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BRIEF REPORT

H5N1 highly pathogenic avian influenza virus isolated from conjunctiva of a whooper swan with neurological signs

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Abstract An H5N1 highly pathogenic avian influenza virus was isolated from conjunctiva of a whooper swan with neurological signs, which was captured during the latest H5N1 HPAI outbreak in Japan. The conjunctival swab contained a larger amount of the virus in comparison with the tracheal swab. This is the first report on H5N1 virus isolation from the conjunctiva of a wild bird, and the result may suggest the conjunctival swab to be a critical sample for H5N1 HPAIV detection in waterfowl. Phylogenetic analysis of the HA gene indicated that the virus falls into H5N1 clade 2.3.2.1.

Wild birds, especially waterfowl and shorebirds, are considered natural reservoirs for avian influenza virus (AIV), and it is known that these birds can carry various subtypes of AIV with little or perhaps no impact on their health [20]. However, in 2002, outbreaks of H5N1 highly pathogenic AIV (HPAIV) started in Hong Kong, where deaths of many wild birds were recorded [5, 17]. Since then, Japan has experienced three H5N1 outbreaks. The third outbreak, which occurred during the winter season between 2010 and 2011, was the worst ever experienced in this country, affecting 24 chicken farms in nine prefectures. Prior to and during the big poultry outbreaks, thousands of wild birds of various species were found dead or moribund. Importantly, isolation of the H5N1 virus from the fecal samples of ducks at Lake Onuma, Hokkaido, in October 2010 is regarded as the earliest case of viral isolation. Subsequently, the H5N1 virus was isolated from 63 wild birds in 17 prefectures throughout Japan [16]. There has been an increasing interest in the ecology of wild birds, since AIVs are known to spread during their migration period [12, 20]. The latest H5N1 highly pathogenic avian influenza (HPAI) outbreak in Japan indeed highlighted the importance of AIV surveillance in wild birds that can carry viruses between geographic areas. In AIV surveillance, cloacal and tracheal swabs are generally collected, but we describe here the isolation of H5N1 HPAIV from a conjunctival swab from a whooper swan with neurological signs that was captured in Japan.

In early 2011, an emaciated whooper swan showing neurological signs, including uncontrollable head shaking, was captured by a surveillance team of the Ministry of the Environment in Hamanaka, Hokkaido. Screening tests using rapid immunochromatographic diagnostic kits for humans, such as Espline Influenza A&B-N (Fujirebio Inc., Tokyo, Japan) and Rapid Testa FLUII (Sekisui Medical Co. Ltd., Tokyo, Japan), which detect the internal protein of influenza A virus, were negative for cloacal and tracheal swabs collected from the diseased swan. In addition to these swabs, a conjunctival swab was taken from the swan at our request, and all three samples were sent to our laboratory for further investigation.

Virological and genetic analyses on the samples were performed as described previously [3]. Real-time reverse transcription polymerase chain reaction (RRT-PCR) for the

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matrix gene was positive for the cloacal, tracheal and conjunctival samples. However, the conjunctival sample had a lower C_t value (29.5) than the tracheal (33.2) and cloacal (39.4) samples. The virus was isolated from embryonating chicken eggs inoculated in the allantoic cavity with the original tracheal and conjunctival samples, but not the cloacal sample. However, we were only able to obtain a viral titer from the conjunctival swab sample ($10^{3.8}$ EID_{50}/ml). The virus was isolated from two of the three eggs inoculated with the undiluted tracheal sample, but not from any of the eggs inoculated with diluted tracheal samples. Therefore, calculation of the virus titer by the Behrens–Kärber method was not applicable for the results on the tracheal sample. The conjunctival sample was confirmed to contain the H5N1 virus by subtype specific RT-PCR using the H5 primer and the N1 primer reported by Lee et al. [8] and Qiu et al. [13], respectively (Table 1). The virus isolated from the conjunctival swab was designated as A/whooper swan/Hamanaka/2011 (H5N1) (Ws/HN/11).

