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New Genotype of Avian Influenza H5N1 Viruses Isolated from Tree Sparrows in China

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The 2004 outbreaks of highly pathogenic avian influenza H5N1 disease in China led to a great poultry loss and society attention. A survey of avian influenza viruses was conducted on tree sparrows (*Passer montanus*) collected in China in 2004. Four viruses were isolated from free-living tree sparrows. The results of the whole-genome analysis indicated that an H5N1 virus with a new genotype is circulating among tree sparrows. The hemagglutinin and neuraminidase genes of the new genotype were derived from Gs/Gd/96-like viruses and the nuclear protein gene descended from the 2001 genotype A H5N1 viruses, while the other inner genes originated from an unknown influenza virus. In experimental infection, all four viruses were highly pathogenic to chickens but not pathogenic to ducks or mice. The four tree sparrow viruses were different from the 2003 tree sparrow strain (genotype Z) in Hong Kong. The results suggested that H5N1 viruses might be distributed widely in tree sparrows.

Highly pathogenic H5N1 influenza virus has caused serious poultry loss, and since 1997 it has been reported to cause human deaths. It has been determined that H5N1 influenza viruses have undergone reassortment in recent years (12, 17). The Hong Kong influenza H5N1 virus that infected humans in 1997 was confirmed to be a reassortant virus that had acquired the hemagglutinin (HA) gene from A/goose/GuanGdong/1/96 (Gs/Gd/96; H5N1)-like viruses, the neuraminidase (NA) gene from A/teal/HongKong/W312/97 (Teal/HK/W312/97; H6N1)-like viruses, and the internal genes from A/quail/HongKong/G1/97(Qa/HK/G1/97; H9N2)-like or Teal/HK/W312/97 viruses (1, 5). Multiple genotypes of H5N1 were detected from 2001 to 2004, which were designated A, B, C, D, E, V, W, X, Y, Z, and Z+ (6, 12). Since 2002, genotype Z has been the dominant H5N1 virus in southern China and was responsible for the 2003-2004 outbreaks in Asia (12).

Although the aquatic bird has been considered a natural reservoir for avian influenza viruses (20), in recent years H5N1 viruses have also been isolated from terrestrial birds. From 2002 to 2004, genotype Z viruses were isolated from a feral pigeon (*Columba livia*), a tree sparrow (*Passer montanus*), and a peregrine falcon (*Falco peregrinus*) (12), and two genotype V viruses were isolated from crows in Japan (13). The systematic surveillance of avian H5N1 influenza viruses in resident birds has not been well established. As the tree sparrow is a very common terrestrial bird in China and has frequent contact with humans, we conducted a survey of epidemic avian influenza viruses in tree sparrows in the Henan province of China in 2004. Four H5N1 avian influenza viruses were isolated. All the viruses were sequenced, and their pathogenicity to chickens, ducks, and mice were tested. The results indicated that they were new-genotype H5N1 viruses and highly pathogenic to chickens.

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MATERIALS AND METHODS

Virus isolation. A survey of epidemic avian influenza virus in tree sparrows was conducted in Pingyu country in the Henan province of China in May 2004. Cloacal swabs were collected from 38 captured free-living tree sparrows and were eluted with 0.5 ml of phosphate-buffered saline (PBS). After centrifugation at 6,000 × *g*/min for 5 min at 4°C, the supernatant was identified by sandwich enzyme-linked immunosorbent assay with an avian influenza virus antigen detection kit (Keqian Ltd.). The antigen-positive supernatant of cloacal swabs was mixed with an equal volume of PBS containing antibiotics (penicillin G, 4,000 U/ml; streptomycin sulfate, 800 U/ml) for 4 h at 4°C and was inoculated into the allantoic cavities of 10-day-old, specific-pathogen-free, embryonated eggs (Beijing MERIAL Ltd.). After incubation at 37°C for 48 to 72 h, the allantoic fluid of the inoculated eggs was collected. Fifty percent egg infectious dose (EID_{50}) titers were calculated by the method of Reed-Muench. Allantoic fluid containing virus stocks had been stored at −70°C before being used.

