Indigenous sources of 2007–2008 H5N1 avian influenza outbreaks in Thailand

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Outbreaks of H5N1 avian influenza show strong seasonality. It is not clear where the source of virus originates from in each new outbreak season. This study sought to understand the nature of viral resurgence in recent outbreak seasons in Thailand, where the epidemic is relatively well controlled. In such a situation, indigenous viruses surviving the inter-outbreak season would have to pass through a bottleneck. In order to look for evidence of the bottleneck effect, viral genome sequences from recent outbreaks in the country were analysed. H5N1 avian influenza viruses were isolated from six outbreaks in the rainy season and winter of 2007 through to early 2008. Most of the outbreaks were in the Yom–Nan River basin in the southern part of the northern region of the country. Sequences of these viral isolates were identified as clade 1, genotype Z, similar to viruses from previous years in the central region of the country. The sequences clustered into two groups, one of which was closely related to viruses isolated from the same area in July 2006. These analyses indicated that there was a strong bottleneck effect on the virus population and that only a few lineages remained in the area. In addition, evidence of reassortment among these viruses was found. These indicated re-emergence of viruses from a small pool of indigenous sources that had been silently perpetuated over the dry summer months. Therefore, an approach to eradicate H5N1 avian influenza from the area by eliminating these local reservoirs may be feasible and should be seriously considered.

INTRODUCTION

Thailand has suffered several rounds of H5N1 avian influenza (AI) outbreaks in poultry. The outbreaks in 2004–2005 were explosive and caused severe economic loss to the poultry industry. Poultry outbreaks, as well as human cases, in Thailand have a clear seasonal pattern. Each year, cases appear with the start of the rainy season in July, peak at the end of the rainy season and the start of winter in September–October, and disappear with the start of summer in February (Tiensin et al., 2005). The outbreaks in 2004–2005 were widespread, covering all regions of the country (Buranathai et al., 2007; Tiensin et al., 2005). After massive campaigns and vigorous outbreak control by culling and movement restrictions, the epidemic subsided. Subsequent outbreaks in 2006 and later were limited to small clusters of backyard chickens and ducks. These outbreaks were concentrated in the central part (lower northern region) of the country and were caused by clade 1 viruses. Although a few limited outbreaks were reported in the north-eastern region of the country, the epidemic was effectively controlled by movement restrictions and culling. An approach to eradicate H5N1 avian influenza from the area by eliminating these local reservoirs may be feasible and should be seriously considered.

The GenBank/EMBL/DDBJ accession numbers for the avian influenza sequences are EU233413–EU233420, EU497919–EU497921, EU547798–EU547801, EU669187–EU669201, EU676306–EU676321 and EU875388–EU875397; details are available with the online version of this paper.
country bordering Laos in July 2006 and January 2007, they were caused by a different group of viruses, belong to clade 2 (Chutinimitkul et al., 2007). Interaction or exchange of the clade 1 and clade 2 viruses between these two areas has never been detected. In this study, we confined our analyses to the outbreaks in the central part of the country, which has been suffering repeated H5N1 AI outbreaks since the beginning of the epidemic in 2003–2004. The provinces with repeated H5N1 outbreaks in poultry in 2006–2008 include Sukhothai, Phitsanulok, Pichit and Nakhonsawan in the lower northern region. In the rainy season and winter of 2007 through to early 2008, there were several reports of backyard chicken die-off in these provinces. Despite intensive control and the apparent complete disappearance of outbreak in the dry summer months, outbreaks reappeared in the rainy season of 2006 and 2007. This indicated either the existence of locally persistent viral reservoirs or the reintroduction of viruses from other countries. Although introduction of a new viral strain (clade 2.3.4) into the north-eastern region of the country was reported, the viral sequences from the north/central region remained clade 1, genotype Z (Chutinimitkul et al., 2007; Keawcharoen et al., 2005). This indicated that yearly re-emerging viruses in central Thailand belong to a similar lineage and suggested that they originate from a locally persistent reservoir (Amonsin et al., 2006; Buranathai et al., 2007). The disappearance of the virus in summer suggested that there might be only a small number of viruses surviving the inter-outbreak period, causing a bottleneck effect. Although previously published analyses of viral sequences in 2004–2005, when the epidemic was extensive and did not completely subside in summer, did not suggest any bottleneck effect (Amonsin et al., 2006; Buranathai et al., 2007), we questioned whether the situation would be different in 2006–2008, when the epidemic was better controlled. In order to understand the nature of viral perpetuation and resurgence, we analysed nucleotide sequences from viruses isolated between 2006 and 2008 in the lower northern provinces of Thailand, where outbreaks have taken place repeatedly in every outbreak season.

