Detections (% of Total Samples) of Respiratory Viruses Among Influenza-Like Illness (ILI) Patients and Asymptomatic Individuals

	IAV	IBV	VRS	HMPV	PIV-1	PIV-2	PIV-3	PIV-4	ICV	HRhV/EnV	SARS-CoV	HCoV-OC43	HCoV-229E	HCoV-HKU1	HCoV-NL63	AdV	HBoV	Total
ILI patients	0	3 (1.3)	6 (2.6)	2 (0.8)	6 (2.6)	3 (1.3)	17 (7.3)	0	1 (0.4)	36 (15.4)	0	10 (4.3)	2 (0.8)	2 (0.8)	4 (1.7)	3 (1.3)	1 (0.4)	83 (35.5)
Asymptomatic individuals	0	0	0	0	0	0	0	0	0	8 (8.9)	0	0	0	0	0	0	1 (1.1)	9 (10)

TABLE IIIb. Number of Detections (% of Total Samples) of Single Viral Infections by Age Group Among Influenza-Like Illness (ILI) Patients/*Asymptomatic Individuals*

Age groups	0–4 years	5–10 years	11–20 years	21–30 years	31–40 years	41–50 years	>50 years	Total
Age characteristics	Average: 1.1; 95 CI: 0.9–1 Average: 2.9; 95 CI: 2.0–3.7	Average: 6.6; 95 CI: 6.1–7.1 Average: 7.2; 95 CI: 6.4–8.2	Average: 14.7; 95 CI: 13.7–15.7 Average: 15.4; 95 CI: 13.8–16.9	Average: 24.5; 95 CI: 23.7–25.3 Average: 28.2; 95 CI: 26.8–29.6	Average: 35.8; 95 CI: 34.5–37.0 Average: 35; 95 CI: 33.2–36.8	Average: 44.9; 95 CI: 43.5–46.2 Average: 45.4; 95 CI: 43.7–47.2	Average: 60.9; 95 CI: 57.5–64.4 Average: 59.3; 95 CI: 55.1–63.6	
Sample size	40/8	45/11	38/15	36/18	29/15	22/11	24/12	234/90
IAV	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
IBV	0/0	0/0	0/0	1 (2.6)/0	0/0	0/0	1 (4.2)/0	2 (0.8)/0
VRS	1 (2.5)/0	2 (4.4)/0	0/0	0/0	0/0	0/0	0/0	3 (1.3)/0
HMPV	0/0	0/0	1 (2.6)/0	0/0	1 (3.4)/0	0/0	0/0	2 (0.8)/0
PIV-1	0/0	2 (4.4)/0	2 (5.3)/0	1 (2.8)/0	0/0	0/0	0/0	5 (2.1)/0
PIV-2	0/0	1 (2.2)/0	1 (2.6)/0	0/0	0/0	0/0	0/0	2 (0.8)/0
PIV-3	2 (5)/0	3 (6.7)/0	2 (5.3)/0	1 (2.8)/0	0/0	1 (4.5)/0	0/0	9 (3.8)/0
PIV-4	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
ICV	0/0	0/0	0/0	0/0	0/0	1 (4.5)/0	0/0	1 (0.4)/0
HRhV/EnV	7 (17.5)/0	9 (20)/3 (27.3)	6 (15.8)/2 (13.3)	4 (11.1)/3 (16.7)	0/0	1 (4.5)/0	2 (8.3)/0	29 (12.4)/8 (8.9)
SARS-CoV	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
HCoV-OC43	3 (7.5)/0	4 (8.9)/0	1 (2.6)/0	1 (2.8)/0	0/0	0/0	0/0	9 (3.8)/0
HCoV-229E	0/0	0/0	1 (2.6)/0	0/0	0/0	0/0	0/0	1 (0.4)/0
HCoV-HKU1	0/0	0/0	0/0	0/0	0/0	0/0	0/0	2 (0.8)/0
HCoV-NL63	1 (2.5)/0	2 (4.4)/0	0/0	1 (2.8)/0	1 (3.4)/0	0/0	0/0	2 (0.8)/0
AdV	0/0	0/0	0/0	0/0	0/0	0/0	0/0	1 (0.4)/0
HBoV	0/0	0/1 (9.1)	1 (2.6)/0	0/0	0/0	0/0	0/0	1 (0.4)/1 (1.1)
Total	14 (35)/0	23 (51.1)/4 (36.4)	15 (39.5)/2 (13.3)	9 (25)/3 (16.7)	2 (6.9)/0	3 (13.6)/0	3 (12.5)/0	69 (26.1)/9 (10)

