Short Report: Dual Infection of Novel Influenza Viruses A/H1N1 and A/H3N2 in a Cluster of Cambodian Patients

Christopher A. Myers, Matthew R. Kasper, Chadwick Y. Yasuda, Chin Savuth, David J. Spiro, Rebecca Halpin, Dennis J. Faix, Robert Coon, Shannon D. Putnam, Thomas F. Wierzba, and Patrick J. Blair*

Naval Health Research Center, San Diego, California; US Naval Medical Research Unit No. 2, Phnom Penh, Kingdom of Cambodia; National Institute of Public Health, Ministry of Health, Phnom Penh, Kingdom of Cambodia; J. Craig Venter Institute, Rockville, Maryland

Abstract. During the early months of 2009, a novel influenza A/H1N1 virus (pH1N1) emerged in Mexico and quickly spread across the globe. In October 2009, a 23-year-old male residing in central Cambodia was diagnosed with pH1N1. Subsequently, a cluster of four influenza-like illness cases developed involving three children who resided in his home and the children’s school teacher. Base composition analysis of internal genes using reverse transcriptase polymerase chain reaction and electrospray ionization mass spectrometry revealed that specimens from two of the secondary victims were coinfected with influenza A/H3N2 and pH1N1. Phylogenetic analysis of the hemagglutinin genes from these isolated viruses showed that they were closely related to existing pH1N1 and A/H3N2 viruses circulating in the region. Genetic recombination was not evident within plaque-purified viral isolates on full genome sequencing. This incident confirms dual influenza virus infections and highlights the risk of zoonotic and seasonal influenza viruses to coinfect and possibly reassort where they cocirculate.

Globally, influenza remains a leading cause of human morbidity and mortality, largely as a result of the virus’s inherent evasiveness from the immune response. Coinfection of viruses in birds or mammals, such as swine, increases the chance for the emergence of new variants. Novel viruses can emerge within a population, evade immunity, and result in local epidemics or in some instances, pandemics. However, recombination among subtypes remains rare. In early 2009, a novel influenza A/H1N1 virus (pH1N1) emerged in Mexico. By October 2009, pH1N1 had become the predominant influenza subtype infecting populations in most areas of the world. Notwithstanding, in Southeast Asia, seasonal influenza viruses as well as the avian influenza virus A/H5N1 continued to circulate [World Health Organization (WHO) Pandemic (H1N1) 2009–Update 82; http://www.who.int/csr/don/2010_01_08/en/index.html]. In the Southeast Asian nation of Cambodia, we and others have shown that cases of influenza peak with the monsoon between the months of July and December.

In early October 2009, a 23-year-old man from central Cambodia presented to the Ta Khmau health clinic with influenza-like symptoms (Table 1). Real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assays to detect influenza A and B viruses were used to diagnose pH1N1 infection. The patient received treatment to alleviate symptoms and recovered at his residence.

On October 14, 2009, three male (M) children, ages 13, 8, and 4 years, who lived in the same home as the suspected index case presented at the health clinic with fever (39°C), cough, sore throat, headache, and symptoms of either nausea or vomiting (Table 1). The students attended classes in a single-room school. They reported neither recent extended contact with animals nor travel. A day after their illness, their teacher reported febrile illness, and samples were obtained. Specimens from one of three school children and the teacher indicated dual infection with both seasonal A/H3N2 and pH1N1 viruses.

Virus isolation from collected clinical specimens was performed in Madin-Darby canine kidney (MDCK) cells and shell vials. Isolated viruses were analyzed on the Ibis T5000 by ESI-MS (Ibis Biosciences, Inc., Carlsbad, CA) analysis to generate a specific mass measurement for each amplified PCR product. Primer sequences and other PCR components were as previously described. The base composition signature for the product was then compared with known sequences in a database to generate an internally verified identification. Analyses from six regions of the influenza genome confirmed pH1N1 infection in the 23-year-old man and dual A/H3N2 and pH1N1 infection in specimens from one of three children (8/M) and the teacher (24/female [F]). Only A/H3N2 viruses were evident in samples from the 4/M and 13/M victims. Analysis did not discern recombinated signatures in gene segments within the isolated viruses (Table 2).

