In February 2011, a mother and her child from Banteay Meanchey Province, Cambodia, were diagnosed, post-mortem, with avian influenza A(H5N1) virus infection. A field investigation was conducted by teams from the Cambodian Ministry of Health, the World Health Organization and the Institut Pasteur in Cambodia. Nasopharyngeal, throat and serum specimens collected from 11 household or three neighbour contacts including two suspect cases tested negative by reverse transcriptase–polymerase chain reaction (RT-PCR) for A(H5N1). Follow-up sera from the 11 household contacts also tested negative for A(H5N1) antibodies. Twenty-six HCW who were exposed to the cases without taking adequate personal protective measures self-monitored and none developed symptoms within the two following weeks. An unknown number of passengers travelling with the cases on a minibus while they were symptomatic could not be traced but no clusters of severe respiratory illnesses were detected through the Cambodian surveillance systems in the two weeks after that. The likely cause of the fatal infection in the mother and the child was common-source exposure in Preah Sdach District, Prey Veng Province. Human-to-human transmission of A(H5N1) virus was unlikely but genetic susceptibility is suspected. Clusters of A(H5N1) virus infection should be systematically investigated to rule out any human-to-human transmission.

Introduction
The first human cases of the ongoing avian influenza A(H5N1) virus epidemic were detected in 2003 in Guangdong, China [1]. Clusters of confirmed human cases have been described in the past in Azerbaijan [2], mainland China [3], Egypt [4], Hong Kong [5], Indonesia [6], Pakistan [7,8] Thailand [9], Turkey [10] and Vietnam [11]. In at least one Indonesian cluster, transmission of A(H5N1) may have occurred through contact with environmentally-contaminated material [12]. However, human-to-human transmission in each of these clusters could not be definitively excluded.

In Cambodia, the first human cases of influenza A(H5N1) were described in 2005 [13]. As of 31 March 2014, there have been 56 confirmed A(H5N1) cases in humans with 37 deaths in 14 provinces and in Phnom Penh, including a mother-and-child cluster described herein [14]. In 2011, two different lineages of influenza A(H5N1) virus co-circulated in Cambodia: lineage 5 which circulated in Cambodia and south Vietnam and lineage 6 believed to be endemic to Cambodia only [15]. Viruses from lineage 6 have also been isolated from other sporadic human and poultry cases (IPC data, not shown).

The event
On 20 February 2011, a fatal influenza A(H5N1) virus infection was laboratory-confirmed by the national influenza center at Institut Pasteur in Cambodia (IPC). The deceased, a child who resided in Banteay Meanchey Province, in western Cambodia, had visited Prey Veng Province, in eastern Cambodia, with his parents before the onset of symptoms (Figure 1) [16]. Following the notification, the Rapid Response Team (RRT) of the Ministry of Health (MOH) of Cambodia visited the child’s village of residence in western Cambodia and the admitting hospital in Siem Reap Province the following day. His hospital record was reviewed and the child’s father, grandmother and healthcare workers (HCW) who had taken care of him were interviewed. Initial investigations revealed that the 19-year-old mother of the child had reportedly died from severe respiratory infection in another district hospital in the same province on 12 February 2011. The RRT subsequently also visited the hospital where the mother had died and obtained clinical information of the mother from hospital staff who provided care before her death. Some stored serum from the mother, retrieved at the hospital and sent to...
IPC, tested positive for influenza A(H5N1) virus antibodies on 22 February 2011.

Additional field investigations were subsequently conducted in Banteay Meanchey and Prey Veng Provinces by staff from the MOH, the World Health Organization (WHO), and IPC to determine the magnitude of the cluster and possible sources of infection, and investigate the possibility of person-to-person transmission. This report summarises the clinical, laboratory, and epidemiological findings of the investigation of this first laboratory-confirmed human A(H5N1) cluster in Cambodia.

**Methods**

**Clinical and epidemiological investigations**

Clinical and epidemiological data of the cases were collected by reviewing available medical records at admitting health facilities, and by interviewing household members, relatives, caregivers, other villagers, and HCW in Banteay Meanchey, Siem Reap or Prey Veng Provinces.

