# Influenza A/H5N1 virus infection in humans in Cambodia

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## Abstract

*Background:* Between January 2005 and April 2006, six patients of influenza A/H5N1 virus infection were reported in Cambodia, all with fatal outcome.
*Objectives:* We describe the virological findings of these six H5N1 patients in association with clinical and epidemiologic findings.
*Study design:* Broncho-alveolar lavage, nasopharyngeal, throat and rectal swabs and sera were cultured for virus isolation and viral load quantified in clinical specimens by real-time RT-PCR. We compared sequences obtained from different body sites within the same patient to detect viral quasi-species.
*Results:* H5N1 virus strains isolated in Cambodia belong to genotype Z, clade 1 viruses. H5N1 viruses were isolated from serum and rectal swab specimens in two patients. The haemagglutinin gene sequences of the virus in different body sites did not differ. Amino acid substitutions known to be associated with a change in virus binding were not observed.
*Conclusion:* The high frequency of virus isolation from serum and faecal swabs highlights that H5N1 is likely to be a disseminated infection in humans and this has implications for antiviral treatment, biosafety in clinical laboratories and on risks for nosocomial and human-to-human transmission. There were no tissue-specific adaptive mutations in the HA gene from viruses isolated from different organs.
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*Keywords:* Avian influenza virus; H5N1; Cambodia; Tissue tropism; Virus dissemination

## 1. Introduction

During January 2005–April 2006, six patients with H5N1 disease were confirmed in Cambodia, four in 2005 and two in 2006. In this study, we describe the virological findings of these patients in association with clinical and epidemiologic findings.

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## 2. Methods

### 2.1. Specimen collection

Broncho-alveolar lavage, nasopharyngeal, throat, rectal swab specimens and sera were collected at the Cambodian reference hospitals except for patients 1 and 4 who died in Vietnamese hospitals, near the Vietnam–Cambodia border. Swabs were transported to the Institut Pasteur in Cambodia or to the Institut Pasteur in Ho Chi Minh City in virus transport medium (VTM) at 4 °C. All samples were collected on admission and where possible, second specimens were obtained for confirmation of the diagnosis. Nasopharyngeal and throat swab and sera were subsequently systematically collected among close contacts which included household members and health care workers.

### 2.2. RNA extraction and PCR

Viral RNA extraction was performed in a biosafety level 2 laboratory with enhanced containment practices. Nucleic acid was extracted with the use of viral RNA kit (QIAamp, Qiagen®) and tested by conventional reverse-transcriptase-polymerase-chain reaction (RT-PCR) and real-time RT-PCR. Real-time RT-PCR for H5 used H5 specific primer pairs and probe (detailed protocol can be provided upon request). H5 quantified synthetic RNA was used as internal control and to determine the viral load. M and N1 genes were amplified by RT-PCR according WHO recommendations (WHO, 2004).

### 2.3. Sequencing

For direct sequencing of viral nucleic acids from clinical specimens, gene fragments were amplified and sequenced with the use of Big Dye terminator cycle sequencing kit (Ver-

sion 3.1, Applied Biosystems) on an ABI3730XL system. In selected clinical specimens, the PCR amplicons were cloned into a Topo plasmid vector and multiple clones sequenced to detect evidence of viral quasi-species.

### 2.4. Virus isolation

Virus isolation was carried out by inoculation on to Madin-Darby canine kidney (MDCK) cells in a biosafety level 3 laboratory.

### 2.5. Serology

Sera were tested by the Hong Kong Government Virus Unit Laboratory for detection of anti-H5 neutralising antibodies by microneutralisation assay and confirmatory Western blot assay. A positive serology was defined as an antibody titre equal or greater than 80 with a confirmatory Western blot assay (Rowe et al., 1999).

