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Rapid Communication

Characterization of a highly pathogenic avian influenza H5N1 virus sublineage in poultry seized at ports of entry into Vietnam☆

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ABSTRACT

Highly pathogenic avian influenza H5N1 virus was detected in poultry seized at two ports of entry located in Lang Son Province, Vietnam. Sequence analysis of the hemagglutinin (HA) genes from five H5N1 virus isolates and ten PCR amplicons from chicken cloacal samples revealed their close phylogenetic relationship to clade 7 H5N1 HA genes. However, these HA genes exhibited extensive genetic divergence at both the nucleotide and amino acid levels in comparison to previously described clade 7 viruses; e.g., A/chicken/Shanxi/2/2006. In addition, hemagglutination inhibition tests revealed antigenic differences between these and previously isolated H5N1 viruses from Vietnam. These results indicate that viruses with clade 7 HA are evolving rapidly in poultry in Southeast Asia.

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Introduction

Highly pathogenic avian influenza (HPAI) H5N1 virus continues to circulate in poultry populations of many countries in the Middle East, Africa, and Asia (FAO, 2008a). The virus spreads to new areas from endemic regions causing outbreaks in poultry and aquatic birds. Migratory birds and movement of poultry and/or poultry products have been proposed as two major mechanisms of geographic spread (Kilpatrick et al., 2006). These mechanisms of virus spread to a new area are most often inferred by phylogenetic analysis of sequence data, and subsequently tracing known migratory bird routes or patterns of international poultry trade linking the geographic sites involved (Wallace et al., 2007). Intensive global H5N1 virologic surveillance in birds and phylogenetic analysis of the viral HA have revealed the presence of locally or regionally evolved virus lineages at each geographic location (Ducatez et al., 2007; Nguyen et al., 2008; Salzberg et al., 2007; Smith et al., 2006). Vietnam, in particular, implemented a comprehensive HPAI surveillance program in poultry in 2004, which provided an understanding of the spatiotemporal evolution of H5N1 in the country (Wan et al., 2008). These and additional phylogeographic analyses of H5N1 in Vietnam have demonstrated that multiple sublineages (clades) of the virus were introduced into the country in the recent past (Nguyen et al., 2008; Smith et al., 2006). Although exotic avian influenza (AI) viruses are most likely introduced into a country by either international poultry movement or bird migration (Chen et al., 2006; Ducatez et al., 2007; Salzberg et al., 2007; Wang et al., 2008a), few studies provide direct evidence to support either claim. While migratory birds are likely to have played a major role in the spread of clade 2.2 viruses into Europe and Africa following the Qinghai Lake outbreaks in 2005 (Wang et al., 2008b), some studies have implicated legally traded poultry and its products in the movement of H5N1 between regions (Mase et al., 2005; Tumpey et al., 2002). Illegal international movement of live birds has also been considered as a potential mechanism of spread (Lee et al., 2007). HPAI H5N1 of Asian origin was identified in two live crested-hawk eagles confiscated at the Brussels airport in

☆ The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention or the Agency for Toxic Substances and Disease Registry.

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December 2004 (Steensels et al., 2007). As reported herein, the detection of an exotic clade of H5N1 in poultry seized at north-eastern ports of entry into Vietnam highlights the importance of international border control and other barriers to control the geographic spread of the virus.

Results

Virological surveillance of imported poultry at ports of entry

To control the spread of highly pathogenic avian influenza (HPAI) H5N1 virus Vietnam has implemented trade restrictions on the importation of poultry and/or poultry products from H5N1 infected countries. These regulations are enforced at sanitary control stations at all land, air and sea ports of entry into Vietnam. In particular, imported illegal birds seized at border control stations in Lang Son Province are transported to regional or national quarantine departments where cloacal swabs are collected. The swabs are placed in containers with viral transport medium and refrigerated until shipped to the National Centre for Veterinary Diagnostics (NCVD), Hanoi, Vietnam for diagnostic testing. From January to May of 2008 NCVD received 495 cloacal swab samples from chickens confiscated at two border crossing checkpoints between Guangxi Province, China and Lang Son Province, Vietnam (Table 1). The two checkpoints were located in separate administrative districts (Cao Loc and Loc Binh) in Lang Son Province. Over this time period, 17 of 495 swabs from confiscated chickens tested positive for influenza A matrix gene RNA by real-time RT-PCR used in screening. Further real-time RT-PCR testing using subtype-specific primers and probes identified 15 of the 17 matrix positive swabs as containing the H5 hemagglutinin (HA) and N1 neuraminidase (NA) (Table 1). The remaining two matrix positive samples received from Lang Son Province border checkpoints yielded low pathogenic avian influenza subtype H9N2 viruses (data not shown).

Viral isolation, sequencing and phylogenetic analysis

Five of the 15 samples cultured for virus isolation yielded replicating virus (Table 1). The HA gene of each of these viruses was then sequenced following RNA extraction and amplification by RT-PCR. The remaining 10 swabs without viable virus were also used for RNA extraction and PCR amplification for sequence analysis of the HA gene (Table 1). The nucleotide sequences of the HA genes of five H5N1 virus isolates, as well as ten HA genes derived from swab samples, were deposited in public databases (Table 1).

