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Characterization of H5N1 Highly Pathogenic Avian Influenza Virus Isolated from a Mountain Hawk Eagle in Japan

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ABSTRACT. On January 4, 2007, an emaciated mountain hawk-eagle was found in Kumamoto Prefecture, Japan. Highly pathogenic avian influenza (HPAI) virus subtype H5N1 was isolated from both tracheal and cloacal swabs of the dead bird. On January 13, an outbreak of HPAI, caused by H5N1 strain, occurred in a chicken farm in Miyazaki Prefecture. Within three weeks, three additional outbreaks had occurred (two in Miyazaki Prefecture and one in Okayama Prefecture). To investigate the relationship between the hawk-eagle isolate and chicken isolates, we studied the virus growth, pathogenicity, and phylogenetic information of this hawk-eagle isolate. The highest virus titer was found in the brain ($10^{7.25}$ EID_{50}/g), followed by trachea and muscle ($10^{2.65}$ and $10^{2.50}$ EID_{50}/g, respectively). Sequence analysis at the hemagglutinin (HA) cleavage site of this isolate revealed a typical virulent-type sequence, R-R-R-K-K-R. Phylogenetic analysis demonstrated that the hawk-eagle isolate belongs to Qinghai Lake type virus group. A homology search of the HA gene also showed major similarity (more than 99%) to the Miyazaki and Okayama isolates in 2007 and also Korean isolates in 2006. These results suggest that Qinghai Lake type H5N1 HPAI virus was newly introduced from Asian Continent into Japan, and had already present in natural environment of Kyusyu district in the beginning of January 2007.
KEY WORDS: avian influenza virus, H5N1, HPAI, mountain hawk-eagle.

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The first outbreak of highly pathogenic avian influenza (HPAI) was reported in 1878 in Italy, and the disease had also been reported in Scotland, South Africa, U.S.A., Britain, Australia, Ireland, Belgium, Holland, France, Russia, Canada, Israel, Hungary, Japan, China, Indonesia, Thailand, and Vietnam [17]. After causing the first human fatalities in Hong Kong in 1997 [13], HPAI has been taken far more seriously as a problem in the poultry industry and as a global hazard to public health. As of September 2009 WHO report, worldwide human cases and death due to H5N1 avian influenza infection number 442 and 262, respectively world wide [16].

HPAI has also been detected in Asian countries, including Japan. H5N1 HPAI virus was first isolated in Japan in 2003 [10]. Between the end of December 2003 and March 2004, four outbreaks of H5N1 virus occurred in birds in three prefectures separated by 150–450 km, and these incidents involved three chicken farms and a group of chickens reared as pets. H5N1 virus isolation from dead crows was also reported in 2004. These viruses were reported antigenically similar to and were closely related to the A/chicken/Shantou/4231/2003 [10].

Subsequently, Japan had been free of H5N1 avian influenza, until it re-emerged on 2007. On January 4, 2007, a sick mountain hawk-eagle was found at Sagaramura, Kumamoto Prefecture of Kyushu Island, and later died. On January 13, an outbreak of HPAI, caused by the H5N1 strain, occurred in a chicken farm in Miyazaki Prefecture, which is about 900 km south west of Tokyo [5]. The outbreak killed approximately 3,800 chickens over a span of three days and a further remaining 8,200 birds were culled. Within three weeks, three additional outbreaks had occurred, two outbreaks affecting chickens in Miyazaki and one in Okayama Prefecture [11].

In May to July 2005, there were outbreaks of H5N1 HPAI virus at Qinghai Lake in China, causing the death of thousands of wild migratory water birds [15, 18]. Similarly, in the winter of 2006–2007, there were several outbreaks of HPAI virus (H5N1) among domestic poultry and in migratory bird habitats in South Korea [6]. The Miyazaki and Okayama isolates were found genetically similar to Korean and Qinghai Lake strains, as well as Mongolian strains isolated in 2006 [5]. To investigate the relationship between the hawk-eagle isolate and chicken isolates in Japan, as well as Korean and Qinghai Lake strains, we studied the virus growth, pathogenicity and phylogenetic data of the hawk-eagle isolate.

