BRIEF REPORT

Genetic characterization of H9N2 avian influenza virus in plateau pikas in the Qinghai Lake region of China

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Abstract Qinghai Lake is a major migratory-bird breeding site that has experienced several highly pathogenic avian influenza virus (AIV) epizootics. Plateau pikas (*Ochotona curzoniae*) have previously been implicated in the ecology of avian influenza virus in this region. We first isolated an H9N2 AIV (A/Pika/Menyuan/01/2008) from plateau pikas between November 2008 and October 2009. Sequence analysis showed that the A/Pika/Menyuan/01/2008 AIV was closely related to the H9N2 AIV strain (A/Turkey/Wisconsin/ 1/1966). Our findings suggested that plateau pikas may contribute to AIV epidemiology in the Qinghai Lake region.

Keywords Qinghai Lake · Plateau pika · Avian influenza virus · H9N2

Introduction

H9N2 avian influenza virus (AIV) was first isolated in Guangdong Province of China between November 1992 and May 1994 [1]. Following the initial emergence of this virus in China, widespread transmission of H9N2 AIV was observed in chickens, but vaccination campaigns helped to control the virus in poultry. Currently, H9N2 AIV is endemic in multiple avian species in different regions. multipleT100059023.0999792(Qn)-544.18.5909998891.25189995TL9.9

Materials and methods

Sample collection and virus identification

To identify AIV infections in wild pikas at Qinghai Lake between November 2008 and October 2009, tissue samples were collected from plateau pikas in Haibei, Huangnan, Hainan, Haixi, Guoluo and Yushu Prefecture of Qinghai Province (Fig. 1). Tissue samples were stored at -80 °C prior to inoculation into 10-day-old specific-pathogen-free (SPF) embryonated chicken eggs and propagation (3 passages). The allantoic fluids with positive hemagglutination activity were harvested and stored at -80 °C. Subtyping of the positive samples was done by RT-PCR [5, 6].

Genome sequence analysis

The genomic sequences of the virus isolates were determined as described previously [6]. Briefly, total RNA was extracted from the allantoic fluid using TRIzol® LS Reagent (Invitrogen, Carlsbad, CA, USA), and RT-PCR was then performed using a OneStep RT-PCR Kit (QIAGEN, Hilden, Germany) with primers specific for the H9N2 virus HA gene HA-F 5′, CTCAGGGAGCAAAAGCAGGGG-3′; HA-R 5′, GGACATGGCCCAGAATAGGAAG-3′) and the NA gene NA-F 5′, ATGAATCCAAATCAGAAG-3′; NA-R 5′ CTACTTGTCAATGGTGAATG-3′). The PCR products were cloned into the pMD18-T vector and then sequenced. Multiple sequence alignments were made using the Clustal W program of the BioEdit software package (version 5.0.9). Phylogenetic analysis was carried out using MEGA (version 4).

Nucleotide sequence accession number

The nucleotide sequence of the H9N2 virus in this study has been deposited in the GenBank database (accession number shown in Table 1).

Results

Only one virus was isolated from 817 tissue samples collected from 138 pikas. The virus was isolated from the kidney tissue of one pika in Menyuan County of Qinghai

