Environment: a potential source of animal and human infection with influenza A (H5N1) virus

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Background Very little is known regarding the persistence of highly pathogenic avian influenza H5N1 viruses in natural settings during outbreaks in tropical countries, although environmental factors may well play a role in the persistence and in the transmission of H5N1 virus.

Objective To investigate various environmental compartments surrounding outbreak areas as potential sources for H5N1 virus transmission.

Methods Environmental specimens were collected following outbreaks of avian influenza in Cambodia between April 2007 and February 2010. The methods used to concentrate H5N1 virus from water samples were based either on agglutination of the virus with chicken red blood cells or on adsorption on glass wool, followed by an elution-concentration step. An elution-concentration method was used for mud specimens. All samples that tested positive by real-time RT-PCRs (qRT-PCRs) targeting the HA5, M and NA1 genes were inoculated into embryonated hen eggs for virus isolation.

Results Of a total of 246 samples, 46 (19%) tested positive for H5N1 by qRT-PCRs. Viral RNA was frequently detected in dust, mud and soil samples from the farms’ environment (respectively, 46%, 31% and 15%). Samples collected from ponds gave a lower proportion of positive samples (6%) as compared to those collected from the farms (24%). In only one sample, infectious virus particles were successfully isolated.

Conclusion During H5N1 virus outbreaks, numerous environmental samples surrounding outbreak areas are contaminated by the virus and may act as potential sources for human and/or animal contamination.

Keywords Cambodia, environment, H5N1 virus, influenza, outbreaks, transmission risk.
without direct contact with infected poultry is associated with an increased risk of human infection.\textsuperscript{23–27} The exact role of the environment in the transmission of H5N1 virus remains poorly understood. Few authors have described the survival of H5N1 virus in water, soil or various surfaces in laboratory-controlled conditions with temperatures usually ranging from 0 to 25°C,\textsuperscript{15,16,28,29} but very little is known regarding the persistence of the virus in natural settings where outbreaks regularly occur, for example, in tropical countries where average temperatures can reach over 35°C in the shade. The purpose of this study was to investigate various environmental components as potential reservoirs for H5N1 virus and thus as potential sources for human and animal contamination.

**Materials and methods**

**Sample collection**

In response to the notification of confirmed cases of H5N1 infection in humans or poultry, we conducted four investigations in the households of the index cases and in the surrounding vicinities. Environmental specimens were collected in five households of three Cambodian provinces between April 2007 and February 2010 (Figure 1). These samples included water – collected in sterile tubes and containers – mud, aquatic plants and animals, poultry feathers, various domestic animal faeces and soil collected in sterile tubes, and dust swabs, moistened with viral transport medium (VTM) prior to collection and storage in VTM tubes afterwards. All specimens were kept at 4°C while being transferred to the laboratory within few hours and then stored at −80°C until testing.

**BioSafety statement**

All tests conducted on the samples were performed within the Bio-Safety level 3 Laboratory (BSL3) of Institut Pasteur in Cambodia.

**Concentration of H5N1 virus in water**

Two methods of influenza virus concentration in water were used. The first one was based on the biological property of the virus to agglutinate chicken red blood cells (CRBCs) as described previously\textsuperscript{30} and was used to test small volumes of water (<50 ml). The second method consisted in an adsorption step on glass wool, followed by an elution step with a beef extract solution at alkaline pH, in combination with a final concentration step with polyethylene glycol (PEG), and was optimized for large volumes of water (up to 10 l).\textsuperscript{31} The final concentrates obtained were used for nucleic acid extraction, HA5 haemagglutinin gene amplification and virus isolation.

**Concentration of H5N1 virus from mud and soil specimens**

Mud and soil specimens (5 g) were eluted with 25 ml of 10% beef extract solution at pH 7, followed by a PEG-pre-
cipitation step for virus and RNA concentration. The mud eluates and concentrates were then used for RNA extraction or inoculation into embryonated hen eggs for infectious viral particles isolation.