MATERIALS AND METHODS

Virus isolation: Cloacal and pharyngeal swabs were taken from the mountain hawk-eagle recovered from Sagaramura, Kumamoto Prefecture, Japan. Swabs were dipped and squeezed in 500 μl of nutrient broth (Nissui, Tokyo, Japan) fortified with 10,000 units of penicillin-streptomycin. The swab suspensions were centrifuged (2,200 × g for 5 min) and 0.1 ml of suspension was inoculated in 10-day-old embryonated chicken eggs. After 48 hr of incubation at

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37°C, followed by chilling at 4°C, hemagglutination test was performed, and the hemagglutinating agents were confirmed to be type A influenza viruses using a commercial rapid antigen assay kit. Viruses were subtyped as H5N1 based on a panel of antiserum, and the strain yielded from pharyngeal swab was designated as A/mountain hawk-eagle/Kumamoto/1/2007 (MHE/Kumamoto/07).

Virus titration of the infected tissues: Tissue samples (0.1 g) from brain, trachea and muscle were macerated in 900 μl of phosphate buffer saline (PBS) fortified with 5,000 units of penicillin-streptomycin. Tissue suspensions were centrifuged (2,200 × *g* for 5min) and 0.1 m*l* of suspension was inoculated in 10-day-old embryonated chicken eggs. The 50% egg infectious dose (EID_{50}) titer was determined as described by Reed and Muench [12]. All experiments with the H5N1 isolates were performed in a Biosafety Level 3 laboratory, Tottori University.

Genome sequencing: All eight gene segments of A/mountain hawk-eagle/Kumamoto/1/07 (MHE/Kumamoto/07) were sequenced and compared with the Korean, Qinghai, and Japanese strains, along with viral sequences available at the GenBank. Viral RNA was extracted from virus-infected allantoic fluid samples by QIA amp viral RNA mini kit (QIAGEN, Valencia, CA, U.S.A.) according to the manufacturer's protocol. Reverse transcription (RT) was done by Prime script reverse transcription kit (TaKaRa, Otsu, Japan) by using Uni-12. Briefly, 5 μl of viral RNA was mixed with 1 μl (2 pmol) of primer, 1 μl of dNTP mixture (10 mM each) and RNase-free distilled water up to a final volume of 10 μl. The mixture was heated at 65°C for 5 min, followed by immediate cooling on ice. Ten microliters of above sample was mixed with 4 μl of 5X Primescript buffer, 0.5 μl of RNase inhibitor (20 units), 1 μl of Reverse transcriptase (200 units) and RNase-free distilled water up to a final volume of 20 μl (4.5 μl), and was subjected to the reaction at 30°C for 10 min and 50°C for 45 min. The resulting cDNA was used in subsequent PCR amplifications with gene-specific primers (sequence are available upon request). PCR products were extracted and examined by 2% agarose gel electrophoresis, stained by ethidium bromide and purified by QIA quick Gel extraction kit (QIAGEN) according to the manufacture's protocol. DNA sequencing was performed using a commercially available kit (Big Dye terminator version 3.1 sequencing kit, Applied Biosystems, Foster city, CA, U.S.A.) and Thermal cycler-dice (Takara Bio.). Extended sequencing products were purified before sequencing analysis was performed with a 3130 Genetic Analyzer (Applied Biosystems). Sequence data of MHE/Kumamoto/07 were submitted to the GenBank Nucleotide sequence Database and are listed under Accession No. AB525188 to AB525195.

Phylogenetic analysis: DNA sequence data were edited and aligned using BioEdit version 7.0.8.0 software [4]. Phylogenetic analyses of HA gene was performed applying the clustal W method using MEGA 4 software [14]. The phylogenetic trees were constructed with Kimura two parameter nucleotide model. The robustness of the grouping in the neighbor joining analysis was assessed with 1,000 bootstrap resamplings.