TABLE IIIc. Number of Detections (% of Total Samples) of Viruses Detected in Co-Infections Among Influenza-Like Illness (ILI) Patients/*Asymptomatic Individuals* for Each Age Group

Age groups	0–4 years	5–10 years	11–20 years	21–30 years	31–40 years	41–50 years	>50 years	Total
Age characteristics	Average: 1.1; 95 CI: 0.9–1 / Average: 2.9; 95 CI: 2.0–3.7	Average: 6.6; 95 CI: 6.1–7.1 / Average: 7.2; 95 CI: 6.4–8.2	Average: 14.7; 95 CI: 13.7–15.7 / Average: 15.4; 95 CI: 13.8–16.9	Average: 24.5; 95 CI: 23.7–25.3 / Average: 28.2; 95 CI: 26.8–29.6	Average: 35.8; 95 CI: 34.5–37.0 / Average: 35; 95 CI: 33.2–36.8	Average: 44.9; 95 CI: 43.5–46.2 / Average: 45.4; 95 CI: 43.7–47.2	Average: 60.9; 95 CI: 57.5–64.4 / Average: 59.3; 95 CI: 55.1–63.6	
Sample size	40/8	45/11	38/15	36/18	29/15	22/11	24/12	234/90
HRhV + PIV-3	2 (5)/0	1 (2.2)/0	1 (2.6)/0	0/0	0/0	0/0	0/0	4 (1.8)/0
HRhV + HCoV-NL63	1 (2.5)/0	0/0	1 (2.6)/0	0/0	0/0	0/0	0/0	2 (0.9)/0
AdV + PIV-1	1 (2.5)/0	0/0	0/0	0/0	0/0	0/0	0/0	1 (0.4)/0
AdV + PIV-3	1 (2.5)/0	0/0	0/0	0/0	0/0	0/0	0/0	1 (0.4)/0
HCoV-OC43 + PIV-2	0/0	0/0	1 (2.6)/0	0/0	0/0	0/0	0/0	1 (0.4)/0
HRhV + IBV	0/0	0/0	0/0	1 (2.8)/0	0/0	0/0	0/0	1 (0.4)/0
PIV-3 + RSV	1 (2.5)/0	1 (2.2)/0	0/0	0/0	0/0	0/0	0/0	2 (0.9)/0
HRhV + RSV	1 (2.5)/0	0/0	0/0	0/0	0/0	0/0	0/0	1 (0.4)/0
HRhV + PIV-3 + HCoV-229E	1 (2.5)/0	0/0	0/0	0/0	0/0	0/0	0/0	1 (0.4)/0
Total	8 (20)/0	2 (4.4)/0	3 (7.9)/0	1 (2.8)/0	0/0	0/0	0/0	14 (6.0)/0

the control group, extracted nucleic acids were diluted serially 10-fold and re-tested by RT-PCR/PCR. In both the symptomatic group and the control group only one HBoV was detected. The limit of detection by PCR was a dilution of 1:10 in the control group and 1:1,000 in the patient group. For HRhV, no significant differences were observed in the detection limits between asymptomatic individuals and patients with an influenza-like illness (Table V).