Tracing of close contacts was performed by interviewing the household members of the cases and their neighbours in Banteay Meanchey and Prey Veng Provinces, as well as HCW in Banteay Meanchey and Siem Reap Provinces. The purpose was to collect information on types and degrees of exposure to the cases, poultry, and other environmental elements.

Three types of close contacts were defined. Close contacts with the cases referred to those with whom the child had played or those who had taken care of the child and/or the mother for at least 15 minutes and had been within less than one metre distance from the cases while ill. This group included household members (household contacts), neighbours (neighbour contacts) and HCW (HCW contacts). Close contacts with sick or dead poultry referred to those who had touched, carried, defeathered, eviscerated, cleaned or cut any...
sick/dead poultry. Close contacts with the cases and with sick or dead poultry were defined as those who met criteria for inclusion in both groups.

**Case definition**
A suspected influenza A(H5N1) case was defined as a close contact who presented with fever and/or respiratory and digestive signs or symptoms within two weeks after last exposure to the confirmed cases or any sick or dead poultry. A confirmed case was defined as being laboratory-confirmed (see ‘Laboratory methods’).

Nasopharyngeal, throat, and serum specimens were collected from household contacts and suspect cases in Prey Veng and in Banteay Meanchey Provinces, who agreed to be tested for A(H5N1) infection. Follow-up sera from identified contacts was collected more than three weeks later and tested for A(H5N1) antibody. All identified contacts were monitored for two weeks for development of acute respiratory signs and symptoms after their last exposure.

We defined a cluster of A(H5N1) infections as consisting of at least two symptomatic persons with laboratory-confirmed A(H5N1) infection among household members, relatives or other types of contacts.

As part of urgent public health investigations of human A(H5N1) infections in Cambodia, the national ethics committee was informed by the Ministry of Health and fast-track ethics approval was obtained. All participants in the epidemiological investigations gave their informed consent to be interviewed and tested for A(H5N1) infection, and were informed about their laboratory results and their interpretations.

**Laboratory-testing**
Laboratory-testing of samples for influenza A(H1N1) collected from field investigations was performed at IPC, the WHO-designated National Influenza Center in Cambodia. Ribonucleic acid (RNA) was extracted from nasopharyngeal, throat and serum specimens and submitted to quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) tests. Serum samples from contacts were tested for A(H5N1) antibodies by hemagglutination-inhibition (HI) assay [17] and microneutralisation (MN) assay in the biosafety level (BSL) 3 laboratory of the Virology Unit at IPC using the A(H5N1) virus strain isolated from the index case 1. The titre was calculated by the Reed and Muench method [18]. A chemiluminescence-based NA enzyme inhibition assay (NA Star, Applied Biosystems, CA, United States) was used to test the cultured virus for its sensitivity to oseltamivir and zanamivir, as described previously [19].

Laboratory confirmation was defined as a positive PCR and/or virus isolation or a fourfold or greater rise in antibody titre against A(H5N1) in paired sera (acute and follow-up) with the follow-up serum having a titre of 1:80 or higher, or antibody titre of 1:80 or more in a single serum collected at day 14 or later after onset of symptoms, and a titre of 1:160 or greater in HI using horse red blood cells [20,21].

**Results**

**Case descriptions**

**Case 1**
A nine-month-old male infant developed fever and cough in the early morning of 5 February 2011. He and his mother were treated for similar symptoms with over-the-counter paracetamol in a village in Banteay Meanchey Province. Ten days later, on 15 February, he developed dyspnea and was admitted to Jayavarman VII Children Hospital in Siem Reap Province. Clinical evaluation on admission revealed a temperature of 38.2°C, elevated breathing frequency of 48 breaths/min, oxygen saturation ranging from 93%-99% on ambient air, and bilateral wheezing on lung auscultation. A chest radiograph showed air trapping in both lungs with thickening of bronchi. Complete blood cell count showed normal white blood cells including lymphocytes but low haemoglobin of 7.7 g/dl (norm: 13.5–19.5) and hematocrit of 25% (norm: 44.0–64.0), elevated platelets at 461 x 10^9/L (norm: 150 x 10^9–400 x 10^9) and alanine aminotransferase (ALT) at 95 IU/l (norm: 5–41). Aspartate transaminase (AST) was normal. The child was diagnosed with acute bronchitis, anaemia, malnutrition and possible paracetamol overdose. Despite intravenous amoxicillin/clavulanic acid and nebulised terbutaline and salbutamol, his condition deteriorated rapidly; on 17 February respiratory rate was 68 breaths/min and the child was cyanotic without oxygen supplementation and was transferred to the intensive care unit where he was intubated with handbag ventilation performed by family members. A repeat chest radiograph on the same day showed extensive bilateral infiltrates. Repeat blood tests showed aggravation of anaemia with haemoglobin of 6.6 g/dl and impairment of liver function with ALT at 104 IU/l and normal AST.