## 3. Results

The diagnosis of H5N1 infection was confirmed by detecting virus RNA by RT-PCR from broncho-alveolar lavage, nasopharyngeal, throat and rectal swab specimens and from serum collected from 6 hospitalised patients on the day of hospitalisation (Table 1). The median duration between the symptom onset and hospitalisation was 5.7 days (range 2–7) (Table 1). The mean age of the patients was 16 years (range 3–28). Four patients were female. Except for patient 1 who nursed her sick brother, all patients had a history of contact with sick or dead poultry. It is thought that she may have been infected when nursing and wiping out the body of her bother who had died with undiagnosed severe pulmonary infection

Table 1
Clinical and biological information of each H5N1 patient collected on the day of admission at the hospital

| Variable | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 |
|---|---|---|---|---|---|---|
| Time from onset of symptoms to hospitalisation (days) | 7 | 2 | 7 | 6 | 6 | 6 |
| Time from onset of symptoms to death (days) | 10 | 3 | 7 | 7 | 7 | 6 |
| Sex | F | M | F | F | F | M |
| Age (year) | 25 | 28 | 8 | 20 | 3 | 12 |
| Cough | Yes | Yes | Yes | Yes | Yes | Yes |
| Sore throat | NA | No | Yes | NA | NA | NA |
| Dyspnea | Yes | Yes | Yes | Yes | Yes | Yes |
| Presence or history of diarrhoea | Yes | Yes | No | Yes | Yes | No |
| Presence or history of abdominal pain | NA | NA | NA | Yes | Yes | NA |
| History of fever | Yes | Yes | Yes | Yes | Yes | Yes |
| Temperature at admission (°C) | NA | 39.7 | NA | NA | 36.5 | 37.5 |
| Leucocyte count (cells/mm$^3$) | 4700 | 3410 | 4300 | 6000 | 3600 | 4100 |
| Lymphocytes count (cells/mm$^3$) | 1000 | 310 | 1200 | NA | 1700 | 287 |
| Alanine aminotransferase (U/l) | <40 | NA | 690 | NA | 8750 | 216 |
| Aspartate aminotransferase (U/l) | <40 | NA | 99 | NA | 3230 | 60 |
| Viral load in serum (copies of cDNA/ml) | 0 | $8.2 \times 10^5$ | $3.2 \times 10^5$ | $6.3 \times 10^5$ | $4.3 \times 10^6$ | $1.7 \times 10^6$ |
| Nasopharyngeal swab specimen (number cDNA copies/ml of VTM) | $2.9 \times 10^5$ | $1.2 \times 10^6$ | $6 \times 10^5$ | $2.7 \times 10^5$ | $5 \times 10^7$ | $1.7 \times 10^7$ |
| Throat swab specimen (number of cDNA copies/ml of VTM) | NA | $8.7 \times 10^5$ | $4.2 \times 10^5$ | NA | $1.6 \times 10^5$ | $2.5 \times 10^7$ |
| Brocho-alveolar wash (number of cDNA copies/ml) | NA | $1.0 \times 10^7$ | $9.9 \times 10^8$ | NA | NA | $2.4 \times 10^8$ |
| Rectal swab specimen (number of cDNA copies/ml of VTM) | NA | $1.5 \times 10^6$ | $3.4 \times 10^5$ | NA | $1.7 \times 10^6$ | $8.6 \times 10^2$ |

Table 2
Comparison of H5N1 virus gene sequence results obtained from MDCK cell virus isolates, from RT-PCR amplicons directly from specimens and from multiple plasmid clones

| Patient | Specimen | Sequencing after propagation in MDCK cells | Direct sequencing of RT-PCR amplicon or sequencing of cloned plasmids | Comments |
|---|---|---|---|---|
| 1 | Respiratory | No | Direct sequencing (HA, NA) | |
| 2 | Respiratory | Yes | Direct sequencing (PB2, HA, NA, M, NS) and cloned plasmids (all segments) | |
| 3 | Respiratory | No | Direct sequencing (all segments) | Mutation Lys627 on PB2 |
| 4 | Respiratory | No | Direct sequencing (HA) | |
| 5 | Respiratory | Yes | Direct sequencing (PB2, HA, NP, NA, M, NS) | Deletion Arg position -6 cleavage site HA |
| | | | Cloned plasmids (all segments) | Deletion Arg position -6 cleavage site HA (9/9 clones) and mutation 701N (1/9 clones) |
| | Serum | Yes | Cloned plasmids (HA) | Deletion Arg position -6 cleavage site HA (20/20 clones) |
| | | No | Cloned plasmids (HA) | Deletion Arg position -6 cleavage site HA (20/20 clones) and mutation E324G (1/20 clones) |
| | Rectum | Yes | Cloned plasmids (HA) | Deletion Arg position -6 cleavage site HA (20/20 clones) |
| 6 | Respiratory | No | Direct sequencing (HA, NP, NA, M, NS) | No deletion at the cleavage site |
| | Serum | No | Cloned plasmids (HA) | No deletion at the cleavage site (15/15 clones) |
| | | Yes | Cloned plasmids (HA) | Deletion Arg position -6 cleavage site HA (15/15 clones) |
| | Rectum | Yes | Cloned plasmids (HA) | Deletion Arg position -6 cleavage site HA (15/15 clones) |

2 days prior to her symptoms onset and who was cremated before specimens could have been collected.