The entire genome sequence of Ws/HN/11 was compared with those of other H5N1 HPAIVs. All of the genes of this isolate were similar to those of other H5N1 viruses isolated in Japan in 2011 [16], and they were also closely related to those of Mongolian and Korean strains isolated in 2009-2010 and 2010-2011, respectively (Table 2). The HA cleavage site sequence of Ws/HN/11 has the typical sequence motif of a virulent type, SPQRERRRKR/GLF. The NA gene of Ws/HN/11 has a deletion of 20 amino acids from positions 49 to 68, which has been reported to be a marker of a highly virulent virus [9]. In the NS gene of Ws/HN/11, there is a deletion of five amino acids at position 80-84, indicating high virulence as reported in other H5N1 strains [10]. The genetic features in the NA and NS genes were common to all of the H5N1 HPAIVs isolated in Japan during 2010 and 2011 (data not shown).

Table 1 Isolation of A/whooper swan/Hamanaka/2011 (H5N1) from the conjunctival swab of the diseased whooper swan

Swab sample	Ct value in RRT-PCR[a]	RT-PCR[b]		Virus titer (log EID_{50}/ml)
		H5	N1	
Cloacal	39.4	Negative	Negative	<1.5[c]
Tracheal	33.2	Negative	Negative	<1.5[d]
Conjunctival	29.5	Positive	Positive	3.8

[a] Real-time RT-PCR for the matrix gene

[b] RT-PCR using swab samples for subtyping avian influenza viruses

[c] No virus was isolated from the three eggs inoculated with the undiluted sample

[d] The virus was isolated from two of the three eggs inoculated with the undiluted sample, but not from any eggs with diluted samples. Therefore, calculation of the virus titer by the Behrens–Kärber method was not applicable for the results

Table 2 Sequence comparison between A/whooper swan/Hamanaka/2011 (H5N1) and other H5N1 strains isolated in Japan (2011), Mongolia (2009–2010), and Korea (2010–2011)

Gene segment	Nucleotide identity (%) with A/whooper swan/Hamanaka/2011[a]		
	Mongolian strains	Korean strains	Japanese strain[b]
PB2	99.17[c]	99.60[e]	99.95
PB1	99.08[c]	99.34[f]	100.00
PA	99.50[c]	99.62[e]	99.95
HA	99.42[d]	99.41[g]	99.88
NP	99.88[c]	99.19[e]	100.00
NA	99.63[d]	99.11[e]	100.00
M	99.07[c]	99.06[f]	99.89
NS	99.63[c]	99.75[f]	100.00

[a] Nucleotide sequence of A/whooper swan/Hamanaka/2011 (H5N1) are available in GenBank with the accession numbers CY110735 to CY110742

[b] Compared with A/whooper swan/Hokkaido/4/2011

[c] Compared with A/whooper swan/Mongolia/6/2009

[d] Compared with A/whooper swan/Mongolia/21/2010

[e] Compared with A/mallard duck/Korea/W404/2011

[f] Compared with A/mallard/Korea/1195/2010

[g] Compared with A/mandarin duck/Korea/K10-485/2010

Phylogenetic analysis of the HA gene clearly indicated that Ws/HN/11 belongs to the Eurasian lineage. The isolate fell into clade 2.3.2.1, similar to other H5N1 strains isolated in Japan, Mongolia, and Korea between 2008 and 2011 [16]. The H5N1 strains isolated in Japan in 2008 form a single branch separated from the 2011 strains including Ws/HN/11 in clade 2.3.2.1 (Fig. 1). The H5N1 strains isolated in Japan can be divided into three disparate clades: 2.5, 2.2 and 2.3.2.1. The results seem to suggest that H5N1 viruses circulating in Asian countries are undergoing genetic change, possibly by antigenic drift and/or positive selection under immunological pressures as described by Sakoda et al. [16]. Continuous efforts for characterizing the viral genes of the influenza virus variants should be of extreme importance.