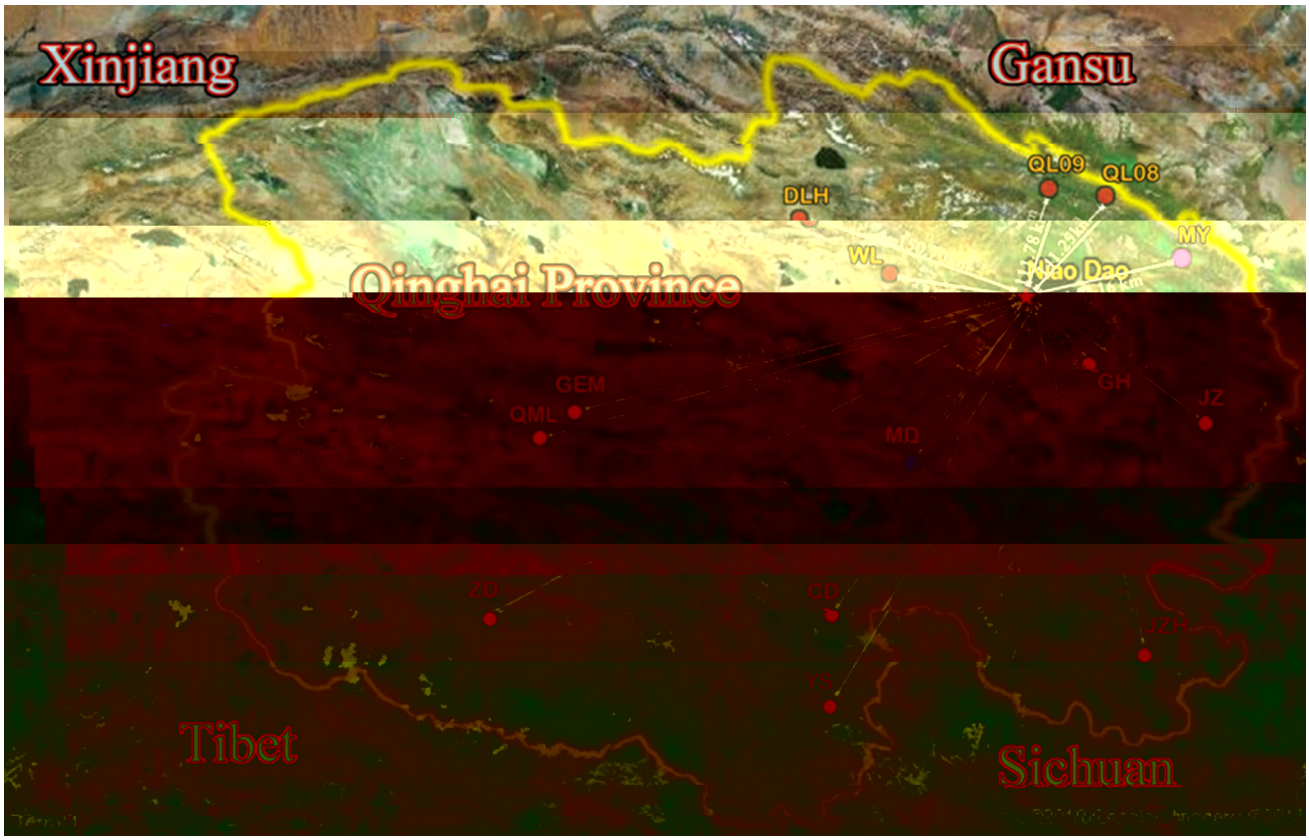

Fig. 1 Plateau pika sampling sites in Qinghai Province, China, between 2008 and 2009. Round red symbols indicate sites of pika sample collection. The pink point indicates the site of the isolated H9N2 virus. QL, Qilian; WL, Wulan; DLH, Delingha; GEM, Ge'ermu; ZD, Zhiduo; YS, Yushu; JZ, Jiuzhi; MD, Maduo; GH, Gonghe; MY, Menyuan; CD, Chengduo; JZH, Jianzha; QML, Qumalai (color figure online)

Table 1 Genetic similarity of A/Pika/Menyuan/01/2008 (H9N2) to the most closely related H9N2 viruses

Gene	Virus with the highest nucleotide sequence identity	Source	Identity	GenBank accession no.
HA	A/turkey/Wisconsin/1/1966(H9N2)	Avian	99.4%	CY014663
	A/chicken/Heilongjiang/35/2000(H9N2)	Avian	98.9%	DQ064366
NA	A/turkey/Wisconsin/1/1966(H9N2)	Avian	99%	CY087826
PB2	A/turkey/Wisconsin/1/1966(H9N2)	Avian	99.8%	CY014670
PB1	A/turkey/Wisconsin/1/1966(H9N2)	Avian	99.5%	CY014669
PA	A/turkey/Wisconsin/1/1966(H9N2)	Avian	99.6%	CY014668
NP	A/chicken/Heilongjiang/35/2000(H9N2)	Avian	99.6%	DQ064447
M	A/turkey/Wisconsin/1/1966(H9N2)	Avian	99.9%	CY014664
NS	A/turkey/Wisconsin/1/1966(H9N2)	Avian	99%	CY087828

Province in December 2008. Subtype analysis showed that the isolated virus belonged to the H9N2 subtype of avian influenza virus, and it was named A/Pika/Menyuan/01/2008 (H9N2, Pk/MY/1/08). After three passages in chicken eggs, the HA titer of the Pk/MY/01/08 was 2^3.