RESULTS

In order to examine the tissue tropism of the H5N1 HPAI isolate, MHE/Kumamoto/07, virus titrations in tissue and swab samples from the mountain hawk-eagle was carried out. The highest titer of MHE/Kumamoto/07 was found in the brain ($10^{7.25}$ EID_{50}/g) (Table 1) followed by the trachea and muscle ($10^{2.65}$ and $10^{2.50}$ EID_{50}/g, respectively). Cloacal and tracheal swabs had titers $10^{5.50}$ and $10^{4.50}$ EID_{50}/m*l*, respectively.

In order to investigate the relation between MHE/Kumamoto/07 and other highly pathogenic H5N1 viruses, a homology search of the 8 gene segments of the MHE/Kumamoto/07 was performed. The results showed that each gene of the strain is very similar to those in Qinghai Lake type strains, but were more closely related to the Korean strains (Table 2). In fact, the Korean strains (ASAN/5/06 and K/IS/06) were found to be identical (100%) to MHE/Kumamoto/07 in terms of NA gene. HA was found to be 99.0% identical to Qinghai Lake strain,

Table 1. Virus titers in different tissues of infected mountain hawk-eagle

Tissue	Virus titer (log EID_{50}/g)
Brain	7.25
Trachea	2.65
Muscle	2.50
Tracheal Swab	4.50*
Cloacal Swab	5.50*

* log EID_{50}/m*l*.

Table 2. Genetic homology of mountain hawk-eagle strain with other H5N1 Qinghai lake type strains

Gene segment	Qinghai strain	Korean strain		Mongolian strain
		ASAN/5/06	K/IS/06	SW/MG/06
PB2	99.2 (Q0510/05)	99.9	99.5	99.6
PB1	99.3 (Q0510/05)	99.8	99.1	99.4
PA	99.1 (Q5/05)	99.5	99.2	99.5
HA	99.0 (Q5/05)	99.8	99.2	99.6
NA	99.3 (Q0510/05)	100	99.2	99.5
NP	99.4 (Q60/05)	99.8	99.4	99.5
M	99.3 (Q0510/05)	99.7	99.4	99.5
NS	98.9 (Q60/05)	99.7	99.2	99.6

Strain abbreviations are follows: A/bar-headed goose/Qinghai/0510/2005 (Q0510/05), A/bar-headed goose/Qinghai/5/2005(Q5/05), A/bar-headed goose/Qinghai/60/2005 (Q60/05), A/duck/Korea/Asan/5/2006 (ASAN/5/06), A/chicken/Korea/IS/2006(K/IS/06), A/whooper swan/Mongolia /220/06 (SW/MG/06).