Rapid influenza diagnostic kits for humans have been utilized in field surveillance for AIVs and in the frontline laboratories fighting against AIVs in Japan. Essentially, those kits were developed to detect the nucleoprotein or matrix protein of influenza A virus, which are known to be conserved not only in human influenza viruses but also in AIVs. However, the sensitivity of the kit to swab samples from animals would be lowered by genetic incompatibilities in the viruses, and further by the presence of feces and/or unrelated proteins in the samples, as reported previously [15]. In this study, the rapid tests failed to detect AIV from the tracheal and cloacal swab

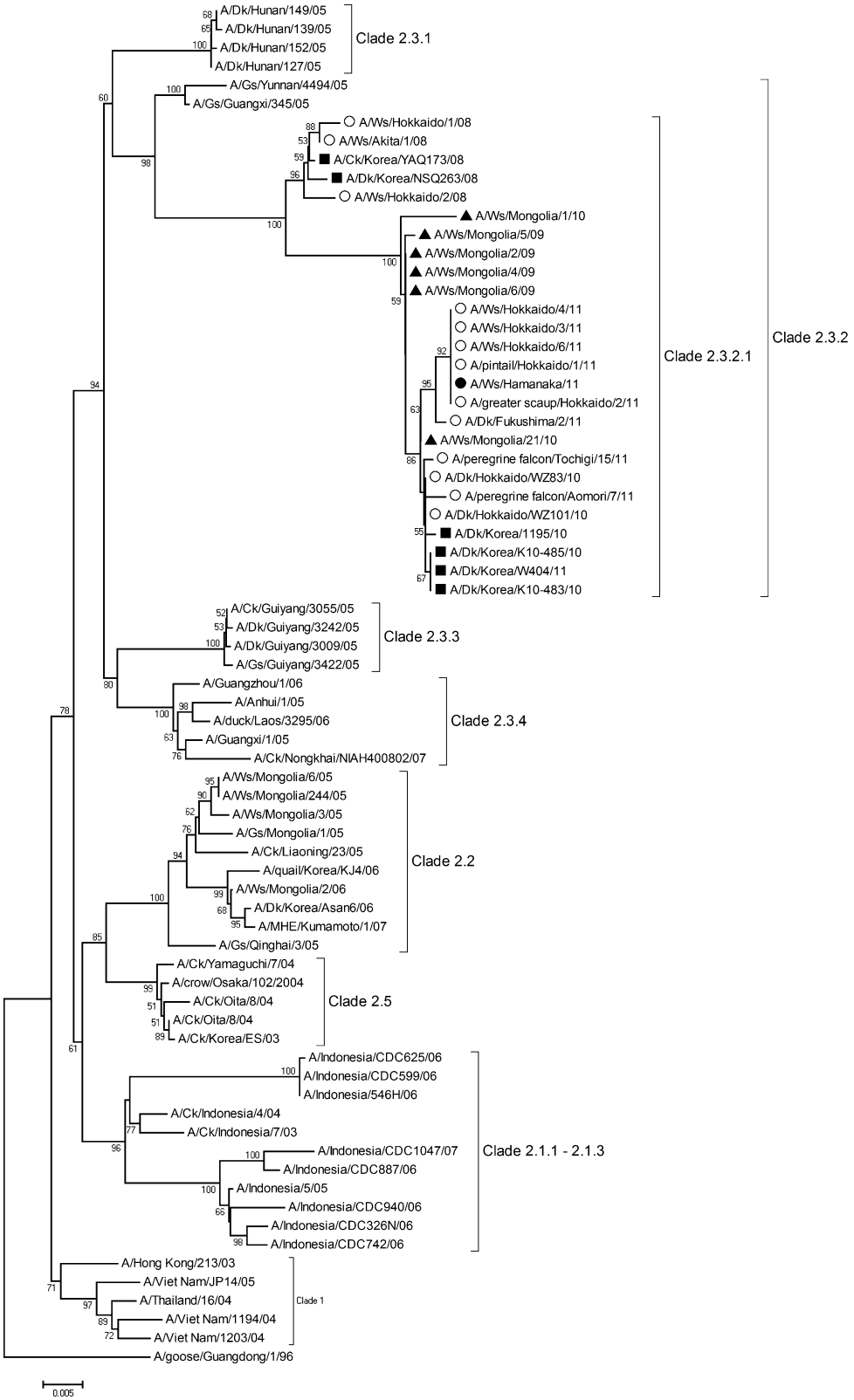

Fig. 1 Phylogenetic analysis of the full-length HA genes of A/whooper swan/Hamanaka/2011 (H5N1) indicated by ●, H5N1 strains isolated after 2008 from Japan (○), Mongolia (▲), and Korea (■), and other representative strains of H5N1. The evolutionary history was inferred using the neighbor-joining method. Numbers at each branch point indicate bootstrap values ≥50 % in the bootstrap interior branch test. The evolutionary distances were computed using the Tamura-Nei method and are in units of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1581 positions in the final dataset. Phylogenetic analysis was conducted in MEGA4. The scale bar indicates 0.005 nucleotide substitutions per site. Ck, chicken; Dk, duck; Gs, goose; MHE, mountain hawk-eagle

samples of the H5N1 infected whooper swan; however, the conjunctival sample was not tested using the rapid tests. Thus, this study verified the problem of the sensitivity of rapid diagnostic kits and revealed an urgent need for a more sensitive rapid diagnostic tool for AI control and pandemic preparedness.

The H5N1 virus was isolated from the conjunctival and tracheal swabs, but not from the cloacal swab of the diseased whooper swan. Unexpectedly, the conjunctival swab contained a larger amount of the virus in comparison with the tracheal swab (Table 1). Cloacal and tracheal or oropharyngeal swabs are usually used for the surveillance and diagnosis of AIVs. However, the present result may suggest the conjunctival swab to be a critical sample for H5N1 HPAIV detection in waterfowl. It was reported that the detection rate of low-pathogenic AIV in wild mallards was much higher in cloacal than in oropharyngeal samples [6]. In contrast, in