RNA extraction and nucleotide sequencing. Viral RNA was extracted from virus-infected allantoic fluid with Trizol reagent (Invitrogen). The cDNAs were amplified by the TaKaRa rTAQ enzyme (TaKaRa Bio) with avian influenza virus primers (10). After being purified with Montage PCR cleanup filter plates (Millipore Corporation), the PCR products were used for sequencing with an Amersham ET dye terminator kit (Amersham Pharmacia Bio) and ABI PRISM 370 DNA sequencer (PE Applied Biosystems). The sequencing primers were available at the website http://www.genomics.org.cn/AI/index.jsp. All sequence data were edited by BioEdit version 5.0.9 and aligned by Clustal X (version 1.8). Phylogenetic trees were generated with MEGA version 2.0.

Pathogenicity tests. To determine the pathogenicity of the virus isolated, the viruses were inoculated into chickens, ducks, and mice. Groups of eight specific-pathogen-free, 6-week-old chickens (Beijing MERIAL Ltd.) were tested according to the recommendation of the Office International des Épizooties (OIE). Each chicken was intravenously injected with 0.2 ml of a 1:10 dilution of allantoic fluid containing virus, and mortality was observed over a 10-day period. Groups of eight 3-week-old ducks were inoculated intranasally with 0.1 ml of allantoic fluid, and mortality was also observed over a 10-day period. Groups of 10 6- to

TABLE 1. Percentages of sequence similarity between the tree sparrow virus isolates and other influenza viruses

Segment	Virus with the highest similarity[a]	% Similarity
PB2	A/Pheasant/Ireland/PV18/97 (H9N2)	94
PB1	A/swine/Hong Kong/126/82 (H3N2)	94
PA	A/swine/Hong Kong/81/78 (H3N2)	94
HA	A/chicken/Jilin/9/04 (H5N1)	97
NP	A/Duck/Hong Kong/289/78 (H9N2)	98
NA	A/Duck/Guangxi/50/01 (H5N1)	96
M	A/Duck/Hong Kong/698/79 (H5N3)	98
NS	A/Duck/Hong Kong/610/79 (H9N2)	98

[a] Percentages of sequence similarity were calculated based on the nucleotide sequences of the complete open reading frames of eight genes, with Ts/HN/2/04 as the representative strain. The nucleotide sequences of Ts/HN/2/04 were compared to those in GenBank.

8-week-old female BALB/c mice (Experimental Animal Center of Hubei Control Disease Center, Wuhan, China) were inoculated intranasally with the virus-allantoic fluid in a volume of 50 μl and were observed daily for 14 days for signs of disease. On the third day, three mice of each group were killed purposely, and the EID_{50}s of the viruses in the lungs and brains were determined. The remaining mice were monitored daily for mortality. All the animal experiments were performed in a biosafety level 3 laboratory, and the inoculated viral doses of the allantoic fluid were $10^{5.5}$ to $10^{6.5}$ EID_{50}s. Control groups of chickens, ducks, and mice were inoculated with PBS.

Nucleotide sequence accession numbers. All sequences have been deposited in GenBank. The accession numbers are AY741215 to AY741222 and DQ073399 to DQ073422.

RESULTS

Virus isolation and molecular characterization. Among the 38 cloacal swabs from captured free-living tree sparrows, 25 tested avian influenza virus antigen positive by enzyme-linked immunosorbent assay. These 25 samples were inoculated into embryonated eggs, and four avian influenza viruses were isolated. They were named A/Tree sparrow/Henan/1/04 (H5N1) (Ts/HN/1/04) to A/Tree sparrow/Henan/4/04 (H5N1) (Ts/HN/4/04). Each of eight gene segments of the four viruses was sequenced. The four isolates shared a high homology with each other (96% to 99%), except in their NA and PB2 genes. For the NA gene, only Ts/HN/4/04 did not contain a 20-amino-acid (aa) deletion in the stalk of the NA molecule, so the homology between Ts/HN/4/04 and other sparrow viruses was low (91% to 92%). The homology between the PB2 genes of Ts/HN/2/04 and Ts/HN/3/04 was 99%, but the homology between Ts/HN/1/04 and Ts/HN/2/04 (or Ts/HN/3/04) was 91%, while that between Ts/HN/4/04 and Ts/HN/2/04 (or Ts/HN/3/04) was 95%. Nucleotide sequence analysis revealed that they originated from different sources (Table 1.).