**METHODS**

**Outbreaks.** We investigated six episodes of poultry die-off. All but one occurred in backyard chicken flocks with limited spread. One outbreak in the Chumsaeng district of Nakhonsawan province was in a broiler chicken farm. These outbreaks were located in the Yom–Nan River basin. The Yom and Nan Rivers are branches of the Chaopraya River, the main river of the central plain of Thailand. The locations of the outbreaks are shown in Fig. 1, and characteristics of the outbreaks are given in Table 1.

**Virus isolation and sequencing.** Tracheal and cloacal swabs collected from backyard chickens and ducks were inoculated into Madin–Darby canine kidney cells. The subtype of viruses was identified by RT-PCR using H5- and N1-specific primers (Lee et al., 2001). RT-PCR was performed on RNA samples extracted from haemagglutination-positive culture supernatants and the PCR

![Fig. 1. Location of H5N1 outbreaks in poultry in 2007–2008, with the date of sample collection from the outbreaks.](http://vir.sgmjournals.org)
products were directly sequenced as described previously (Puthavathana et al., 2005). The sequences of A/chicken/Thailand/PC168/06, A/chicken/Thailand/PC170/06 and A/duck/Phitsanulok/NIAH6-5-0001/07 were obtained from GenBank. All sequences determined in this study were submitted to GenBank; the accession numbers are shown in Supplementary Table S1, available in JGV Online.

Phylogenetic reconstruction. Sequence alignment was carried out using MUSCLE version 3.6 and adjusted manually using BioEdit version 7.0.9. The maximum-likelihood (ML) method from the PhyML package was applied to generate a phylogenetic tree of the aligned sequences. Bootstrap analyses were performed on the ML trees for 1000 pseudo-replicates.

RESULTS

We sequenced all of the genomic fragments of the isolated viruses. The sequences were inspected for unusual changes. We found no mutations at key determinant residues, including receptor-binding preference in the haemagglutinin (HA) gene at positions 138, 186, 196 and 226–228 (H3 numbering system) (Auewarakul et al., 2007; Stevens et al., 2006; Yamada et al., 2006); adaptation to mammalian hosts and growth at lower temperatures in the polymerase basic gene (PB2) at position 627 (Hatta et al., 2007); innate immunity escape and cytokine induction in the non-structural gene (NS1) at position 92 (Lipatov et al., 2005; Seo et al., 2004); and oseltamivir resistance in the neuraminidase (NA) gene at position 274 (Le et al., 2005).

We performed phylogenetic analyses of all of the genomic segments using the ML algorithm. All of the sequences clustered with clade 1, genotype Z viruses isolated previously in the central region of Thailand, and could be clearly separated from the previously reported clade 2.3.4 from north-eastern Thailand (Chutinimitkul et al., 2007) (Fig. 2). When we looked at the relationship among the newly isolated viruses, the HA sequences of ICRC-V143 and ICRC-V213 did not appear to be more related to each other than to other Thailand sequences (Fig. 2). This was unexpected, as the two viruses were isolated from neighbouring areas over consecutive time periods. In contrast, sequences with clear epidemiological linkage clustered closely and formed separated branches, e.g. sequences from open-billed storks in Nakhonsawan in 2005, which indicated a common origin of viruses within the same outbreak cluster. On the other hand, the HA sequence of ICRC-V213 was closely related to that of ICRC-V195 (Fig. 2). The two viruses were isolated in the same week of August 2007 but from areas about 60 km apart. Surprisingly, the patterns of relationship among these three viruses were not consistent for some other genomic segments. The polymerase subunits PB1 and PA of ICRC-V213 were closely related to those of ICRC-V143 rather than ICRC-V195. This suggested that ICRC-V213 could be a reassortant between ICRC-V143- and ICRC-V195-like viruses. Interestingly, the HA, matrix (M),