Due to the sudden deterioration of his condition, history of fatal respiratory illness in his mother five days earlier and recent reports of sick and dead poultry in a village in Prey Veng Province where the family visited for a month before onset, A(H5N1) virus infection was suspected. Nasopharyngeal (NP) swabs and serum were collected on 17 February and sent to IPC. Oseltamivir 3mg/kg twice daily was started immediately after specimen collection. However, the child died on the same day only a few hours after transfer to ICU. On 20 February, IPC notified health authorities that nasopharyngeal swabs tested positive for A(H5N1) virus by qRT-PCR.

**Case 2**
A 19-year-old female, the biological mother of Case 1, developed fever and headache in the evening of 4 February 2011. A day later, she developed cough and mild dyspnea and received over-the-counter...
paracetamol which she took for three days. Still, her dyspnea continued to worsen and on 8 February she was admitted to a private clinic in the capital of Banteay Meanchey Province. On admission, she presented with a temperature of 38.5°C, productive cough, chest pain and shortness of breath. A chest radiograph showed bilateral infiltrates and pleural effusion. She received a third-generation cephalosporin and a chest drain was placed in her right hemithorax. Five days later, on 12 February, she was transferred to a district hospital for severe dyspnea where she died two hours after admission. No respiratory specimens were collected before her death. Stored serum that had been collected on 12 February, was retrieved at the hospital and tested at IPC on 22 February, after samples from Case 1 had tested positive for A(H5N1). The serum of the patient was analysed by qRT-PCR and tested positive for A(H5N1) virus with a viral load of $8.7 \times 10^3$ equivalent cDNA copies/mL.

Exposure histories of cases

On 3 January, the two cases and three other household family members (the child’s father, aunt and grandmother) travelled from their home village in Banteay Meanchey Province to spend the Lunar New Year with relatives in a village in Preah Sdach District, Prey Veng Province. From 7 January onwards, poultry in the family and two neighbouring households began to die. By 20 February, all chickens in the three households including the relatives’ 20 chickens had died. None of the ducks in any of the households became ill or died (Figure 2).

Case 2 was reportedly present with Case 1 at all times. Case 1 sat, played and crawled on the ground at their relatives’ and neighbours’ homes. However, none of the cases visited the pond on the homestead or had direct contact with any sick, dead or slaughtered poultry. Both cases reportedly spent time in areas surrounding the three households where chickens had died, including areas contaminated with feathers, feces and discarded waste of sick or dead poultry.

Contact tracing

A total of 48 contacts were identified in Prey Veng, Banteay Meanchey and Siem Reap Provinces (Table), none of them blood relatives of Case 2. Eight were household contacts who had close contact with sick or dead poultry, and two provided home care to Case 2 in the evening of 4 February (for less than 24 hours) after her onset of symptoms. All eight contacts were asymptomatic during the two-week period after their last exposure. A nasopharyngeal swab and acute serum sample were collected from each of the seven household contacts who were present during the investigation on 23 February; samples could not be taken from one contact who was away at school. Follow-up sera were collected on 29 March from all eight household contacts. All tested negative for A(H5N1) virus and anti-A(H5N1) antibodies.