The HA genes from the four tree sparrows all have the same multiple basic amino acids (RRKKR) at the connecting peptide between HA1 and HA2, which was considered a characteristic of influenza viruses that are highly pathogenic for chickens (11). All amino acids relevant to receptor binding (aa 91, 130 to 134, 149, 151, 179, 186, 190 to 191, and 220 to 225) were identical to those of Gs/Gd/1/96 (3). Therefore, it is likely that these viruses bind to cellular receptors with 2,3-NeuAcGal linkages, as has been shown for Gs/Gd/1/96 (7).

The HA protein of Ts/HN/1/04, Ts/HN/2/04, and Ts/HN/3/04 had an additional glycosylation site (aa 170 to 172) at the head of the HA molecule and contained a 20-aa deletion in the stalk of the NA molecule, while Ts/HN/4/04 did not. The additional glycosylation site in the HA protein and the deletion of 20 aa in the NA protein were also dominant in other 2004 viruses isolated from poultry and human, which was suggested to be connected with the adaptation of viruses for spreading more efficiently in terrestrial domestic poultry (15).

Four tree sparrow viruses had a 5-aa deletion (aa 80 to 84) in the middle of the NS molecule but did not have a mutation of Glu92 in the NS1 protein or Lys627 in the PB2 protein, which had been suggested to be associated with the increased virulence of H5N1 viruses in mice (4, 8). Moreover, the four viruses did not have mutations at the amino acids Ser31, Leu26, Val27, and Ala30 in the transmembrane region of the M2 protein, which occur in many genotype Z viruses and which have been proved to be associated with amantadine resistance (18).

Phylogenetic analysis. Phylogenetic analysis revealed that the HA genes of tree sparrows formed a branch in the phylogenetic tree with 2000-2004 isolates from Asia (genotypes Z, Z+, Y, A, B, C, D, E, and X). The HK/97 isolates formed another branch. Both branches originated from Gs/Gd/96-like viruses (Fig. 1).

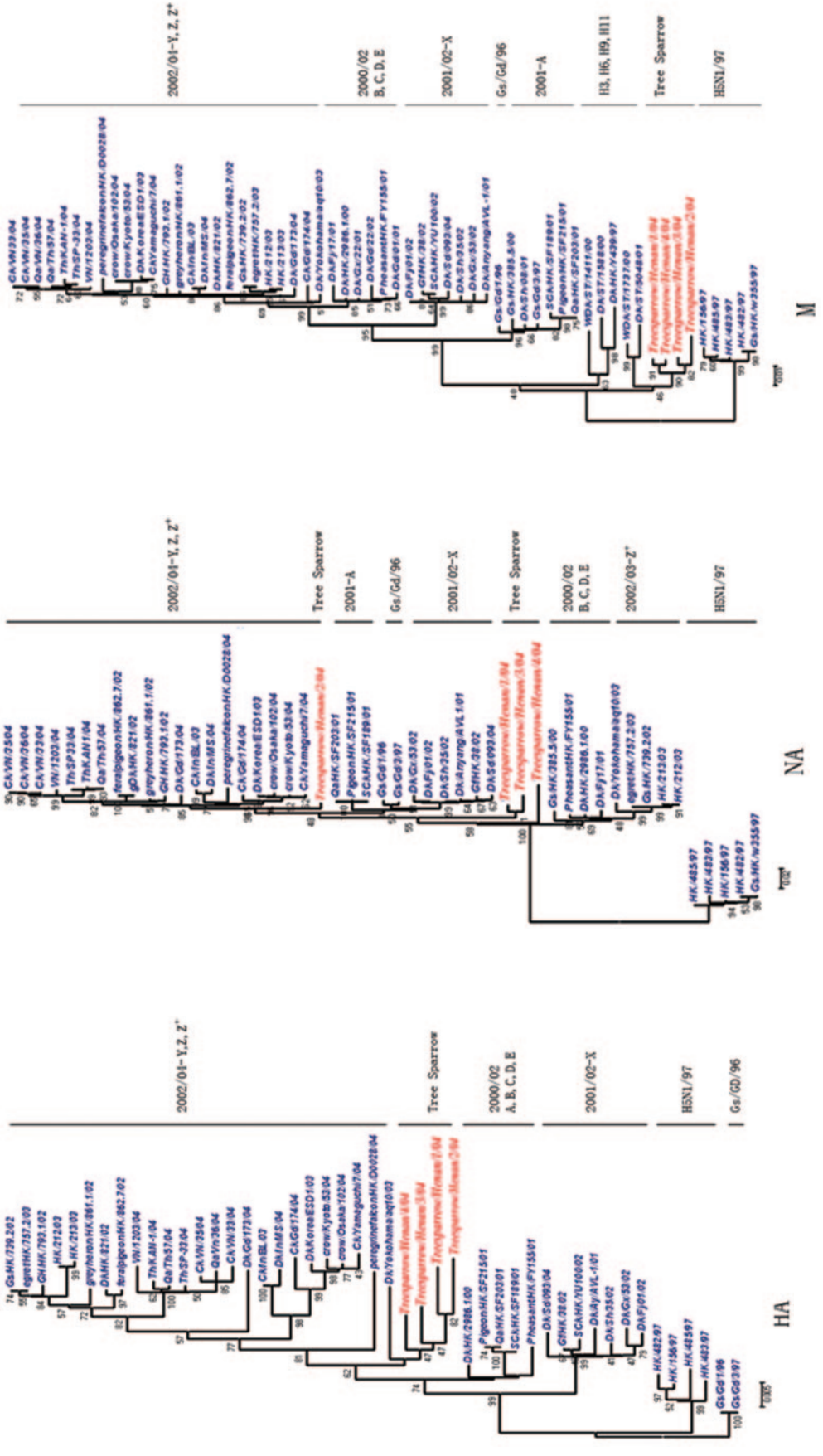
The NA gene tree showed that tree sparrows branched with genotypes A, B, C, D, E, X, Y, Z, and Z+ and with Gs/Gd/96 and formed a branch of avian influenza viruses. Isolates of HK/97 formed a separate branch. Unlike the other three tree sparrow isolates, Ts/HN/02 clustered into the branch of genotype Y, Z, and Z+ viruses of 2002 to 2004 (Fig. 1).