Lymphopenia ($<1000$ cells/mm$^3$) was observed in two of five patients and elevated liver enzymes in three of four patients. While viral load (i.e. number of copies of complementary DNA (cDNA) per millilitre of VTM) of parallel nasopharyngeal swabs and throat swabs of four patients did not differ (Table 1), in three patients who also had parallel broncho-alveolar lavage specimens available, the broncho-alveolar lavage specimen had 10–100-fold higher viral loads even though larger rinsing volumes were used for broncho-alveolar lavage specimens. Viral RNA was detectable in serum of five of six patients with viral loads ranging from $3.2 \times 10^5$ to $4.3 \times 10^6$ cDNA copies/ml. The four patients with RT-PCR positive faecal swabs had virus load ranging from $8.6 \times 10^2$ to $1.7 \times 10^6$ cDNA copies/ml of VTM. The patient with low faecal viral load was constipated upon admission at the hospital. Virus isolates were obtained from respiratory samples in all six patients and from two sera and two rectal specimens (patients 5 and 6).

The titres of neutralising antibodies were $<80$ but this was not unexpected since these patients died within the first 2 weeks of illness. Patient's contacts, including relatives ($n = 10$), health care workers ($n = 28$) and close contact villagers ($n = 42$) were found seronegative by microneutralisation test. All these individuals were also clinically monitored for 10 days and systematically tested for H5N1 detection by real-time and conventional RT-PCR on nasopharyngeal and throat swab specimens. Results were all negative.

Full genome sequencing of the virus isolates from the respiratory tract of all six patients and from the serum and faeces of patients 5 and 6 was attempted. The phylogenetic analysis showed that Cambodian H5N1 belonged to genotype Z, clade 1 and were closely related to viruses isolated from southern Vietnam in 2005 (data not shown). The viruses from patients 1 to 4 contained the motif of multiple basic amino acids (PQRERRRKKR↓ G) at the cleavage site of the haemagglutinin (HA) molecule indicating that these were highly pathogenic avian influenza viruses. Two patients (patient 5 and 6) had a deletion of one Arginine at position $-6$ from the cleavage site. In order to investigate whether the deletion at position $-6$ from the cleavage site was tissue specific, we compared the sequences of the HA protein from H5N1 viruses isolated from nose, throat, rectum and serum in these two patients (Table 2). For patient 5, the deletion was present in MDCK cell grown virus isolates from respiratory and rectal swab specimens, from the virus isolate from the serum as well as from all 20 cDNA clones derived from direct PCR amplification from the serum. However, for patient 6, the deletion was only observed in virus isolates from serum and rectal specimens but not in sequences obtained by direct sequencing from respiratory specimens or from the 15 cDNA clones derived from direct RT-PCR amplification of the HA gene extracted from the serum. Amino acid substitutions known to be associated with a change in virus binding from sialic acid $\alpha2,3$ galactose (SA$\alpha2,3$ Gal) to SA$\alpha2,6$ Gal were not observed (Yamada et al., 2006). But a substitution Ser123Thr occurred in the HA of the virus obtained from the respiratory specimen of patient 3.

We sequenced the PB2 gene in three patients (patients 2, 3 and 5). The virus isolated from patient 3, in 2005, showed the mutation Lys627 in the PB2 gene. One of six independent plasmid clones sequenced from virus in respiratory specimens in patient 5 had a 701Asn mutation. None

of the Cambodian viruses had a glutamate at the amino acid position 92 in the NS1 protein (Seo et al., 2002).

As with other clade 1 viruses in Vietnam and Thailand, the gene coding for the M2 protein of all the Cambodian virus isolates have Leu26Ile and Ser31Asn amino-acid substitutions that are associated with resistance of the M2 ion channel-blocking adamantine derivatives (amantadine, rimantadine) (WHO, 2005; Cheung et al., 2006). The mutation H274Y on the neuraminidase (NA) gene which is associated with resistance to oseltamivir (Tamiflu®) was not found (Smith et al., 2006).