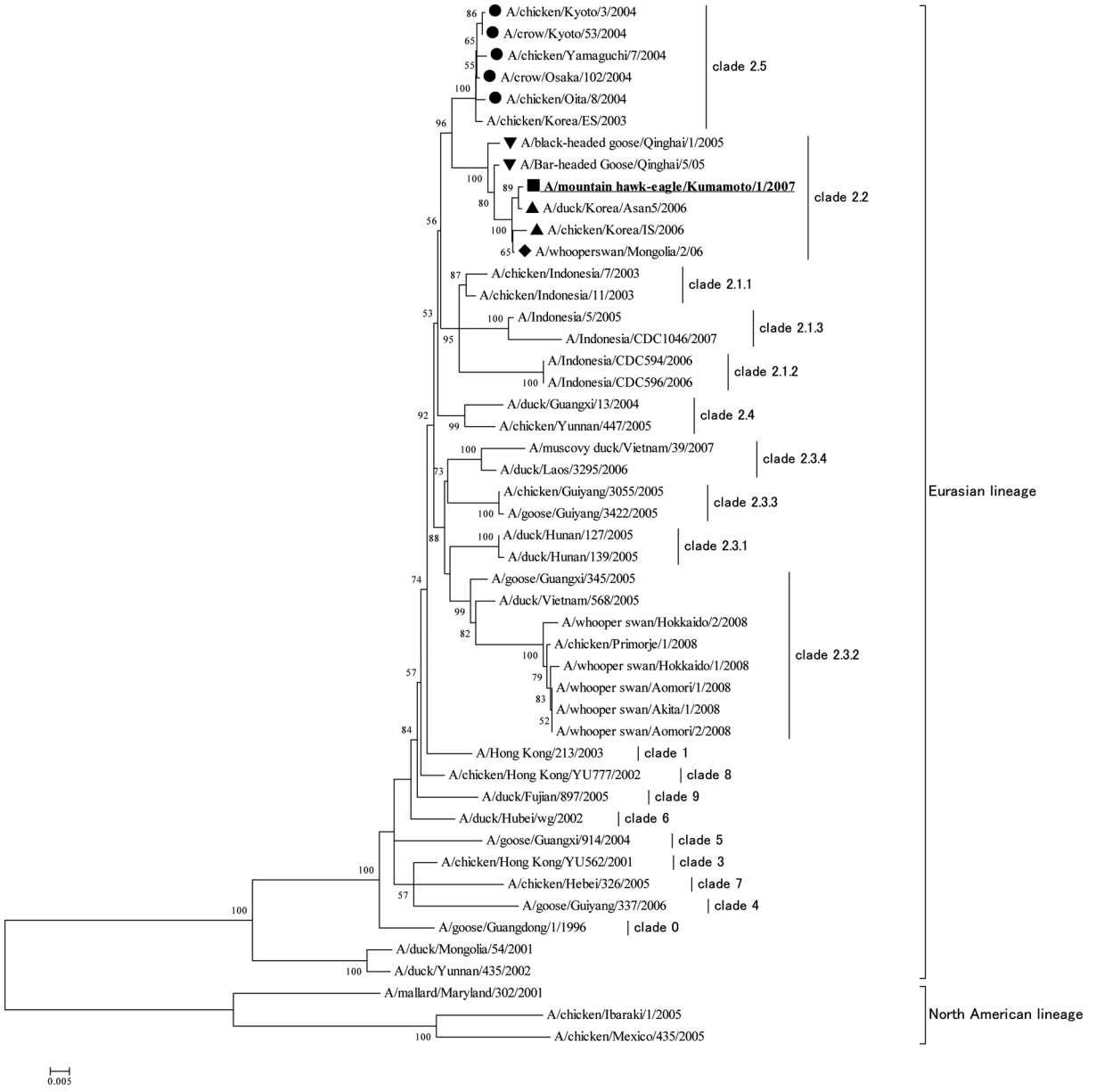

Fig. 1. Phylogenetic analyses of HA segment of MHE/Kumamoto/07 and other H5N1 influenza viruses from Asia, Europe and Africa including the Qinghai Lake, Korean, Japanese and Mongolian strains. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1,000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. Analyses were based on the nucleotides of coding region of all the segments. The evolutionary distances were computed using the Kimura 2-parameter method [3] and are in the units of the number of base substitutions per site. [■: MHE/Kumamoto/07, ▼: Qinghai lake strains (2005), ▲: Korean strains (2006), ●: Japanese strains (2004), ◆: Mongolian strain (2006)].

99.8–99.2% identical to the Korean and 99.6% to the Mongolian strain. PB2 had 99.2% identity to Qinghai Lake strain and 99.9 to 99.5% identity to Korean strains. PB1 was also found to be 99.8–99.1% identical to Korean strains and 99.4% to Mongolian strain and 99.3% to the Qinghai Lake strain. Similarly, PA also showed 99.1% identity to the Qinghai strain and higher identity (99.5–99.2%) to the Korean and Mongolian strain (99.5%). NP gene was found to be 98.9% identical to Qinghai strain and, like other genes, was found to be more identical to Korean strains (99.8–99.4%) and Mongolian strain (99.5%). The NS and M gene segments also shows high identity to the Qinghai Lake strains, and higher identity to the Korean and Mongolian strains, similarly to other gene segments (Table 2).