Three other household contacts (father, aunt and grandmother of Case 1) who were also exposed to sick or dead poultry over one month in the village in Prey Veng Province, travelled back to Banteay Mean Chey Province with the cases. These three individuals were the most exposed to the cases after the onset of their symptoms. They cared for them while they were sick, performed hand-bagging ventilation during hospitalisation, and washed Case 2’s corpse. However, no acute serum samples or nasopharyngeal swabs were collected from them as they had already left the village in Prey Veng by the time the team started the investigation. Follow-up sera were collected on 29 March from all eight household contacts. All tested negative for A(H5N1) virus and anti-A(H5N1) antibodies.

**Figure 2**

Sequence of exposure and clinical events in two confirmed human cases of influenza A(H5N1) virus infection, Prey Veng cluster, Cambodia, 2011
Ten neighbour contacts in Prey Veng Province had repeated direct exposures to sick or dead poultry through touching, carrying, defeathering, eviscerating, cleaning, cutting, or chopping sick or dead chickens. They had no contact with the cases after symptoms onset but had contact with contaminated environments by playing on the ground (for children), dusting or wiping chairs or day beds, clearing trash from the ground contaminated by sick or dead chickens, for more than one month. Eight of them were symptom-free during two weeks after their last exposure to the cases. Two children aged seven and 10 were symptomatic (cough and runny nose) but did not develop severe respiratory disease; both recovered completely after several days. Nasopharyngeal swabs and acute sera were collected from both during the investigation on 23 February, and follow-up sera were collected on 29 March, all of which tested negative for A(H5N1) virus and anti-A(H5N1) antibodies, respectively. No further testing was performed during the cluster investigation in the remaining eight neighbour contacts in Prey Veng as they were not considered exposed to the cases after their symptoms onset.

A total of 26 HCW took care of both confirmed cases in Banteay Mean Chey and Siem Reap Provinces. None of them had any exposure to sick or dead poultry, but had multiple exposures with the cases during their hospitalisations between one to seven days. Of the 26, several had performed high-risk procedures such as endotracheal intubation, nasopharyngeal aspiration or chest drain insertion with no appropriate personal protective equipment (PPE) before A(H5N1) virus infection was suspected. All HCW contacts chose to not get tested and preferred to self-monitor for symptoms for two weeks after their last exposure; none of them developed any symptoms during the observation period.

One neighbour contact in Banteay Mean Chey helped to wash Case 2’s corpse; she reported having washed her hands with soap and water after washing the corpse and she was not aware of any exposure to sick or dead poultry. She did not develop symptoms during the two weeks after the last exposure to the case and despite exposure she was not sampled during the initial investigation of the RRT because the investigation team did not return to the village in Banteay Mean Chey for this purpose.

On 5 February, one day after the onset of Case 2’s symptoms, the family returned home on a crowded 12-seat minibus with approximately 15 other passengers and both cases developed cough during the 12-hour trip. The fellow passengers were unknown to the family members travelling with the cases. In the absence of passenger lists, contact tracing was not possible.

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Table 1
Contact tracing results in cluster of two cases of confirmed influenza A(H5N1) virus infection, Cambodia, 2011

<table>
<thead>
<tr>
<th>Close contact</th>
<th>Relationship to cases (Province)</th>
<th>Number of contacts</th>
<th>Duration of exposure</th>
<th>Number tested</th>
<th>Results (PCR and/or IgM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No symptoms</td>
<td>With symptoms</td>
<td>To sick/dead poultry</td>
<td>To cases after onset</td>
</tr>
<tr>
<td>With cases and</td>
<td>Household (Prey Veng)</td>
<td>8</td>
<td>8</td>
<td>&gt;1 month</td>
<td>&lt;24 hours</td>
</tr>
<tr>
<td>sick or dead poultry</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>poultry</td>
<td>Household (Prey Veng, Banteay Mean Chey)</td>
<td>3c</td>
<td>3</td>
<td>1 month</td>
<td>14 days</td>
</tr>
<tr>
<td>With symptoms</td>
<td>Neighbour (Prey Veng)</td>
<td>8</td>
<td>8</td>
<td>&gt;1 month</td>
<td>None</td>
</tr>
<tr>
<td>only</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With the cases</td>
<td>HCW (Siem Reap, Banteay Mean Chey)</td>
<td>26d</td>
<td>3</td>
<td>None</td>
<td>7 days</td>
</tr>
<tr>
<td>only</td>
<td>Neighbour (Banteay Mean Chey)</td>
<td>1</td>
<td>1</td>
<td>None</td>
<td>Multiple exposure ranging from 1 to 3 days</td>
</tr>
<tr>
<td>Passengers on</td>
<td>Passengers on minibus from Prey Veng to Banteay Mean Cheanckey</td>
<td>Unknowna</td>
<td>Unknowna</td>
<td>Unknowna</td>
<td>12 hours</td>
</tr>
</tbody>
</table>