To fully characterize the gene segments of dual-infected individuals, single-passaged viruses from two of the patients and six of corresponding purified virus plaques were processed for pyrosequencing using a specialized multisegment RT-PCR procedure to amplify the genome of all subtypes of the influenza A virus through degenerate primers. Sequencing, genome assembly, and closure reactions were performed as previously described. Complete genomes (> 99% open reading frame [ORF]) were obtained for all eight segments of each virus isolate. A complete ORF region (100% genome length) was obtained for all isolates. Sequences for the hemagglutinin (HA) segment from the isolates were compared with known sequences (data not shown). Relative to A/Perth/16/2009 (H3N2), the H3N2 vaccine component for 2010 and 2011, a total of 3 aa substitutions were seen in the area sequenced, I25V, P162T, and S214I, with an overall similarity of 99%. The latter mutation corresponds to a previously identified antibody combining site. More complete datasets for recent swine strains allowed for a fuller comparison of pH1N1 HA sequences. Comparison between the pH1N1 reference strain, A/California/04/2009, and the full genome sequence of isolate material from 4/M revealed 4 aa changes from the vaccine strain (P100S, S220T, I338V, and Y528H), with an overall similarity of 99.3% over 566 aa. Phylogenetic analysis of the HA genes of all analyzed plaques revealed a single genetic sequence for both the A/H3N2 and pH1N1 strains. None of the isolated plaques showed evidence of recombination between...
pH1N1 and A/H3N2, and all had full sequences for the eight influenza segments from both strains.

Herein, we describe a cluster of influenza-like illness (ILI) cases at a school in central Cambodia. Among the afflicted, two were coinfected with A/H3N2 and pH1N1 influenza viruses. The finding of coinfections has rarely been reported. A recently study of over 2,000 clinical samples found no dual infection. However, coinfection of pH1N1 and A/H3N2 has been reported in a 38-year-old woman from Singapore, and mixed infection was also evident in six individuals after an outbreak of influenza at a college campus near Beijing, China. A more recent New Zealand study collected and screened 1,044 clinical samples during the pandemic and found 11 coinfections with A/H1N1 seasonal viruses. Transmission of pH1N1 at a time when seasonal influenza viruses were circulating in Cambodia resulted in coinfection and raised the possibility of reassortment. The generation of novel influenza viruses through reassortment has occurred when zoonotic viruses mix in birds, swine, and humans, and gene segments are reshuffled. Pandemic strains often are the result of emerging viruses from reservoirs to which humans have little or no immunity. The A/H2N2 1957 and A/H3N2 1968 pandemics occurred after reassortment between human and avian strains. The 1957 virus was generated when A/H1N1 1918 reassorted with avian viruses to pick up new PB1, HA, and neuraminidase (NA) segments. Similarly, the novel virus isolated from ILI cases in southern California in April 2009 contained genetic elements from four different sources, including North American swine influenza viruses, North American avian influenza viruses, human influenza viruses, and a Eurasian swine influenza virus. In our analysis, recombination was not detected in viruses isolated from the Cambodian patients.

The clinical disease within the dual A/H3N2 and A/H1N1 Cambodian patients did not result in hospitalization nor did these patients’ disease seem more severe than the disease in the other patients with influenza. Clinical findings were broad, including upper respiratory and gastrointestinal symptoms. None of five patients in this outbreak had been vaccinated against either seasonal or pH1N1 influenza infections. Indeed, in rural Cambodia, little seasonal influenza vaccination is conducted, and use of therapeutics such as neuraminidase inhibitors is rare; thus community-wide immunity is lacking.

Southeast Asia has proven to be a critical region for the adaptation and emergence of variants of seasonal influenza viruses as well as an area of zoonotic virus transmission in humans. Cases of A/H5N1 have largely been restricted to the Near East and southeast Asia, with Cambodia suffering 15 confirmed human cases and 13 fatalities since 2005. The endemicity of A/H5N1 in poultry in many areas of southeast Asia provides increased opportunity for human exposure and adaptation of a lethal virus suitable for sustained human transmission. Our findings emphasize the importance

### Table 1
Demographics of Cambodian cases involved in influenza cluster

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)/sex</th>
<th>Disease onset</th>
<th>Date sampled</th>
<th>Occupation</th>
<th>Clinical findings*</th>
</tr>
</thead>
</table>

*Recorded on date of sample collection.