Database similarity searches (NCBI, http://blast.ncbi.nlm.nih.gov/Blast; LANL, https://flu.lanl.gov/blast) using these HA gene sequences indicated that they were most closely related to two previously described H5N1 viruses; A/chicken/Hebei/326/2005 and A/chicken/Shanxi/2/2006 viruses (on average 96.1% and 96.3% HA gene nucleotide sequence identity, respectively). Phylogenetic analysis performed to investigate the ancestry of the 15 viral HA sequences revealed that the HA gene of each virus clustered within a subgroup of previously reported clade 7 HA genes (Fig. 1). Notably, 13 of the 15 HA genes branched from one of two distinct nodes in the tree (Fig. 1; provisionally designated as NCVD groups A and B). In addition, two samples (A/chicken/Vietnam/NCVD-016/2008 and A/chicken/Vietnam/NCVD-swab19/2008) appeared to occupy intermediate positions in the phylogenetic tree, closer to the roots of the two groups. There was no clear association between the phylogenetic clustering of each group and the epidemiologic information such as port of entry or date of swab specimen collection that may have suggested why two discrete groups of viruses were identified (Table 1). However, the geographic origins of the infected poultry could not be determined.

The evolutionary distances between NCVD group A and B HA genes suggest extensive diversity within this sublineage of the clade 7 genes, as indicated by the length of the branches on the phylogenetic tree (Fig. 1), as well as their average nucleotide distance from the nearest clade 7 HA progenitor (i.e., A/chicken/Shanxi/2/2006). To quantify the genetic divergence between these and other H5N1 viruses, pairwise comparisons of the percentage nucleotide and amino acid divergence were generated (Table 2). Representative viral strains from each of the major H5N1 clades were included in this analysis to provide references with which to compare the degree of genetic divergence within and between the newly identified HA genes and previously reported clades (WHO/OIE/FAO, 2008). The HA genes of NCVD groups A or B displayed very little within group nucleotide or deduced amino acid sequence divergence with an overall average of 0.39% nucleotide divergence and 0.38% amino acid divergence. In contrast, divergence between these two groups reached an average of 4.05% and 5.69% at the nucleotide and amino acid levels across the HA gene coding region (Table 2). This high degree of divergence equated to ≥30 amino

Table 1
H5N1 viral isolates and RNA from chicken swabs collected at ports of entry into Vietnam.

Viral isolates[a]	Port of entry (entity, district, province)	Collection date	Clade/group	Subtype	GenBank accession number for HA gene
A/chicken/Vietnam/NCVD-016/2008	Sub-DAH, Cao Loc, Lang Son	Feb-08	7/NCVD	H5N1	FJ842476
A/chicken/Vietnam/NCVD-03/2008	Sub-DAH, Cao Loc, Lang Son	Feb-08	7/NCVD group B	H5N1	FJ842481
A/chicken/Vietnam/NCVD-04/2008	Sub-DAH, Cao Loc, Lang Son	Feb-08	7/NCVD group B	H5N1	FJ842482
A/chicken/Vietnam/NCVD-05/2008	Sub-DAH, Cao Loc, Lang Son	Feb-08	7/NCVD group B	H5N1	FJ842483
A/chicken/Vietnam/NCVD-093/2008	Sub-DAH, Cao Loc, Lang Son	Apr-08	7/NCVD group A	H5N1	FJ842480
HA gene DNA amplicon from viral RNA[a]					
A/chicken/Vietnam/NCVD-swab06/2008	Sub-DAH, Cao Loc, Lang Son	Feb-08	7/NCVD group B	H5N1	FJ842484
A/chicken/Vietnam/NCVD-swab15/2008	DAH-RQD, Loc Binh, Lang Son	Mar-08	7/NCVD group A	H5N1	FJ842477
A/chicken/Vietnam/NCVD-swab16/2008	Sub-DAH, Loc Binh, Lang Son	Apr-08	7/NCVD group B	H5N1	FJ842485
A/chicken/Vietnam/NCVD-swab17/2008	Sub-DAH, Loc Binh, Lang Son	Apr-08	7/NCVD group A	H5N1	FJ842478
A/chicken/Vietnam/NCVD-swab18/2008	Sub-DAH, Loc Binh, Lang Son	Apr-08	7/NCVD group A	H5N1	FJ842479
A/chicken/Vietnam/NCVD-swab19/2008	Sub-DAH, Cao Loc, Lang Son	Apr-08	7/NCVD	H5N1	FJ842486
A/chicken/Vietnam/NCVD-swab20/2008	Sub-DAH, Cao Loc, Lang Son	Apr-08	7/NCVD group B	H5N1	FJ842487
A/chicken/Vietnam/NCVD-swab24/2008	DAH-RQD, Loc Binh, Lang Son	Apr-08	7/NCVD group B	H5N1	FJ842488
A/chicken/Vietnam/NCVD-swab25/2008	DAH-RQD, Loc Binh, Lang Son	Apr-08	7/NCVD group B	H5N1	FJ842489
A/chicken/Vietnam/NCVD-swab26/2008	DAH-RQD, Loc Binh, Lang Son	Apr-08	7/NCVD group B	H5N1	FJ842490

Sub-DAH, Lang Son Provincial Sub-Department of Animal Health.
DAH-RQD, Department of Animal Health-Regional Quarantine Department.
[a] From cloacal swab specimen.