HCW: healthcare workers; Ig: immunoglobulin; n.a.: not applicable; NP: nasopharyngial; PCR: polymerase chain reaction.

a NP swabs tested by PCR.
b One contact was at school during the investigation and was not available for sampling.
c Father, aunt and grandmother of Case 1 travelled with the cases back to Banteay Mean Cheay Province, and were exposed for up to 14 days.
d HCW chose not to be tested but self-monitor for 14 days instead.
e Unknown to cases and, therefore contact tracing was not possible.
Genome analysis of the virus

Whole-genome sequence analysis of the virus isolated from the child (A/Cambodia/V0219301/2011(H5N1), GenBank accession numbers: JN588806, JN588857, JN588879, JN588827, JN588926, JN588911, JN588896, JN588863) did not demonstrate any mutation associated with higher virulence or adaptation to human receptors. Phylogenetic analysis of the HA sequence of the virus revealed that the strain isolated from this patient belongs to clade 1.1, lineage 6. This clade had been detected in 19 previous sporadic influenza A(H5N1) cases in Cambodia (IPC, unpublished data). Chemiluminescence-based NA enzyme inhibition assay results demonstrated that the virus was sensitive to both antiviral drugs with IC50 at 0.46 nM and 2.15 nM for oseltamivir and zanamivir, respectively.

Discussion

Since the spread of A(H5N1) avian influenza in Asia beginning in 2003, over 500 confirmed human cases of severe respiratory disease due to A(H5N1) virus infection have been reported [22,23]. The vast majority of these human A(H5N1) infections have been sporadic occurrences involving direct contact with sick or dead poultry through culling, discarding, plucking or preparing food as the likely mode of transmission [2,22,24–26]. However, a small number of confirmed cases reportedly did not have direct poultry contact. In these cases, exposure to environments contaminated by infected poultry, as well as person-to-person transmission in some clusters, could not be ruled out [6,8,9,11,27–29].

Of the 56 laboratory-confirmed cases in Cambodia as of 31 March 2014, the two cases in this report represent the first confirmed human cluster of A(H5N1) infections in this country. Symptoms of respiratory illness in Case 2 began less than 24 hours before onset of illness in Case 1. The incubation period for A(H5N1) virus infection in humans typically ranges from two to five days [24,30] but may be up to 17 days in cases of low-dose exposure such as to live poultry in a market [31]. It was unlikely that Case 2 was the source of infection to Case 1 or vice versa, based on the short interval between symptom onsets of the cases.

Transmission of A(H5N1) virus from patients to HCW or asymptomatic or subclinical A(H5N1) virus infections are rare but have occurred in the past [32–35]. Despite the presence of symptoms during travel and during home as well as hospital care, there was no clinical or laboratory evidence of person-to-person transmission from Case 1 or Case 2 to neighbours, family members or unprotected HCW. Passengers on the minibus were exposed to both cases for around 12 hours after symptom onset (mild fever and cough). Since they could not be identified follow-up and collection of samples for laboratory-testing was not possible, human-to-human transmission from this cluster to the fellow passengers could not be completely ruled out. However, no cases of severe and acute respiratory infection, suspected or confirmed to be influenza A(H5N1), were reported across Cambodia through the event-based surveillance system during the two weeks after the exposure.