Homogenization of other solid samples (plants, straw, aquatic animals)
All aquatic animals (fish, snails, insects) and plants collected from ponds, as well as straw samples collected from poultry cages, went through an homogenization step using the MagNa Lyser Instrument (Roche Diagnostics, Mannheim, Germany) for three runs of 50 seconds at 5000 g. Supernatants were then used for RNA and virus detection.

Total nucleic acid extraction and amplification by real-time RT-PCR
All samples processed as described above were mixed with an antibiotics solution (dilution 1/10) prior to further RNA extraction or virus isolation to reduce the number of contaminating organisms in the samples. Either MagNa Pure LC Total Nucleic Acid Isolation Kit (Roche Diagnostics) with the MagNa Pure LC Instrument (Roche Diagnostics GmbH, Mannheim, Germany) or QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) was then used for viral RNA extractions on all eluted/concentrated/homogenized samples (200 μl), following the manufacturers’ recommendations. Quantitative real-time RT-PCRs (qRT-PCRs) targeting the haemagglutinin (HA5), matrix (M) and neuraminidase (NA1) genes were performed on the extracted RNA, as described previously. The qRT-PCR designed for HA5 detection was used to screen all the samples as it was the most sensitive of the three qRT-PCRs. The qRT-PCR targeting M gene was used only to confirm results on the specimens that tested positive for HA5, and the NA1 qRT-PCR was meant to confirm the virus subtype. A sample was declared positive for H5N1 virus when it tested positive with the three different qRT-PCRs.

Virus isolation in embryonated hen eggs
All samples that tested positive by qRT-PCR for all HA5, NA1 and M genes were subsequently inoculated into specific pathogen-free (SPF) embryonated hen eggs, each sample being inoculated into three eggs. The eggs were then incubated for 48 hours at 37°C and chilled overnight at 4°C. Amnio-allantoic fluids (AAF) were harvested, and standard haemagglutination (HA) tests were performed to confirm the presence of virus. HA tests were performed in 96-well microtitre plates with 0.75% guinea pig red blood cells and serial twofold dilutions of AAF. Negative HA tests led to additional passages on eggs. A maximum of three passages were performed for each sample. Positive HA test was confirmed by HA5 qRT-PCR for virus identification.

Results
Between April 2007 and February 2010 during the investigation of four outbreaks of H5N1 virus in poultry, a total of 246 environmental specimens were collected in five households of three Cambodian provinces (Figure 1). Among these samples, 178 were collected from the farms’ environment and 68 were collected exclusively from nearby ponds. Of the 246 samples collected, 46 (19%) tested positive by qRT-PCR targeting the HA5, NA1 and M genes (Table 1 and S1), out of which only one contained infectious H5N1 particles. At the time of investigation following the report of a human case, all poultry were already dead or the few surviving ducks already tested negative (data not shown).

H5N1 virus detection in specimens collected from farms’ environment
H5N1 virus RNA was frequently detected in dust (including specimens collected inside the houses), soil and puddle mud samples obtained from the farms’ environment (Table 1 and S1). These specimens often contained high numbers of RNA copies, with mean viral loads of 1.2 × 10⁴ RNA copies per ml of dust supernatant, 3.1 × 10⁴ RNA copies per gram of soil and 8.9 × 10⁵ RNA copies per gram of puddle mud. A third of the samples collected from duck cages tested positive by qRT-PCR. In particular, most of the few available duck feathers and straw specimens collected in the duck cages tested positive. As for the water samples, only two samples tested positive by qRT-PCR: one came from a puddle and the other was a sample of drinking water collected from a container used by ducks (Figure 1, Table 1 and S1). Overall, of the 178 samples collected from within the farms’ environment tested positive for H5N1 virus RNA. The highest viral loads were observed in contaminated straw (4.9 × 10⁵ RNA copies per gram), puddle mud (4.5 × 10⁵ RNA copies per gram) and duck drinking water (2 × 10⁵ RNA copies per ml). Among these 42 specimens, there was only one for which virus isolation was successful. This infectious strain was isolated from a specimen of water collected from a puddle in household 4 (Figure 1). In this household, the last poultry death was reported 2 days prior to sampling date. The viral load measured by HA5 qRT-PCR in this water specimen was surprisingly low (10 copies per millilitre).