experimental infections with recent H5N1 HPAIVs from Asia in wild waterfowl, the virus isolation rate was higher in oropharyngeal swabs with higher titers than in cloacal swabs [1, 7, 18], suggesting that the digestive tract of waterfowl is not the main replication site of recent H5N1 HPAIVs. Interestingly, the most commonly observed sign of H5N1 virus infection in mallards was cloudy eyes [18]. The eye also seems to be one of the targets for HPAIV replication in other waterfowl [2], chickens [11] and humans [4, 19]. Although this study describes only one case of a diseased swan, the viral antigens were detectable from the conjunctival swabs of other diseased swans and ducks, but not from cloacal or tracheal swabs, when using the rapid influenza diagnostic kit. Between January and April in 2010, in the southeastern region of Hokkaido, including Hamanaka, where the whooper swan described in this paper was captured, the government or local authority captured a total of 42 dead or emaciated wild birds. Most of the birds were waterfowl such as swans and ducks, but raptors and cranes were also included. The cloacal and tracheal swabs collected from those birds were tested using rapid kits at the site of capture, and they were also forwarded to the authorized laboratory for genetic analysis. Among the 42 birds, 10 were confirmed by genetic analysis to be positive for H5N1 infection. The bird species with the positive results were either swans or ducks. Out of the 10 cases, four were negative or undetermined because of a marginal result when the cloacal and tracheal swabs were tested using the rapid tests. Among the four negative cases, conjunctival swab samples were, by chance, collected from two whooper swans and one duck. All three conjunctival samples showed positive results in the rapid tests. Another interesting finding is that two additional whooper swans that were captured alive, in addition to the one described in this paper, showed neurological signs [14].

Additional, larger-scale studies on conjunctival swab samples collected from infected wild birds or poultry would evaluate the reliability of a conjunctival swab for AIV diagnosis and surveillance. Such studies might also clarify the possible relationship between neurological signs and the viral replication in the conjunctiva of waterfowl infected with HPAIV.

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