Analysis of the internal protein genes shows that four H5N1 sparrow viruses originated from multiple avian influenza viruses. The PB2 genes of Ts/HN/2/04 and Ts/HN/3/04 were almost identical, but they were different from Ts/HN/1/04 and Ts/HN/4/04. Ts/HN/1/04 and Ts/HN/4/04 formed a branch together with genotypes A, B, C, D, E, Y, Z, Z+, and X and with Gs/Gd/96. This branch was distinctly related to the branch of Ts/HN/2/04 and Ts/HN/3/04 and the branch of H5N1/97 (Fig. 1). Interestingly, the PB2 gene of DK/Yolohama/aq10/03, which was a genotypically unique H5N1 influenza virus isolated from duck meat in 2003 (14), was found to be most closely related to that of the tree sparrows (Fig. 1).

In the phylogenetic tree of the PB1 gene, the four sparrow viruses formed a separate fork and clustered with the branch of genotypes A, B, C, D, E, Y, Z, and Z+ and the branches of Gs/Gd/96 and HK/97. According to the phylogenetic tree, the PB1 gene of tree sparrows might be the ancestor of genotypes A, B, C, D, E, Y, Z, and Z+ and of Gs/Gd/96 and HK/97. The PB1 gene of genotype X is distinctly related to those of all the above-named genotypes (Fig. 1).

In the phylogenetic tree of the PA gene, the four sparrow viruses formed a separate branch. The branch was distinctly related to the branch of genotypes A, B, C, D, E, Y, Z, Z+, and X and to the branch of Gs/Gd/96, the branch of H5N1/97, and that of Gf/HK/38/02 (Fig. 1).

In the phylogenetic tree of the NP gene, the four sparrow viruses formed a separate fork and clustered with the branch of genotypes B, C, E, Y, Z, Z+, and X, the branch of HK/97, and that of DK/Yolohama/aq10/03 (14). The NP genes of the above-named genotypes were similar to those of genotype A and Gs/Gd/96 but distinctly related to that of genotype D (Fig. 1).