H5N1 virus detection in specimens collected from ponds
Of a total of 68 samples collected from ponds, four tested positive for H5N1 virus RNA. Samples collected from ponds appeared to give a lower proportion of positive results by HA5 qRT-PCR (6%) as compared to samples collected elsewhere in the farms.
### Table 1. Influenza A H5N1 virus detection in environmental and animal specimens collected in five households*

| Source                          | Sample type            | Household identification* | No. of specimens tested positive by qRT-PCR (%)** | Total no. of samples tested positive by qRT-PCR (%)** | Viral load in copies of HA5 RNA per gram or per millilitre | No of samples positive by virus isolation |
|---------------------------------|------------------------|---------------------------|---------------------------------------------------|-----------------------------------------------------|-------------------------------------------------------------|----------------------------------------|
| **Farms**                       | Dust                   | 1                         | 1/2 (50)                                          | 16/35 (46)                                          | 1.2 x 10^4 - 8.7 x 10^4                                      | 58 - 0                                  |
|                                 |                        | 2                         | 6/14 (43)                                         |                                                     |                                                             |                                        |
|                                 |                        | 3                         | 3/4 (75)                                          |                                                     |                                                             |                                        |
|                                 |                        | 4                         | 0/5 (0)                                           |                                                     |                                                             |                                        |
|                                 |                        | 5                         | 6/10 (60)                                         |                                                     |                                                             |                                        |
| **Soil**                        |                        | 1                         | 2/9 (22)                                          | 12/81 (15)                                          | 3.1 x 10^4 - 3 x 10^5                                      | 68 - 0                                  |
|                                 |                        | 2                         | 5/13 (38)                                         |                                                     |                                                             |                                        |
|                                 |                        | 3                         | 4/30 (13)                                         |                                                     |                                                             |                                        |
|                                 |                        | 4                         | 0/14 (0)                                          |                                                     |                                                             |                                        |
|                                 |                        | 5                         | 1/15 (6)                                          |                                                     |                                                             |                                        |
| **Mud**                         |                        | 1                         | 1/4 (25)                                          | 6/19 (31)                                          | 8.9 x 10^4 - 4.5 x 10^5                                    | 108 - 0                                 |
|                                 |                        | 2                         | 1/5 (20)                                          |                                                     |                                                             |                                        |
|                                 |                        | 4                         | 0/6 (0)                                           |                                                     |                                                             |                                        |
|                                 |                        | 5                         | 4/4 (100)                                         |                                                     |                                                             |                                        |
| **Water (puddles, wells)**      |                        | 1                         | 0/1 (0)                                           | 1/14 (7)                                           | 10 - NA - NA - NA                                         | 0 - 0 - 0 - 0                           |
|                                 |                        | 2                         | 0/6 (0)                                           |                                                     |                                                             |                                        |
|                                 |                        | 3                         | 0/2 (0)                                           |                                                     |                                                             |                                        |
|                                 |                        | 4                         | 1/5 (20)                                          |                                                     |                                                             |                                        |
| **Drinking water collected inside duck cages** | | | | | | |
| **Straw in duck cages**         |                        | 1                         | 2/4 (50)                                          | 2/4 (50)                                           | 2.5 x 10^4 - 4.9 x 10^5                                    | 9000 - 0                                |
|                                 |                        | 2                         | 1/1 (100)                                         | 2/2                                                | 320 - 473                                               | 167 - 0                                 |
| **Duck tracheal swabs**         |                        | 2                         | 1/1 (100)                                         | 1/4 (25)                                           | 147 - NA - NA - NA                                       | 0 - 0 - 0 - 0                           |
| **Duck faeces**                 |                        | 1                         | 0/5 (0)                                           | 0/7 (0)                                            | NA - NA - NA - NA                                        | 0 - 0 - 0 - 0                           |
| **Domestic animal’s faeces/rectal swab (dogs, bovines)** | | | | | | |
| **Ponds**                       | Mud                    | 1                         | 0/8 (0)                                           | 2/24 (8)                                           | 3050 - 5000 - 1100                                       | 0 - 0 - 1100                            |
|                                 |                        | 2                         | 2/7 (28)                                          |                                                     |                                                             |                                        |
|                                 |                        | 3                         | 0/3 (33)                                          |                                                     |                                                             |                                        |
|                                 |                        | 5                         | 0/6 (0)                                           |                                                     |                                                             |                                        |
| **Water**                       |                        | 1                         | 0/6 (0)                                           | 0/16 (0)                                           | NA - NA - NA - NA                                        | 0 - 0 - 0 - 0                           |
|                                 |                        | 2                         | 0/1 (0)                                           |                                                     |                                                             |                                        |
|                                 |                        | 3                         | 0/6 (0)                                           |                                                     |                                                             |                                        |
|                                 |                        | 5                         | 0/3 (0)                                           |                                                     |                                                             |                                        |
| **Aquatic plants**              |                        | 2                         | 0/1 (0)                                           | 1/4 (25)                                           | 10^4 - NA - NA - NA                                       | 0 - 0 - 0 - 0                           |
|                                 |                        | 3                         | 1/0 (1)                                           |                                                     |                                                             |                                        |
|                                 |                        | 5                         | 1/2 (50)                                          |                                                     |                                                             |                                        |
| **Aquatic animals**             | Fishes, shells, snails, insects, etc. | 1 | 0/13 (0)                                          | 1/24 (4)                                           | 2500 - NA - NA - NA                                       | 0 - 0 - 0 - 0                           |
|                                 |                        | 2                         | 1/4 (25)                                          |                                                     |                                                             |                                        |
|                                 |                        | 3                         | 0/5 (0)                                           |                                                     |                                                             |                                        |
|                                 |                        | 5                         | 0/2 (0)                                           |                                                     |                                                             |                                        |
| **Total**                       |                        |                           |                                                    |                                                    |                                                             |                                        |