## 4. Discussion

The clinical presentation of the Cambodian patients is similar to that reported elsewhere (Hien et al., 2004; Kandun et al., 2006). The high case fatality rate is likely a reflection of the late hospital admission of the Cambodian cases. Fever was only present on admission in one patient. This observation may have been associated with shock syndrome as part of the severe respiratory distress or antipyretic intake before admission to the intensive care units (ICU); the latter information was unfortunately not reported in the medical records.

Despite the late presentation in the course of the illness, H5N1 virus was detected in all patients and in all the specimens tested, except for the serum of one patient. The viral loads in the clinical specimens of our patients are comparable with those reported in Vietnamese patients with a fatal outcome (de Jong et al., 2006). Our data emphasizes the diagnostic value of testing faecal and serum specimens as well as respiratory specimens for viral RNA. In agreement with a previous report on two patients (de Jong et al., 2006), we found viral load in the broncho-alveolar lavage was higher than in upper respiratory specimens highlighting that lower respiratory specimens are likely to be the most productive for viral diagnosis. These findings are in agreement with the observations that H5N1 viruses can replicate in the lower respiratory tract (Shinya et al., 2006).

H5N1 RNA was detected in sera and rectal swabs from patients examined with typical respiratory presentation of H5N1 disease. More importantly, infectious virus was isolated from 2 of these 5 sera and from 2 of 4 faecal specimens indicating that H5N1 virus is replicating in sites outside of the respiratory tract. The isolation of H5N1 virus from serum was only previously reported in two other patients (de Jong et al., 2006; Chutinimitkul et al., 2006). Similarly, virus isolation from faeces was only previously reported from two patients (Uiprasertkul et al., 2005; de Jong et al., 2005). Interestingly, liver enzymes were elevated in patients with viraemia and were normal in one patient who did not have viraemia when bled at day 7. Many patients with H5N1 disease in this and other studies have a watery diarrhoea (Hien et al., 2004; Apisarnthanarak et al., 2004; Beigel et al., 2005). This contrasts with findings in human influenza (Naficy, 1963;

Lehmann and Gust, 1971; Zinserling et al., 1983) and re-enforce the possibility that H5N1 disease in humans may be a disseminated infection. Further clinical and autopsy studies are required to define the extent of virus dissemination in H5N1 disease. Unfortunately, post-mortem examination is not culturally acceptable in Cambodia.

Analysis of the HA gene sequences detected in different tissues (respiratory tract, gastrointestinal tract or blood) in the same individual did not reveal any tissue specific adaptive mutations. Two patients (patients 5 and 6) had a deletion of an Arginine residue in the haemagglutinin cleavage site. Similar deletions were also reported to be present in a few H5N1 isolates from poultry in Korea 2003, Vietnam and Indonesia in 2005 Laos and Malaysia in 2006 (Smith et al., 2006). The cleavage site found in patient 6 seems likely to be an adaptation in vitro culture rather than an organ-specific adaptation. In patient 5, this deletion was found in the respiratory tract, serum and rectum and does not appear to be a tissue specific adaptation. The amino acid substitutions recently reported to be associated with a change in virus binding from sialic acid SA$\alpha$2,3 Gal to SA$\alpha$2,6 Gal were not observed at any of the organ sites. But the mutation of the serine at position 123 in the HA molecule could influence the preference for the $\alpha$2,3 or $\alpha$2,6 linkage by altering the orientation of the 130-loop (Yamada et al., 2006).

The mutation Lys627 in the PB2 gene has been previously associated with increased virulence of H5N1 in mice (Hatta et al., 2001), with increased replication competence at temperature naturally occurring in the human nasopharynx (Massin et al., 2001) and was often found in waterfowl at Qinghai Lake (Chen et al., 2005). The 701Asn mutation (de Jong et al., 2005) was present at low frequency.

In summary, our evidence strengthens the contention that H5N1 is a disseminated infection. Systemic dissemination of H5N1 emphasises the need for systemically bio-available antivirals that can be parenterly administered. In addition, further studies are needed to consider the nosocomial transmission risks and laboratory hazard from blood and faeces in laboratories and in hospital wards. The lack of infection in contacts of our confirmed H5N1 patients further re-enforces the observation that symptomatic or asymptomatic infection in contacts of H5N1 infected patients is rare (Liem et al., 2005; Schultsz et al., 2005; Vong et al., 2006).

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