None of the cases had direct contact with sick or dead poultry prior to illness. However, both were present in a village for 33 days during which poultry died. Retrospective laboratory confirmation of A(H5N1) in these poultry could not be performed because poultry remains were not available at the time of the investigation. However, both cases most likely had common exposure to environments contaminated by presumed A(H5N1) virus-infected poultry or their waste. Case 2 likely touched contaminated surfaces, objects placed on the ground, poultry cages or soil, and did not wash hands at all times before eating or touching food, the mouth, nose or eyes. Case 1 probably sat, crawled, or played on the ground shared by the sick or dead poultry and other animals (pigs, dogs and cows). Interviews confirmed that his hands were barely washed before touching his nose, mouth, eyes, or eating. It was also likely that both cases inhaled dust while sitting, playing, crawling, or working on the ground. Direct hand contact with environments or inhalation of dust contaminated by A(H5N1) virus during poultry outbreaks have been previously identified as risk factors for A(H5N1) virus infection in humans in Cambodia [36,37]. However, due to the lack of environmental sampling in this investigation, this route of transmission to the cases could not be confirmed.

Apart from the mother and her child, no other household contacts in Prey Veng Province became infected. Cases and household members were exposed to the same environment; however, different levels of exposure i.e. different amount or concentration of virus in the environment, number of times contacted with the surrounding environment each day could not be verified. As revealed by interviews, Case 2 had much less exposure to sick or dead poultry compared with other household members, and Case 1 had no direct exposure to sick or dead poultry. Yet they were infected while the other household members were not. This may be due to higher levels of infective virus in the environment than that in the sick or dead poultry, which is unlikely. Alternatively, genetic vulnerability could have been a crucial risk factor.

Investigations of earlier clusters have shown that A(H5N1) virus transmission to less exposed individuals may occur, especially to blood relatives of confirmed cases [6–10,13,27–29,38,39] while more intensely exposed individuals remain uninfected or symptom-free [38,40]. This suggests the possibility of genetic susceptibility as a risk factor for infection [41–43].

The virus isolated from Case 1 belongs to clade 1.1, lineage 6, and did not present mutations associated with higher virulence or adaptation to human receptors (SA-a-2,6-Gal).
Conclusion
The carefully conducted epidemiological investigations by the field teams did not bring forward evidence for human-to-human transmission between the cases and their household, HCW or traveling contacts in the Provinces of Prey Veng or Banteay Meanchey. Exposure to contaminated environment, rather than human-to-human transmission or direct contact with sick or dead poultry, was the probable source of infection in this first confirmed mother-and-child cluster of A(H5N1) infections in Cambodia. We are currently conducting contact tracing and seroprevalence studies thanks to funding from the Office of the Assistant Secretary for Preparedness and Response within the US Department of Health and Human Services. A nested genetic study is ongoing to document whether suspected A(H5N1) susceptibility polymorphisms - which could have been a major risk factor of infection in this cluster - are more frequent in cases with confirmed A(H5N1) infection compared to those confirmed negative in Cambodia.

Human cases of A(H5N1) will likely continue to occur in Cambodia. Early suspicion of A(H5N1) virus infection in humans could lead to timely diagnosis and pharmacologic and supportive therapy to prevent transmission and fatal outcomes. Prompt and thorough case investigations are necessary to identify possible routes of infection, including human-to-human transmission of influenza A(H5N1) virus. Close collaboration between clinicians, virologists and epidemiologists, with strong support from national and local health authorities, remains critically important for accomplishing thorough and successful field investigations.

Acknowledgements
The authors gratefully acknowledge the cooperation of the family members of the A(H5N1) infection cases in Prey Veng and Banteay Meanchey Provinces. In addition, they acknowledge the members of the Rapid Response Team and the healthcare workers who were involved in the investigations and clinical management of the cases for their dedication.

Conflict of interest
None declared.

Authors’ contributions
Nora Chea, Arnaud Tarantola, Nima Asgari: coordinated field investigations of the cluster and wrote the manuscript. Seng Doernyi N, Heng Seng, Chuong Penh field investigated the cluster. Sareth Rit, Sek Mardy: tested the specimens from the clusters and contacts. Philippe Buchy: tested the specimens from the clusters and contacts and wrote the manuscript. Touch Sok, Sovann Ly, Paul Kitsutani, Maria Concepcion Rocos, Vanra Long wrote the manuscript. Denis Laurent, Beat Richner treated the patients and wrote the manuscript.

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