### Table 2
Base composition data from clinical samples

<table>
<thead>
<tr>
<th>Patient</th>
<th>Detection*</th>
<th>Target segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>23/M</td>
<td>pH1n1</td>
<td>PB1, NP, M1, PA, NS1, NS2</td>
</tr>
<tr>
<td>13/M</td>
<td>H3N2</td>
<td>G32/C24/T33, G21/C20/T25, G26/C24/T29, G25/C27/T24, G36/C20/T28, ND</td>
</tr>
<tr>
<td>8/M</td>
<td>H3N1</td>
<td>G30/C23/T34, G21/C20/T25, G27/C24/T29, G24/C26/T25, G25/C19/T29, G28/C15/T27</td>
</tr>
<tr>
<td>8/M</td>
<td>H3N2</td>
<td>G30/C23/T34, G21/C20/T25, G27/C24/T29, G24/C26/T25, G25/C19/T29, G28/C15/T27</td>
</tr>
<tr>
<td>4/M</td>
<td>H3N2</td>
<td>G30/C23/T34, G21/C20/T25, G27/C24/T29, G24/C26/T25, G25/C19/T29, G28/C15/T27</td>
</tr>
<tr>
<td>24/F</td>
<td>pH1n1</td>
<td>G32/C24/T33, G21/C20/T25, G27/C24/T29, G24/C26/T25, G25/C19/T29, G28/C15/T27</td>
</tr>
<tr>
<td>24/F</td>
<td>H3N2</td>
<td>G30/C23/T34, G21/C20/T25, G27/C24/T29, G24/C26/T25, G25/C19/T29, G28/C15/T27</td>
</tr>
</tbody>
</table>

ND = not determined.
*The listing of strain determinations made by the PlexID instrument based on the base compositions detected in the sample.
†The more recent version of the influenza surveillance plate that was used for sample 23/M does not include an NS2 primer pair.
of national and international cooperation to survey for the emergence of novel and/or reassorted influenza viruses.

Received February 16, 2011. Accepted for publication June 6, 2011.

Acknowledgments: This work would not be possible without the substantial daily efforts of the staff at the clinical sites in Kandal Province. The authors thank the laboratories at Naval Health Research Center and J. Craig Venter Institute and the US Naval Medical Research Unit No. 2 and National Institute of Public Health, Kingdom of Cambodia, for their contributions in diagnosing and characterizing resulting viruses. This research has been conducted in compliance with all applicable federal regulations governing the protection of human subjects in research (Protocols NAMRU2:2005.0004 and NHRCL:2010.0007).

Financial support: This work was funded in part by grants from the US Department of Defense Armed Forces Health Surveillance Center division of the Global Emerging Infections Surveillance and Response System (AFHSC/GEIS) and the US Defense Advanced Research Projects Agency (DARPA) under work unit number 60941. A portion of this project was funded by the National Institute of Allergy and Infectious Diseases, National Institute of Health, Department of Health and Human Services under contract number HHSN272200900007C.

Disclaimer: The views expressed in this article are of the authors and do not reflect the official policy or position of the Department of the Navy, the Department of Defense, or the US Government.

Authors’ addresses: Christopher A. Myers, Dennis J. Faix, and Robert Coon, Naval Health Research Center, San Diego, CA, E-mails: Chris.Myers2@med.navy.mil, dennis.faix@med.navy.mil, and robert.coon@med.navy.mil. Matthew R. Kasper, Chadwick Y. Yasuda, Shannon D. Putnam, and Thomas F. Wierzbz, US Naval Medical Research Unit No. 2, Phnom Penh, Kingdom of Cambodia, E-mails: Matthew.Kasper@med.navy.mil, Chai@namru2.org.kh, shan.putnam@med.navy.mil, and twierzbz@NLM.int, Chin Suvath, National Institute of Public Health, Ministry of Health, Phnom Penh, Kingdom of Cambodia, E-mail:savuth_chin@yahoo.com. David J. Spiro and Rebecca Halpin, J. Craig Venter Institute, Rockville, MD, E-mails: david.spiro@nih.gov and rhalpin@jcv.org. Patrick J. Blair, Naval Health Research Center, J. Craig Venter Institute, Rockville, MD, E-mails: david.spiro@nih.gov and rhalpin@jcv.org. David J. Spiro and Rebecca Halpin, Health, Ministry of Health, Phnom Penh, Kingdom of Cambodia, E-mails: Matthew.Kasper@med.navy.mil, Chad@namru2.org.kh, shan.putnam@med.navy.mil, and twierzbz@NLM.int, Chin Suvath, National Institute of Public Health, Ministry of Health, Phnom Penh, Kingdom of Cambodia, E-mail:savuth_chin@yahoo.com. David J. Spiro and Rebecca Halpin, J. Craig Venter Institute, Rockville, MD, E-mails: david.spiro@nih.gov and rhalpin@jcv.org. Patrick J. Blair, Naval Health Research Center, San Diego, CA and US Naval Medical Research Unit No. 2, Phnom Penh, Kingdom of Cambodia, E-mail:patrick.blair@med.navy.mil.

REFERENCES