HA
2002/01-Y, Z, Z+
Tree Sparrow
2000/02
A, B, C, D, E
2001/02-X
H5N1/97
Gs/GD/96
NA
2002/01-Y, Z, Z+
Tree Sparrow
2001-A
Gs/Gd/96
2001/02-X
Tree Sparrow
2000/02
B, C, D, E
2002/03-Z+
H5N1/97
M
2002/01-Y, Z, Z+
2000/02
B, C, D, E
2001/02-X
Gs/Gd/96
2001-A
H3, H6, H9, H11
Tree Sparrow
H5N1/97

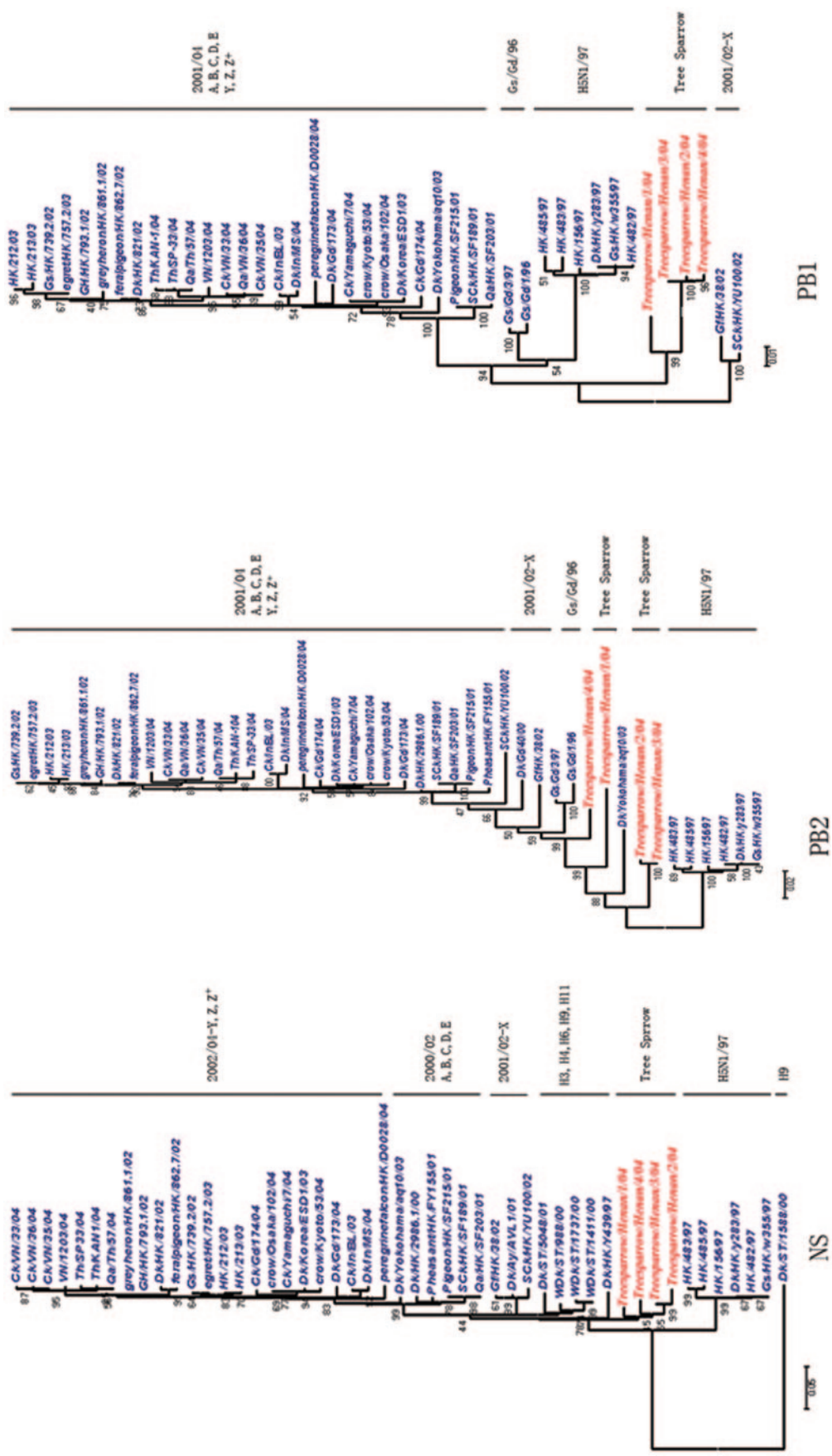


FIG. 1. Phylogenetic trees of virus genes isolated from tree sparrows in China. Trees were generated by using neighbor-joining analysis with the Tamura-Nei-gamma model in the MEGA program (version 2.0). Numbers below branches indicate bootstrap value percentages from 1,000 replicates. Analysis was based on nucleotides 1 to 1,012 (1,012 bp) of the HA gene, 1 to 1,055 (1,055 bp) of the NA gene, 1,058 to 2,227 (1,170 bp) of the PB2 gene, 23 to 1,316 (1,294 bp) of the PB1 gene, 1,450 to 2,148 (699 bp) of the PA gene, 22 to 976 (955 bp) of the NP gene, 25 to 759 (725 bp) of the M gene, and 46 to 685 (640 bp) of the NS gene. The scale bar represents the distance unit between sequence pairs. Th, Thailand; VN, Vietnam; In, Indonesia; HK, Hong Kong; Gd, Guangdong; YN, Yunnan; Sh, Shanghai; Sd, Shandong; ST, Shantou; Ck, chicken; Dk, duck; Qa, quail; Gs, Goose; SCk, silky chicken. The sequences are named in concordance with their GenBank nomenclature. The tree sparrow genes are indicated in red.