Phylogenetic tree of the HA gene shows that the MHE/Kumamoto/07 (bold, underlined and marked by dark square) falls on the same cluster of clade 2.2 as the Qinghai Lake stains (marked by inverted black triangle) and aggregated more closely to the Korean strains (marked by black triangle) and Mongolian strain (marked by black diamond) (Fig. 1). The viruses isolated from Japan on 2004 (A/chicken/Yamaguchi/7/2004, A/chicken/Kyoto/3/2004, A/crow/Kyoto/53/2004, A/crow/Osaka/102/2004 and A/chicken/Oita/8/2004) (marked by dark sphere) belonging to clade 2.5 were found to be away form the cluster shared by the MHE/Kumamoto/07, Mongolian, Korean and Qinghai Lake strains.

The HA cleavage site sequence of the MHE/Kumamoto/07 shows the typical virulent-type sequence, R-R-R-K-K-R, which has been found in the highly pathogenic Qinghai Lake and the Korean strains (Table 3), along with the deletion of the 20 amino acids at the NA stalk region (amino acid residue deleted from position 49 to 68), which is a marker of highly virulent virus [8] in contrast to its precursor virus A/Goose/Guangdong/1/96 (Fig. 2A). This deletion has been found identical with that found in isolates of genotypes of V and Z [7]. The deletions of 5 amino acids at the NS gene from 80–84 residues indicating high virulence which falls on the allele B [9] (Fig. 2B), thus supporting the close identity of this virus to Qinghai Lake type viruses.

Table 3. HA cleavage site sequence of H5N1 HPAI viruses

Strain	Amino acid sequence
	336 350
A/mountain hawk-eagle/Kumamoto/1/07	SPQGERRRKKRGLFG
A/duck/Korea/Asan/5/2006	SPQGERRRKKRGLFG
A/duck/Korea/Asan/6/2006	SPQGERRRKKRGLFG
A/chicken/Korea/IS/2006	SPQGERRRKKRGLFG
A/whooper swan/Mongolia/2/06	SPQGERRRKKRGLFG
A/bar-headed goose/Mongolia/1/05	SPQGERRRKKRGLFG
A/bar-headed goose/Qinghai/0510/05	SPQGERRRKKRGLFG
A/chicken/Yamaguchi/7/2004	SPQRERR - KKRGLFG
A/chicken/Kyoto/3/2004	SPQRERR - KKRGLFG
A/crow/Kyoto/53/2004	SPQRERR - KKRGLFG
A/crow/Osaka/102/2004	SPQRERR - KKRGLFG

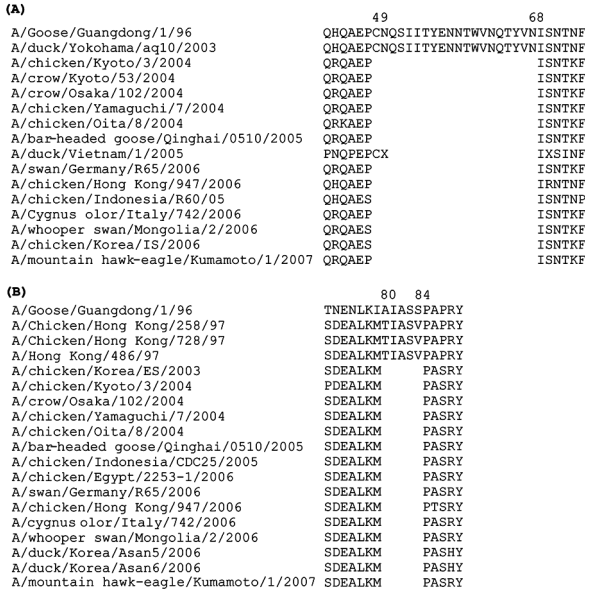

Fig. 2. Alignment comparisons of deduced amino acid sequences of NA stalk region (A) and NS1 protein (B). Twenty amino acid residues (49–68 aa) of the NA proteins have been deleted from the A/mountain hawk-eagle /Kumamoto/1/07 and those of 2003 to 2006 including the Qinghai Lake, Korean, Mongolian and Japanese strains. Five amino acid residues (80–84 aa) have been deleted in NS1 protein of viruses collected from late 2000 to the present.