*Household 1: 6 April 2007 (date of investigation/sample collection), 11 April 2007 (date of last poultry death), 31.5°C [temperature measured at the surface of the water in pond (or in the well for household 4)], pH: 7.5 [pH of the water in pond (or in the well for household 4)], Kampong Cham (province); household 2: 14 December 2008 (date of investigation/sample collection), 7 December 2008 (date of last poultry death), 33°C [temperature measured at the surface of the water in pond (or in the well for household 4)], pH: 7.7 [pH of the water in pond (or in the well for household 4)], Kandal (province); household 3: 17 December 2009 (date of investigation/sample collection), 17 December 2009 (date of last poultry death), 31.7°C [temperature measured at the surface of the water in pond (or in the well for household 4)], pH: 6.85 [pH of the water in pond (or in the well for household 4)], Kampong Cham (province); household 4: 17 December 2009 (date of investigation/sample collection), 15 December 2009 (date of last poultry death), 29.5°C [temperature measured at the surface of the water in pond (or in the well for household 4)], pH: 6.1 [pH of the water in pond (or in the well for household 4)], Kampong Cham (province); household 5: 2 February 2010 (date of investigation/sample collection), 2 February 2010 (date of last poultry death), 34°C [temperature measured at the surface of the water in pond (or in the well for household 4)], pH: 6.9 [pH of the water in pond (or in the well for household 4)], Takeo (province).