<table>
<thead>
<tr>
<th>Location (district, province)</th>
<th>Type of poultry</th>
<th>No. of birds</th>
<th>Viral name</th>
</tr>
</thead>
<tbody>
<tr>
<td>Muang, Phitsanulok</td>
<td>Ducks</td>
<td>207 died (30 backyard chickens, 177 ducks)</td>
<td>A/duck/Thailand/PC-170/2006</td>
</tr>
<tr>
<td>Kongkrilat, Sukhothai</td>
<td>Backyard chickens</td>
<td>Unknown</td>
<td>A/chicken/Thailand/PC168/06, A/chicken/Thailand/PC170/06</td>
</tr>
<tr>
<td>Wangtong, Phitsanulok</td>
<td>Backyard chickens</td>
<td>8 died from 70 backyard chickens (11.4%)</td>
<td>A/chicken/Thailand/ICRC-V143/2007</td>
</tr>
<tr>
<td>Kirirom, Si Sunthai</td>
<td>Backyard chickens</td>
<td>9 died from 70 backyard chickens (13.1%)</td>
<td>A/chicken/Thailand/ICRC-V195/2007</td>
</tr>
<tr>
<td>Chumpon, Phitsanulok</td>
<td>Backyard chickens</td>
<td>408 died from 10,000 backyard chickens (4.08%)</td>
<td>A/chicken/Thailand/ICRC-V195/2007</td>
</tr>
<tr>
<td>Suphanburi</td>
<td>Captive pheasant</td>
<td>127 died from 250 backyard chickens (50.8%)</td>
<td>A/chicken/Thailand/ICRC-V213/2007</td>
</tr>
<tr>
<td>Nakhonsawan</td>
<td>Backyard chickens</td>
<td>1 died from 10 apparent healthy ducks (0.2%)</td>
<td>A/chicken/Thailand/ICRC-V213/2007</td>
</tr>
<tr>
<td>Suphanburi</td>
<td>Captive pheasant</td>
<td>1 died from 10 apparent healthy ducks (0.2%)</td>
<td>A/chicken/Thailand/ICRC-V213/2007</td>
</tr>
</tbody>
</table>

Table 1. Characteristics of the poultry outbreaks in 2006–2008

Data from the Department of Livestock Development (http://www.dld.go.th/home/bird_flu/history.html).

*Data from the Department of Livestock Development (http://www.dld.go.th/home/bird_flu/history.html).
nucleoprotein (NP) and PA genes of ICRC-V195 were closely related to those of A/chicken/Thailand/PC-168/2006 (PC-168), a virus isolated in a neighbouring province, Pichit, 1 year earlier. Moreover, the NA, NS, PB1 and PB2 genes of ICRC-V195 were closely related to those of A/chicken/Thailand/PC-170/06 (PC-170), a virus isolated in the same area and time period as PC-168 (Fig. 2). Although PC-168 and PC-170 were from the same area in the same time period, these two viruses were not more related to each other than to other clade 1 Thailand viruses. This suggested a reassortment event between the PC-168 and PC-170, which might have given rise to ICRC-V195. Another 2007 virus, A/duck/Phitsanulok/NIAH6-5-0001/2007, was identified in January 2007, but its HA and NA sequences did not show any closer relationship to other recent isolates than to other clade 1 isolates, suggesting that this HA/NA lineage may have been extinct after January 2007. However, as sequences of other genomic segments of this virus are not available, we do not know whether the virus passed on some of its other genomic segments to other viruses via reassortment. In January 2008, outbreaks reappeared in Nakhonsawan, Phitsanulok and Supanburi. The viruses A/chicken/Thailand/ICRC-V586/2008, A/chicken/Thailand/ICRC-V618/2008 and A/duck/Thailand/ICRC-V629/2008, and A/peasant/Thailand/VSU-MU-1-SPB/2008 were isolated from Nakhonsawan, Phitsanulok and Supanburi, respectively. The virus A/peasant/Thailand/VSU-MU-1-SPB/2008 was isolated from a zoo in an area where poultry die-off had occurred. All of the genomic segments of this virus clustered closely with those of ICRC-V143, whereas sequences of the other viruses isolated in January 2008 were closely related to those of ICRC-V195. In summary, viruses from a cluster of outbreaks in January 2008 (ICRC-V586, ICRC-V618 and ICRC-V629) appeared to be direct descendents of a virus similar to an isolate from August 2007 (ICRC-V195), which was probably a reassortant of two viruses similar to those identified in July 2006 (PC-168 and PC-170). Another isolate from January 2008 (A/peasant/VSU-MU-1-SPB/2008) seemed to be a descendant of a virus similar to a chicken isolate from June 2007 (ICRC-V143) (Fig. 3). These data indicated that at least two varieties of H5N1 virus persisted silently over the dry summer months and gave rise to outbreaks in the 2007–2008 season, and that reassortment among these viruses may have occurred. The two varieties were probably responsible for all of the outbreaks in season 2007–2008. Other strains from 2004–2005 were not detected after 2006, suggesting that they were extinct, resulting in a strong bottleneck effect on the virus population.

