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. 

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KEY WORDS: multiplex PCR; respiratory virus; influenza-like illness; asymptomatic individuals

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; Mzzgerd, 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

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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 healthcare 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 140μl 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 200μl 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.](image)
### Table I. Primer Sequences Used in the 5 Multiplex PCR/RT-PCR Virus Assay

| Virus          | Assay | Primer designations | Primer sequences (5′–3′) | Expected amplicon size (bp) | Refs.                  |
|----------------|-------|---------------------|--------------------------|-----------------------------|------------------------|
| Multiplex 1    | IAV   | RT-PCR              | A1 (For)                 | CAG AGA CTT GAA GAT GTC TTT | 212                    | Adapted from Bellau-Pujol et al. [2005] |
|                |       |                     | A2 (Rev)                 | GCT CTT TCC ATG TTA TTT GGA(A) ATC | 212 | Adapted from Bellau-Pujol et al. [2005] |
|                |       | Hemi nested A1A3   | (For)                    | CTC TGA CTA AGG GGA TTT TG   | 130 | Bellau-Pujol et al. [2005] |
|                |       |                     | (Rev)                    | GCT CTT TCC ATG TTA GGA(A) ATC | 130 | Bellau-Pujol et al. [2005] |
|                | IBV   | RT-PCR              | B1 (For)                 | GAA AAA TTA CAC TGG TGG TGC GGT G | 365 | Adapted from Bellau-Pujol et al. [2005] |
|                |       |                     | B2B (Rev)                | AGC GGT CTC ATG TTT CTA TGC ATT GA | 260 | Adapted from Bellau-Pujol et al. [2005] |
|                |       | Hemi nested MIB3   | (Rev)                    | CAT GAA AAR TCA CAC TAT T (T) | 260 | Bellau-Pujol et al. [2005] |
|                | RSV   | RT-PCR              | Cane P1 (Rev)            | GAA AAA TTA CAC TGG TGG TGC G | 278 | Adapted from Bellau-Pujol et al. [2005] |
|                |       |                     | Cane P2 (Rev)            | CTG CTG TCA AGT CTA GAC TAC C GAG TATT | 340 | Bellau-Pujol et al. [2005] |
|                |       | Hemi nested VRSi   | (Rev)                    | GGT GAT CTC TGT TAC TCT C | 180 | Bellau-Pujol et al. [2005] |
|                | HMPV  | RT-PCR              | MPV M1a (For)            | GGA GTC CTA CAC AGT AGA C | 537 | Freymuth F [unpublished work] |
|                |       |                     | MPV M2 (Rev)             | GCA GGT CTA ACA GTA GGT C | 410 | Bellau-Pujol et al. [2005] |
| Multiplex 2    | PIV-1 | RT-PCR              | PiS1+ 1 (For)            | CCG GTA ATT TCT CAT ACC TAT G | 317 | Echevarria et al. [1998] |
|                |       |                     | PiS1-1 (Rev)             | CTT TGG AGC GGA CGT GTT AGG | 261 | Echevarria et al. [1998] |
|                |       | Hemi nested PARA2   | (For)                    | CTA GCT GAA CTA GAT GGT G | 340 | Bellau-Pujol et al. [2005] |
|                | PIV-2 | RT-PCR              | PiP2+ 1 (For)            | AAC AAT CTG CTA GAT TTT | 189 | Bellau-Pujol et al. [2005] |
|                |       |                     | PiP2- 1 (Rev)            | ATG TCA GAC AAT GGG GAA AT | 189 | Bellau-Pujol et al. [2005] |
|                |       | Hemi nested PARA3   | (Rev)                    | GCT GTA GAA CAT GAC TTC C | 145 | Bellau-Pujol et al. [2005] |
|                | PIV-3 | RT-PCR              | P3-1 1 (For)             | CTC GAG GTT GTC AGG ATA TAG | 308 | Karron et al. [1994] |
|                |       |                     | P3-2 1 (Rev)             | CTG TGG AGG TGG AAG ACA AGT | 308 | Karron et al. [1994] |
|                |       | Hemi nested PARA3i  | (Rev)                    | GCT GTA GAA CAT GAC TTC C | 145 | Bellau-Pujol et al. [2005] |
|                | PIV-4 | RT-PCR              | Pi4S+ 1 (For)            | CTC GAG GTT GTC AGG ATA TAG | 451 | Aguilar et al. [2000] |
|                |       |                     | Pi4S- 1 (Rev)            | CTG AAC GGG TGG ATC CAT G | 451 | Aguilar et al. [2000] |
| Multiplex 3    | ICV   | RT-PCR              | CHAA (For)               | ACA CTT CCA ACC CAA TTT TG | 485 | Zhang and Evans [1991] |
|                |       |                     | CHAD (Rev)               | CCT GAC ACG AAC TCC C TC AT | 391 | Zhang and Evans [1991] |
|                |       | Hemi nested MICi    | (Rev)                    | GAG GAT TGG GCA ACT ATC CAT | 391 | Bellau-Pujol et al. [2005] |
|                | HRhV  | RT-PCR              | SRH1 1 (For)             | GCA TCI GGY ARY TCC CAC CAN CC | 549 | Savolainen et al. [2002] |
|                |       |                     | SRH2 1 (Rev)             | GGG ACC AAC TAC TTT GGG TGG TGG TCG | 549 | Savolainen et al. [2002] |
|                |       | Hemi nested NESTRHI | 1 (Rev)                  | ATG GGN GCW CAN GTN TCH ANH CA | 450 | Bellau-Pujol et al. [2005] |
|                | SARS- | RT-PCR              | SRHI 1 (For)             | GCA TCI GGY ARY TCC CAC CAN CC | 195 | Bellau-Pujol et al. [2005] |
| CoV            |       |                     | SARS-Bi out-AS (For)     | CAT AAC CAG TCG TGA CAG CTA | 195 | Bellau-Pujol et al. [2005] |
|                |       |                     | SARS-Bi out-S2 (Rev)     | RTC TAG TAC CAA GTG TAA CAT GGT | 110 | Drosten et al. [2003] |
|                |       | Nested SARS-Bi     | in-AS (For)              | CTG TAG AAA ATC CTA GCT GGA | 110 | Drosten et al. [2003] |
|                |       |                     | SARS-Bi in-S (Rev)       | GAA GCT ATT CGT CAC GTG G | 110 | Drosten et al. [2003] |
| Multiplex 4    | HCoV- | RT-PCR              | MF3 1 (For)              | GGC TTA TGT GGC CCC TTA CT | 334 | Vabret et al. [2001] |
| OC43           |       |                     | MF2i 1 (Rev)             | GGC TTA TGT GGC CCC TTA CT | 170 | Vabret et al. [2001] |
|                |       | Hemi nested MF3     | (Rev)                    | GGC TTA TGT GGC CCC TTA CT | 170 | Bellau-Pujol et al. [2005] |
|                |       |                     | MF1 1 (For)              | GGC TTA TGT GGC CCC TTA CT | 170 | Bellau-Pujol et al. [2005] |
Multiplex 5 was developed for the simultaneous detection of the four HCoVs using primers described by others [Vabret et al., 2001, 2005; Woo 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 OneStep 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 OneStep 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 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.