DISCUSSION

In the present study, we characterized the MHE/Kumamoto/07 virus and compared its genome to various Asian strains, including the Japanese strains of 2004, Qinghai Lake strains from 2005 and 2006, and Korean and Mongolian strains from 2006. MHE/Kumamoto/07 was found to be closely related to the Qinghai Lake strains of 2005, and more closely related to the Korean strains of 2006 (Tables 1, 2 and Fig. 1). The typical virulent multibasic R-R-R-K-K-R amino acid sequence at the cleavage site of the HA is suggestive of its high pathogenicity, similarly to the Qinghai Lake and the Korean strains. The PB2 gene of MHE/Kumamoto/07 has the amino acid lysine at the position 627 (data not shown) similar to the Qinghai Lake, Korean and Mongolian strains but different to the 2003–2004 Japanese strains, which has glutamic acid at the position 627. These results suggest that the MHE/Kumamoto/07 was introduced to Japan after 2006, and not the recurrence of the 2004 virus.

Before the HPAI outbreak at the Miyazaki and Okayama Prefectures in Japan, the H5N1 virus was isolated from a female adult mountain hawk-eagle from the roadside of the Sagara village, Kumamoto Prefecture which is 75 km away from the site of the first outbreak in Miyazaki. This strain is genetically very similar to the Miyazaki and the Okayama strains [11], and was also been found to be similar to the Qinghai Lake strain (Table 2). Thus, infection in these two cases was apparently form same source, the virus must have been near the Kyushu area before January 2007. On the other hand, during the 2006 winter season in South Korea, several outbreaks of HPAI virus (H5N1) were confirmed among domestic poultry. Phylogenetic analysis showing all isolates were closely related and belonged to the A/bar-headed goose/Qinghai/5/2005-like lineage, thus suggesting that the Qinghai Lake strains had spread to Korea and might have entered Japan before 2007 [6]. Additionally, during a routine survey for influenza activity in migratory bird habitats in South Korea, H5N1 viruses were isolated from fecal samples in December 2006 before the H5N1 outbreak in chicken farms, suggesting the possible role of migratory birds in the spread of H5N1 HPAI viruses in Asia [6].

Mountain hawk eagle is an endangered raptor, which is

distributed from the foothills of the Himalayas east through China and south through Japan and Taiwan. It is found in the coniferous or mixed forests from 1,000 to 3,500 m in elevation, and can reach 80 cm in length, weighing 3–4 kg, with a 160 to 180 cm wingspan. Its prey includes hares, copper pheasants, snakes, flying small birds, wild rabbits, mice and squirrels [3]. Importantly, the hawk-eagle is a resident bird its habitat range is not more than 25 km; the distance between the place where the hawk was found and the farms of the Miyazaki Prefecture was more than 75 km [11]. Thus, the hawk-eagle was not likely to have delivered the virus, nor did it carry the virus directly from the original source. Although it is unclear whether the mountain hawk-eagle was native to the area where it was recovered, or had moved there from another area, and no clear evidence where is actually became infected, it is more likely to have been infected by consuming infected prey somewhere in Kyushu than to have been infected in Korea and then flown across to Japan.

On the other hand, due to quarantine measures in outbreak countries and the lack of any relationship between farm-related persons or products and outbreak countries, it seems unlikely that human beings brought in the virus into the farms. Although direct evidence of viral dissemination is lacking, the possibility that wild birds and/or wild animals contribute to the spread of H5N1 HPAI viruses cannot be excluded. At the very least, the present results suggest that an H5N1 HPAI virus was already present in the natural environment of Kyusyu before chicken farm outbreaks occurred in the area. To clarify the route of infection, routine monitoring of wild birds, migration surveillance and on-site investigation is important prior to the implementation of the control measures.

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