**DISCUSSION**

A bottleneck effect is an evolutionary event in which a substantial fraction of a population is eliminated, leaving the surviving minority to repopulate the milieu. Population bottlenecks can increase genetic drift. The re-emergence of H5N1 viruses of similar lineage every year in the rainy season after a silent period in the summer suggests that either there is an unrecognized infected reservoir or there is a low-level transmission chain during the summer months. Either way, if the reservoir or the level of the transmission chain during the summer is small enough, there would be a bottleneck effect on the virus population after re-emergence, as only a few viruses would re-emerge each year and give rise to a few branches, leaving other branches extinct. In this case, phylogenetic trees would show subclusters with some chronological and spatial correlations. However, if the reservoir or the transmission chain during summer was large, the emerging virus population structure would be similar to that of the previous season. In this case, phylogenetic trees would show no specific pattern. Previously published phylogenetic analyses of H5N1 sequences from central Thailand in 2004–2005 did not show any specific sublineage within the virus population (Amonsin et al., 2006; Buranathai et al., 2007; Keawcharoen et al., 2005). This suggested that the virus was endemic in the area throughout the year without interruption, despite the brief periods of undetectable transmission during the summer months. In contrast, the outbreaks in 2006–2008 were much smaller in their geographical extent and the number of birds affected. This reduced the size of the virus population in the outbreak season and also in the inter-outbreak period. The virus populations in the summers of 2006 and 2007 were probably small enough to cause a bottleneck effect. Our data support this model. Despite the evidence supporting the existence of a bottleneck, our analyses did not indicate where this bottleneck might be. Mild or asymptomatic infection with limited virus shedding is ideal to render transmission chains undetectable. One of the recent isolates in this study was from an apparently healthy duck, and ducks have been shown to be infected with mild disease by some H5N1 strains (Sturm-Ramirez et al., 2005). Free-grazing duck raising is still common practice in the Yom–Nan River basin, and previous risk analyses have shown that populations of free-grazing ducks correlate well with the risk of AI outbreak (Gilbert et al., 2006, 2007, 2008). It is therefore likely that free-grazing ducks served as the viral reservoir and that limitation of transmission chains within duck populations in inter-outbreak periods caused the observed bottleneck effect. Infection of vaccinated animals can also make the infection asymptomatic and render the transmission undetectable. Although poultry vaccination is prohibited in Thailand, illegal vaccination is believed to be widespread in the area, especially in fighting cocks. However, accurate information on poultry vaccination in Thailand is not available. The extent and role of poultry vaccination in this country needs to be clearly elucidated. Accurate identification of the reservoir and bottleneck requires more detailed surveillance, especially in the inter-outbreak periods. Such intensified surveillance would...
greatly benefit our understanding and future control of H5N1 AI epidemics.

The similarity between ICRC-V143 and A/pheasant/Thailand/VMU-1-SPB/2008 was intriguing, as the locations of the two outbreaks were 280 km apart. This suggests that a long-range carrier, such as birds or the poultry trade, might be involved in the transmission. Understanding how the virus spreads over long distances is crucial to the successful control of the epidemic and deserves further investigation.

H5N1 AI outbreaks have been occurring repeatedly in the Yom–Nan River basin in recent years. The area has a good irrigation system and the water supply is abundant all year round. Rice cultivation is also carried out in the summer in this area, whilst it is limited to the rainy season in most other areas of the country. The largest freshwater lake in Thailand, Bung Borapet, with an area of over 200 km², is also located in this area. Whether these geographical characteristics contribute to the repeated AI outbreaks requires further investigation. The limited geographical area of the sporadic outbreaks in 2006–2008 to the Yom–Nan River basin suggests that future attempts at controlling the virus reservoirs and outbreaks should be focused in this area.

The small pool of viruses surviving inter-outbreak periods may be amplified periodically in the outbreak seasons. It is
plausible that the cycle of low-level perpetuation in the summer and amplification in the rainy season and winter may together play crucial roles in persistence of the virus in this area. Our analyses suggest that the virus population during the inter-outbreak period may be quite small and could be the weak link in the cycle. Targeting this weak link by both extensive surveillance for virus reservoirs in summer and disrupting transmission chains at the end of the outbreak season to prevent the establishment of virus reservoirs may be the most appropriate strategy in the current situation in Thailand. Such a strategy could potentially lead to the eradication of the virus from this country.

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Fig. 3. Schematic diagram showing the predicted evolutionary paths of recent H5N1 isolates in Thailand and the proposed bottlenecks. The linear relationships among viruses were derived from the clustering of their genomic segment sequences in the phylogenetic trees in Fig. 2.

REFERENCES