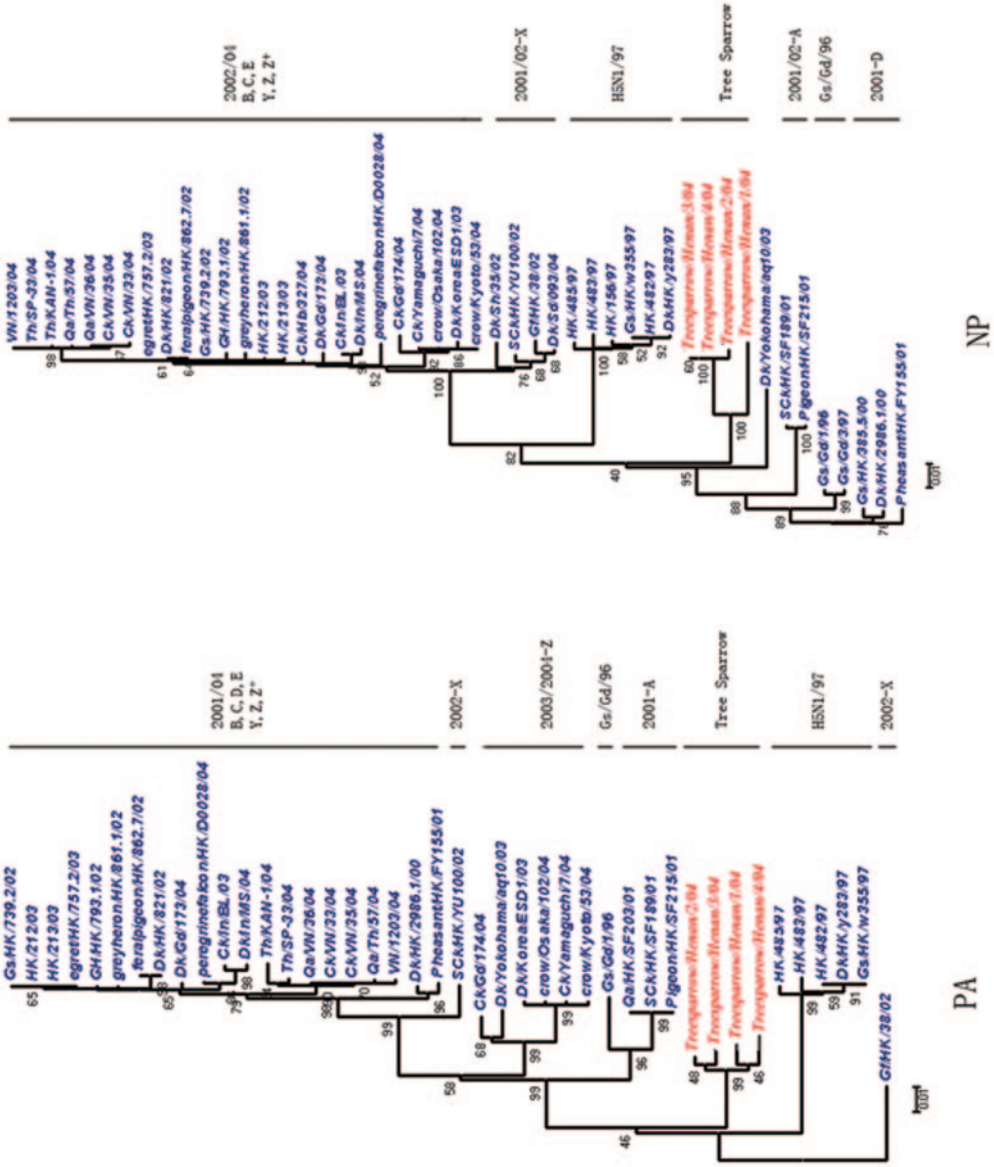


FIG. 1. *Continued.*

TABLE 2. Results of animal infection experiments

Virus	Titer (log_{10} EID_{50})	Avian infection (no. dead/no. inoculated) (no. of days to death)		Mouse infection		
		Chicken	Duck	No. dead/ no. inoculated	Virus in lung (log_{10} EID_{50})	Virus in brain (log_{10} EID_{50})
Ts/HN/1/04	6	8/8(2–5)	0/8	0/10	1.4	No
Ts/HN/2/04	6	8/8(3–4)	0/8	0/10	2.1	1.0
Ts/HN/3/04	5.5	7/8(6–9)	0/8	0/10	1.8	No
Ts/HN/4/04	6.5	8/8(4–5)	0/8	0/10	1.3	No

In the phylogenetic tree of the M gene, the four sparrow viruses formed a separate branch with H3 and H6 subtype viruses. The branch was distinctly related to the branch of genotypes A, B, C, D, E, X, Y, Z, and Z+, that of Gs/Gd/96, and that of HK/97 (Fig. 1). The NS genes of four tree sparrows were distinctly related to that of genotypes A, B, C, D, E, X, Y, Z, and Z+, that of H5N1/97, and that of the H3, H4, H6, H9, and H11 subtypes (Fig. 1).

According to the above-described analysis of phylogenetic relationships, the four tree sparrow isolates were different from other reported H5N1 genotypes. They were reassortants of Gs/Gd/96-like viruses; the outer genes are derived form Gs/Gd/96-like viruses, and the inner genes are derived from unknown influenza viruses resident in wild birds (Fig. 1).

Pathogenicity tests. Four isolates from tree sparrows were inoculated into chickens, ducks, and mice. As shown in Table 2, all the isolates killed seven or eight out of eight infected chickens within 10 days. According to the OIE criteria, all the isolates were highly pathogenic to chickens. It was reported that pathogenicity to duck