**A sample was declared positive when it tested positive with the three qRT-PCRs targeting the HA5, M and NA1 genes.

NA, not applicable.
Avian influenza viruses were reported to have the ability to survive outside the host for a few days up to several months depending on the environmental conditions and viral concentrations. However, our data suggest that in tropical countries, virus inactivation may occur rapidly due to several factors such as heat (temperature in ponds water ranging from 29-5 to 33°C and outside temperature exceeding sometimes 35°C during the investigations), salinity, dryness, ultraviolet radiation and pH. Although the freeze/thaw step included in our collection and testing protocols should only be responsible for a small loss of virus titre, given the already very low viral load detected in some samples (Table 1), this step could partially explain why infectious particles were rarely recovered.

Although viral particles may not be infectious anymore, their RNA is still protected from degradation in the matrix protein and the core and could consequently be detected by qRT-PCR (HA5, NA1 and M genes). As already described in a previous study, PCR inhibitors were detected in almost 30% of the mud samples, but even after serial dilutions, these specimens still tested negative by HA5 qRT-PCR (data not shown). Unsurprisingly, viral loads detected in duck cages were among the highest. However, it is noteworthy that the one sample that tested positive for virus isolation had not been collected from a duck cage but from a puddle water sample, which also contained low quantities of viral RNA (10 RNA copies per ml). These data emphasize the idea that various physico-chemical or microbiological parameters may influence the survival of H5N1 viral particles in natural settings, in ways that are yet to be clarified.

The detection of influenza RNA in 8%, 25% and 4% of mud, aquatic plants and aquatic animals collected from ponds, respectively, should not be regarded as insignificant, especially as these ponds were located nearby (~100 m) the households of the index cases. These results suggest that aquatic sites should be considered as a potential source for human and/or animal infection. Indeed, animals are drinking this water and ducks are swimming in ponds and can therefore contaminate the aquatic environment but also be contaminated. In addition, the ponds are also commonly used by children for playing and swimming, and this behaviour was identified as a risk factor for subclinical human contamination.

The duration of survival of the virus was estimated through the interval of time between the last poultry death and the sample collection. Here, in environmental samples collected 7 days after the last bird’s death, virus RNA was still detected, even though infectious virus could not be isolated in eggs. This was in agreement with our previous findings. This work supports the idea that environmental sampling is a valuable approach to assess the presence and evaluate the extent of the dissemination of influenza viruses in specific geographical or environmental locations. Our findings also demonstrate that during H5N1 virus outbreaks in tropical areas, many environmental components surrounding outbreak areas are widely contaminated by the virus and may act, probably for only a short period of time (during or just after the virus is shed by poultry), as potential sources for human and/or animal contamination as already suggested several times over the last few years. For instance, our data showing a relatively high H5N1 virus detection rate in dust samples raise concerns about a possible airway transmission by inhalation of infectious particles in suspension in the air. In addition, previous studies demonstrated the possibility of H5N1 infection through oral route in mammals, along with the description of an intestinal syndrome in some human cases. This supports the hypothesis that inhalation of infected droplets through direct contact with infected poultry might not be the only possible way of human contamination. Thus, our results underscore the importance for regular surveillance and disinfection of the farms’ environment following avian influenza outbreaks.

Further investigations in outbreak areas and around live bird markets should be carried out to complete the current investigation.
pool of data available on the persistence of HPAI H5N1 virus in natural environment in endemic tropical countries. Indeed, understanding the complete epidemiology of H5N1 virus is important for the prevention of human, wildlife and domestic animal disease caused by this virus. Now that better diagnostic methods have been described for H5N1 detection in water and mud samples, surveillance of H5N1 virus in the environment could be an interesting tool to monitor virus circulation and risk of exposure for humans and animals.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Distribution by sample source of the specimens which tested positive by qRT-PCR for HA5, NA1 and M genes.

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