DISCUSSION

For the purpose of this study, published multiplex RT-PCR methods were optimized and new multiplex PCR/RT-PCR methods were developed using primers described in the literature. In our study, the multiplex approach, by comparison with a "monoplex" PCR or RT-PCR, was sometimes associated with a decrease in sensitivity. Nevertheless, sensitivity was improved after optimization of the Freymuth's protocols, who demonstrated previously that the multiplex RT-PCR represented a significant improvement over conventional methods [Freymuth et al., 2006]. The multiplex 4 and 5 developed for this study also allowed the detection of very low nucleic acid copy numbers, and therefore it was considered that these two methods are reasonably sensitive and specific for the detection of respiratory viruses.

While the case definition was intended to detect primarily influenza viruses, no influenza A cases was observed and IBV and ICV were found in only 4 (1.7%) patients. Recent data demonstrates that the seasonality of influenza virus transmission corresponds mostly to the rainy season and only a few cases can be detected during the dry season [Mardy et al., 2009].

Among specimens collected in patients with influenza-like illness, despite the fact that the case definition used did not include the main symptoms observed during the common cold (often caused by the rhinovirus), the virus detected most frequently was still HRhV (15.4%). Almost all age groups were affected by this virus. These results are consistent with previous findings suggesting that the HRhV are one of the most frequent causes of acute respiratory infections in adults and children with a prevalence varying between 10% to more than 40% (Australia: 44.4%; Finland: 45%; Korea: 33.3%) [Tsolia et al., 2004; Arden et al., 2006; Chung et al., 2007].

Over 11% of the patients were positive for at least one of the PIVs (in comparison with 10.7% in Germany and 11.5% in Korea). PIV-3 is the most frequent of all PIVs and one of the leading causes of respiratory infection in infants and young children. These results are in agreement with the published data [Gröndahl et al., 1999; König et al., 2004; Thomazelli et al., 2007; Yoo et al., 2007]. PIV-1 and PIV-2 were not detected frequently which is also consistent with other studies [Gröndahl et al., 1999; Thomazelli et al., 2007]. PIV-4 was never detected and appears in general to be found rarely
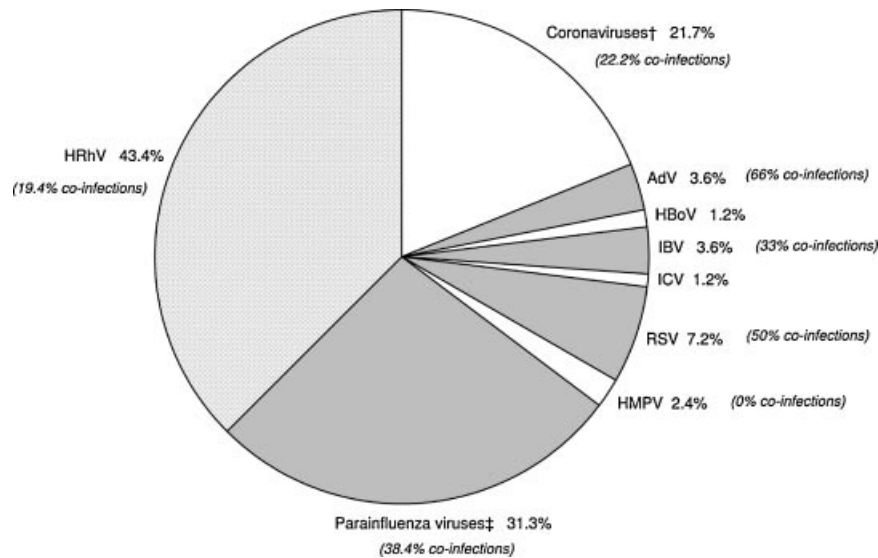
Fig. 2. Viral causes of influenza-like illness among 83 patients (non-mutually exclusive). [†]OC43 (55.5%); HKU1 (11.1%); NL63 (22.2%); 229E(11.1%). [‡]PIV-1 (23%); PIV-2 (11.5%); PIV-3 (65.4%).

among Cambodian patients [Institut Pasteur du Cambodge, unpublished work].