varied according to different H5N1 isolates (16). The result of animal experiments indicated that the tree sparrow isolates were not pathogenic to ducks (Table 2) and so were different from the highly pathogenic viruses isolated from wild aquatic birds in 2002 (16). In the mice experiments, although the viruses were unable to kill mice, they could be detected in all the lungs of mice examined, as well as in the brains of mice infected with Ts/HN/2/04 (Table 2).

DISCUSSION

A survey of epidemic avian influenza viruses in tree sparrows was conducted in Pingyu country, Henan province, China, in May 2004. Four viruses were isolated from 38 free-living tree sparrows and were identified to be the highly pathogenic H5N1 subtype avian influenza virus. By molecular characterization and an analysis of phylogenetic relationships, their genetic characterizations were different from those of the dominant genotype Z viruses and other H5N1 genotype viruses. According to phylogenetic analysis results, only the HA, NA, and NP genes were found connected with other reported genotypes viruses. The HA and NA genes are descended from Gs/Gd/96-like virus, while the NP gene might be from genotype A viruses (Fig. 1 and 2). But other inner genes, such as PB2, PB1, PA, M, and NS, were novel and might have originated from an unknown virus (Fig. 1 and 2). The PB2 gene of DK/Yolohama/aq10/03 might also have originated from this unknown virus. The possible derivation of the tree sparrow viruses and their relationship to other H5N1 genotypes are summarized in Fig. 2. Reassortants were found to be very popular in the origin of influenza viruses (12).