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 10⁵ 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, HCoV-229E, HCoV-HKU1, HCoV-NL63.

**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] |
|               | Hemi nested   | MD3 (Rev)          | CCT GAA CAC CTG AAG CCA AT                       | 230                         | Vabret et al. [2001] |
|               |               | MD2i (Rev)         | CCG TAT CAA CAC TCG TTA TGT GG                   |                             | Bellau-Pujol et al. [2005] |
|               |               | MD1 (For)          | TGG CCC CAT TAA AAA TGT GT                       | 443                         | Bellau-Pujol et al. [2005] |
| HCoV-HKU1     | RT-PCR        | HKU-N-sens3 (For)  | ATC TGA GCG AAA TTA CCA AAC                      |                             | Woo et al. [2005] |
|               |               | HKU1 antisense (Rev) | CCG 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                      | 354                         | Palacious et al. [2003] |
|               | HBoV PCR      | 542R (Rev)         | GAS CTC TGT AAC TAC TAT TAC                     |                             | Allander et al. [2005] |
|               |               |                    | CTC TGT GTT GAC TGA ATA CAG                    |                             |               |

Continued
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 $\chi^2$ 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%) HRhV detections 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). PIs 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 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.

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

| Multiplex | Virus       | Expected amplicon size (bp) | Sensitivity of monoplex PCR/RT-PCR (number of copy/$\mu$l of VTM$^a$) | Sensitivity of multiplex PCR/RT-PCR (number of copy/$\mu$l 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.
### TABLE IIIa. Number of Detections (% of Total Samples) of Respiratory Viruses Among Influenza-Like Illness (ILI) Patients and Asymptomatic Individuals