HCoV was detected in 7.6% of the influenza-like illness patients, which is comparable with data from other authors (France: 5.7%; Hong Kong: 2.1–4.4%), although target populations, inclusion criteria, seasonality, climate environment and diagnostic methods were different [Chiu et al., 2005; Lau et al., 2006; Vabret et al., 2008].

Due to the small sample sizes of each age group and the small number of viral infections, once stratified by age group the results should be interpreted with caution. It seems that HCoV-NL63 and HCoV-229E caused more infections in children <11 years and to a lesser extent in young adults <21 years ($P = 0.054$ and 0.143, respectively), while HCoV-HKU1 had a slight tendency to infect adults ≥21 years ($P = 0.51$). HCoV-

OC43 was detected more often in children than in adults ($P = 0.008$) and was also the most frequent of the four HCoV identified during the study. The predominance of HCoV-OC43 among the HCoV family has already been observed in other Asian countries [Lau et al., 2006].

Only six cases of RSV infection were detected, occurring in children ≤10 years (mean age 2.8 years, range 5 months to 7 years). Three single infections and three co-infections were observed. RSV is usually the most important viral cause of lower respiratory tract infections among infants and young children in both developing and developed countries [Selwyn, 1990]; and also infects the elderly [Falsey et al., 2005]. RSV has a worldwide distribution, with outbreaks occurring yearly with an unusually predictable and regular pattern. In temperate climates, RSV causes annual epidemics during the winter months [Collins et al., 1996], while

TABLE IV. Comparison of Virus Detection Rates Between Age Groups Among Influenza-Like Illness Patients/*Asymptomatic Individuals*

Age group (years)	Sample size	Number of positive cases (%)	P
0–10	85/19	47 (55.3)/4 (21.1)	0.007
≥11	149/71	35 (23.5)/5 (7.0)	0.003

TABLE V. Limit of Detection by RT-PCR After Serial Dilutions of Samples Tested Positive for Rhinovirus in Influenza-Like Illness (ILI) Patients and *Asymptomatic Individuals*

Dilution	ILI patients	Asymptomatic individuals	P-Value
No dilution	33 (100%)	8 (100%)	
1:10	2 (6.1%)	1 (12.5%)	0.5337
1:100	13 (39.4%)	1 (12.5%)	0.1500
1:1,000	8 (24.2%)	2 (25%)	0.9623
1:10,000	7 (21.2%)	3 (37.5%)	0.3354
>1:10,000	3 (9.1%)	1 (12.5%)	0.7713

epidemiological data from tropical regions have shown an association between RSV outbreaks and rainy seasons [Weber et al., 1998; Loscertales et al., 2002]. As is the case for HMPV, the low prevalence of respiratory infections caused by RSV in this study might be explained by the fact that the study was conducted during the dry season, or that the influenza-like illness definition does not fit well with symptoms observed during RSV infections.

According to the literature, HMPV is identified in 3% to nearly 20% of patients who suffer from acute respiratory infections, especially in children (Brazil: 17.8%; Germany: 18%; Hong Kong: 5.5%; Italy: 3,8%; Korea: 4.7%; Singapore: 5.3%) [Peiris et al., 2003; König et al., 2004; Choi et al., 2006; Loo et al., 2007; Thomazelli et al., 2007; Fabbiani et al., 2009]. The seasonal patterns of HMPV infections in tropical countries are not known. This study was conducted during the dry season only, which could explain why only two HMPV infections were detected. Indeed, recent data from a larger study confirmed that HMPV was responsible for infections in Cambodian patients but with a peak of transmission during the rainy season [Institut Pasteur du Cambodge, unpublished work]. In addition, the influenza-like illness case definition is not well adapted for HMPV infections which are usually associated with low or no fever, rhino-pharyngitis, and rarely cough.