In 2003, an avian influenza H5N1 virus was isolated once from a tree sparrow in Hong Kong, and the genotype of this strain was Z. The tree sparrow viruses that we isolated were quite different from the genotype Z virus. The fact that a diversity of H5N1 viruses was isolated from tree sparrows suggested that H5N1 viruses might be distributed widely in tree sparrows and that infections might not be occasional cases. The aquatic birds of the world were considered the natural reservoirs of influenza A viruses. Recently, genotype V and Z H5N1 viruses were isolated from different terrestrial birds (12). Our

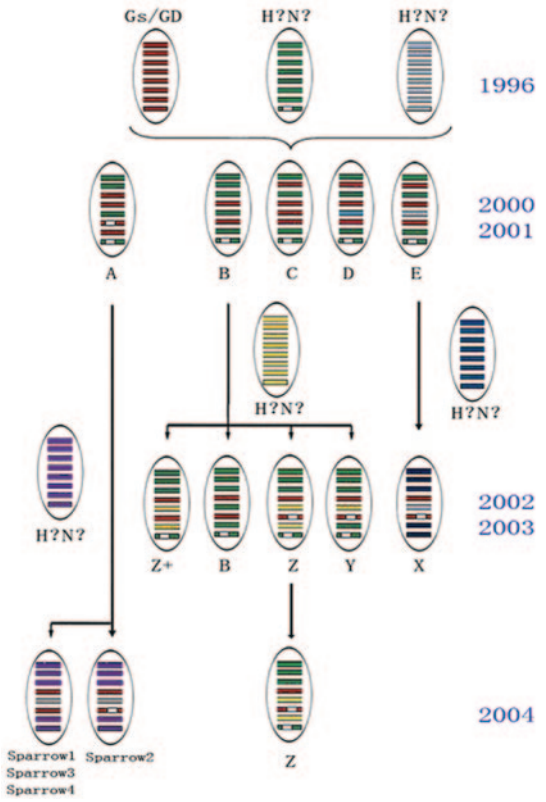


FIG. 2. Possible derivation of the tree sparrow viruses and their relationship to other H5N1 genotypes. The eight gene segments in each schematic virus particle are (from top to bottom) the PB2, PB1, PA, HA, NP, NA, M, and NS genes. Genes of the same lineage are shown in the same color. The capital letters indicate the genotypes and Sparrow 1 to Sparrow 4 indicate the four sparrow isolates.

results indicated that a new genotype H5N1 virus was found in tree sparrows. Multiple genotypes of viruses isolated from terrestrial birds indicated that terrestrial birds may play an important role in the natural reservoir and transmission of influenza virus. The tree sparrows from which the virus was isolated appeared normal when captured, indicating that they were carriers of the viruses instead of dead end hosts.

The origin of the tree sparrow viruses is unclear. The similarity of the HA and NA genes with those of Gs/Gd/96 indicated that they had some connection with aquatic birds. When migrating birds fly to their habitants, viruses that they have can be transmitted to local aquatic and terrestrial birds. In contrast, migrating birds can be infected by the viruses reserved in resident birds. So, viral genes are transmitted between the migrating gene pool and resident gene pool, and new-genotype viruses are created. Tree sparrows are in close contact with aquatic birds and domestic poultry; therefore, the chance of viral infection from other birds is high.

The result of animal experiments indicated that the tree sparrow isolates were highly pathogenic to chickens but not pathogenic to ducks. In mice experiments, the viruses did not kill mice but were detected in the lungs as well as some of the brains. Nowadays, the mechanism of the transmissibility of avian influenza viruses to mammals is not resolved and was proposed to involve multiple viral genes. It is firmly believed that avian gene constellations promote transmission to mammals (19, 21). The possible transmission and adaptation to mammals need to be further studied.

Highly pathogenic H5N1 avian influenza viruses resulted in a windstorm of disease outbreaks in China and other Asian countries in 2004. As there were cases in which viruses were fatal to humans (2, 9), the viruses have been a serious threat to the public health and the poultry industry. Wild birds were considered to be significantly related to the increasing spread of the virus in Asia, and H5N1 viruses with pandemic potential become endemic in regions and were not easily eradicable (12). Tree sparrows are residents and widespread in the whole of China and other Eurasian countries. They are more or less attached to human habitations and are found to have close contact with poultry, wild birds, domestic animals, and human beings. As H5N1 viruses were isolated from these birds, precaution is needed to prevent potential threats to the poultry industry and human health. In the region of China where the H5N1 viruses in this study were isolated, pigs and ducks are housed closely in farming villages. By means of possible transmission between swine and birds, the existence of the H5N1 virus in tree sparrows may serve as a new reservoir for the reemergence of a highly pathogenic avian influenza virus H5N1 outbreak. Additional surveys, especially long-term surveillance of tree sparrows and other wild birds, are necessary.

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