| Viral Cause | ILI patients | Asymptomatic individuals |
|-------------|--------------|--------------------------|
| IAV         | 0            | 0                        |
| IBV         | 3 (1.3)      | 0                        |
| VRS         | 6 (2.6)      | 0                        |
| HMPV        | 2 (0.8)      | 0                        |
| PIV-1       | 17 (7.3)     | 0                        |
| PIV-2       | 0            | 0                        |
| PIV-4       | 1 (0.4)      | 0                        |
| ICV         | 36 (15.4)    | 8 (8.9)                  |
| HRhV/EnV    | 0            | 0                        |
| SARS-CoV    | 10 (4.3)     | 2 (0.8)                  |
| HCoV-OC43   | 2 (0.8)      | 2 (0.8)                  |
| HCoV-229E   | 4 (1.7)      | 3 (1.3)                  |
| HCoV-HKU1   | 3 (1.3)      | 1 (0.4)                  |
| HCoV-NL63   | 83 (35.5)    | 9 (10)                   |
| AdV         | 0            | 0                        |
| HBoV        | 0            | 0                        |
| Total       | 83 (35.5)    | 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 | ILAV | IBV | VRS | HMPV | PIV-1 | PIV-2 | PIV-4 | ICV | HRhV/EnV | SARS-CoV | HCoV-OC43 | HCoV-229E | HCoV-HKU1 | HCoV-NL63 | AdV | HBoV | Total |
|------------|------|-----|-----|------|-------|-------|-------|-----|----------|----------|-----------|-----------|-----------|-----------|----------|------|-------|--------|
| 0–4 years  | 40/8 | 0/0 | 1 (2.5)/0 | 0/0 | 0/0 | 4/0 | 0/0 | 0/0 | 0/0 | 7 (17.5)/0 | 3 (7.5)/0 | 0/0 | 0/0 | 1 (2.5)/0 | 0/0 | 0/0 | 14 (35)/0 |
| 5–10 years | 45/11| 0/0 | 2 (4.4)/0 | 0/0 | 0/0 | 2 (5.3)/0 | 0/0 | 0/0 | 0/0 | 9 (20)/3 | 4 (8.9)/0 | 4/0 | 0/0 | 2 (4.4)/0 | 0/0 | 0/0 | 23 (51.1)/4 |
| 11–20 years| 38/15| 0/0 | 1 (2.6)/0 | 0/0 | 0/0 | 2 (5.3)/0 | 0/0 | 0/0 | 0/0 | 6 (15.8)/2 | 1 (2.6)/0 | 1 (2.8)/0 | 1 (2.8)/0 | 2 (4.4)/0 | 0/0 | 15 (39.5)/2 |
| 21–30 years| 36/18| 0/0 | 1 (2.6)/0 | 0/0 | 0/0 | 1 (2.6)/0 | 0/0 | 0/0 | 0/0 | 4 (11.1)/3 | 1 (2.6)/0 | 1 (2.8)/0 | 1 (2.8)/0 | 0/0 | 0/0 | 9 (25)/3 |
| 31–40 years| 29/15 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 1 (4.5)/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 2 (6.9)/0 |
| 41–50 years| 22/11 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 1 (4.5)/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 3 (13.6)/0 |
| >50 years  | 24/12 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 2 (8.3)/0 | 0/0 | 0/0 | 0/0 | 0/0 | 0/0 | 3 (12.5)/0 |
| Total      | 234/90 | | | | | | | | | | | | | | | | |
### TABLE IIIc. Number of Detections (% of Total Samples) of Viruses Detected in Co-Infections Among Influenza-Like Illness (ILI) Patients/Asymptomatic Individuals

| Age Group | Sample Size | Average | CI: 95% | Limit of Detection |
|-----------|-------------|---------|---------|-------------------|
| 0–4 years | 40/8        | 6.1     | 6.1–7.1 | 1:10              |
| 5–10 years| 45/11       | 13.7    | 13.7–15.7| 1:1,000          |
| >10 years | 38/15       | 26.8    | 26.8–29.6| 1:1,000          |
| Total     | 234/90      | 46.2    | 46.2–49.2| 1:1,000          |

#### 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 used 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 [Gromndahl 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 [Gromndahl et al., 1999; Thomazelli et al., 2007]. PIV-4 was never detected and appears in general to be found rarely...
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%) | 0.5337 |
| 1:10     | 2 (6.1%)    | 1 (12.5%)    | 0.1500 |
| 1:100    | 13 (39.4%)  | 1 (12.5%)    | 0.9623 |
| 1:1,000  | 8 (24.2%)   | 2 (25%)      | 0.3354 |
| 1:10,000 | 7 (21.2%)   | 3 (37.5%)    | 0.7713 |
| >1:10,000| 3 (9.1%)    | 1 (12.5%)    |         |
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%; Italy: 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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contents of the manuscript, including the results or interpretation of publication.

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