Human AdV are usually responsible for up to 13% of acute respiratory tract infections (Australia: 6%; Brazil: 6.8%; Finland: 12%; Germany: 12.9%; Italia: 10.5%) [Gröndahl et al., 1999; Tsolia et al., 2004; Arden et al., 2006; Thomazelli et al., 2007; Fabbiani et al., 2009]. In Cambodia, only three samples (1.3%) were positive for AdV, and all were collected from patients under 5 years old. Similar prevalence was found in other Asian countries (China: 1.7%; Korea: 1.3%; Singapore: 0.3%) [Chung et al., 2007; Loo et al., 2007; Tang et al., 2008].

In Asia, the prevalence of HBoV in children experiencing acute respiratory infections is usually between 5% and 11% [Chung et al., 2006, 2007; Ma et al., 2006; Qu et al., 2007]. In Thailand, one of Cambodia's neighboring countries, a study recruiting patients from all ages showed that 3.9% of samples were positive for HBoV, and more commonly in children <4 years old [Fry et al., 2007]. In Cambodia, only one case was reported. The virus was detected in a 13-year-old adolescent. Additional studies are necessary to document the importance of HBoV as a viral agent responsible for respiratory infections.

Interestingly, HRhV and HBoV were also detected in individuals apparently healthy at the time of sample collection. HRhV appears to be as frequent in the control group as in the patient group, suggesting that these viruses can be detected in the respiratory tract without causing any symptoms and that the detection of these viruses is not necessarily associated with a disease. These results are consistent with other data demonstrating detection of HRhV and HBoV by RT-PCR in 15–30% and 5% of asymptomatic individuals, respectively [van Benten et al., 2003; García-García et al., 2008;

Jartti et al., 2008; Peltola et al., 2008]. Semi-quantitation revealed that the quantity of rhinovirus nucleic acid copies detected in the clinical specimens is similar in both groups and thus can not explain by itself the absence of symptoms in the control group. The number of cases in the present study is limited and the data should therefore be interpreted with caution. Nevertheless, similar findings showing comparable nucleic acid copy numbers in symptomatic and in asymptomatic individuals were obtained previously in Finland using quantitative RT-PCR [Peltola et al., 2008].

Previous studies have already attempted to assess the significance of viruses identified by PCR in asymptomatic subjects [Graat et al., 2003; van Benten et al., 2003; García-García et al., 2008; Jartti et al., 2008; Peltola et al., 2008]. The use of sensitive PCR techniques has obviously increased virus detection rates and not only in symptomatic but also in asymptomatic subjects, and has made the interpretation of positive test results somehow more complicated. The link of causality between virus identification and symptoms remains uncertain, especially when low copy numbers and multiple viruses are detected. Furthermore, at the time of specimen collection, the patient could be in an incubation period and not experiencing any symptoms. In addition, a subject could be asymptomatic whereas the PCR detects remnants of distant infection. For instance, HBoV and HRhV may persist several weeks in nasopharyngeal cavities in convalescent patients [Jartti et al., 2003; Wang et al., 2010]. Nevertheless, some limitations have to be taken into account for this study since the number of enrolled individuals is limited, especially in the asymptomatic group.

CONCLUSION

This is the first study testing patients with influenza-like illness for most respiratory viruses in Cambodia. The objective of the project was to describe the pattern of respiratory virus circulation during the dry season when such viruses are expected to circulate at low levels. Rhinoviruses, coronaviruses and PIVs are the more common viruses detected in patients as well as in asymptomatic persons. Several studies have been conducted previously to evaluate the viral etiologies of acute respiratory infections, but unfortunately few have compared results from symptomatic patients with those from asymptomatic individuals. This study raises the question of the significance of the detection of some respiratory viruses, especially when using highly sensitive detection methods, and of the link between the virus presence and the origin of the illness.

The data should now be completed by conducting an analysis of specimens collected during consecutive rainy seasons in order to ascertain the seasonal variation and to provide a better understanding of the epidemiology and spectrum of illness caused by respiratory viruses in tropical countries like Cambodia.

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