Sequence analysis showed that the Pk/MY/1/08 virus contained the motif PAVSSR/GL at the cleavage site between HA1 and HA2, which is a characteristic of low-pathogenic avian influenza virus (LPAI). Phylogenetic analysis demonstrated that the HA of Pk/MY/1/08 shared 99.4% and 98.9% nucleotide sequence identity with that of A/Turkey/Wisconsin/1/1966 (TY/WI/66) and A/ Chicken/Heilongjiang/35/00, respectively (Fig. 2A). Similar findings were also confirmed for the NA gene, which shared 92.6%-99% nucleotide sequence identity with viruses of the North American TY/Ca/189/66-like lineage (Fig. 2B). There were no amino acid deletions in the stem of the NA protein of the Pk/MY/1/08 virus.

Phylogenetic analysis indicated that all of the internal genes of Pk/MY/1/08 had the genetic characteristics of members of the TY/Ca/189/66-like lineage (data not shown). The internal genes of Pk/MY/1/08 showed high nucleic sequence similarity to those of the Ty/Ca/189/66-like strains. The polymerase genes (PB2, PB1 and PA), M genes, and NS genes shared 99.5%-99.8%, 99.9%, and 99% nucleotide sequence identity, respectively, with those of the A/Turkey/Wisconsin/1/1966, whereas the NP gene shared 99.6% identity with that of the A/chicken/Heilongjiang/35/2000. Residues 26-34 of the M2 protein correspond to the LVIAASIIG motif found in ion channels, and no mutation was found at position 31, which is associated with the resistance phenotype of influenza A virus. The presence of 627E and 701D in PB2 indicated that Pk/MY/1/08 is a strain with low virulence that has not acquired mutations for adaptation to the mammalian host. Like those of the pika H5N1 viruses isolated in 2005, the NS gene of Pk/MY/1/08 belongs to the Y439-like lineage.

Discussion

Qinghai Lake is an important breeding site for migratory birds in the central Asian-India flyway. Therefore, infected wild birds may transmit AIVs to other birds or mammals in the Qinghai Lake region. Plateau pikas live in close proximity to wild birds in this ecosystem. Following the first outbreak of H5N1 HPAIV in migratory birds at Qinghai Lake in 2005, we detected HPAI H5N1 viruses in pikas around Qinghai Lake [6]. This finding raises concern that plateau pikas may play a role in the transmission of AIVs at Qinghai Lake and provide a potential opportunity for AIVs to adapt to mammals. Notably, other groups have demonstrated that wild lagomorphs are susceptible to multiple subtypes of influenza A virus [8, 9] and that some of them have the potential to transmit these viruses back to waterfowl [10].

Researchers from the United States and China have shown that wild birds migrate from Qinghai Lake to Mongolia, Eastern Asia, and the Bay of Bengal [11]. Qinghai Lake, which is the largest salt lake in China, is an important breeding site for migratory birds on the Central Asia-India migratory route. A previous study showed evidence of HPAI H5N1 virus in plateau pikas and suggested that this lagomorph may have the potential to transmit AIVs to domestic mammals and humans [6]. Therefore, we speculate that pikas may serve as a potential spillover host of AIV in the Qinghai Lake region and may have the potential to transmit influenza A viruses to other species. Notably, others have shown that cottontail rabbits (*Sylvilagus* sp.) were able to transmit avian AIV to mallards that shared their living space [10].

Five H9N2 influenza viruses were isolated from the environment at a live poultry market in the Qinghai Lake region in 2012 [12]. In our study, we isolated the Pk/MY/1/08 strain of AIV, which has high sequence similarity to members of the North American lineage A/Turkey/Wisconsin/1/1966. However, the reason for this similarity is unclear. A similar result was found in a previous study, in

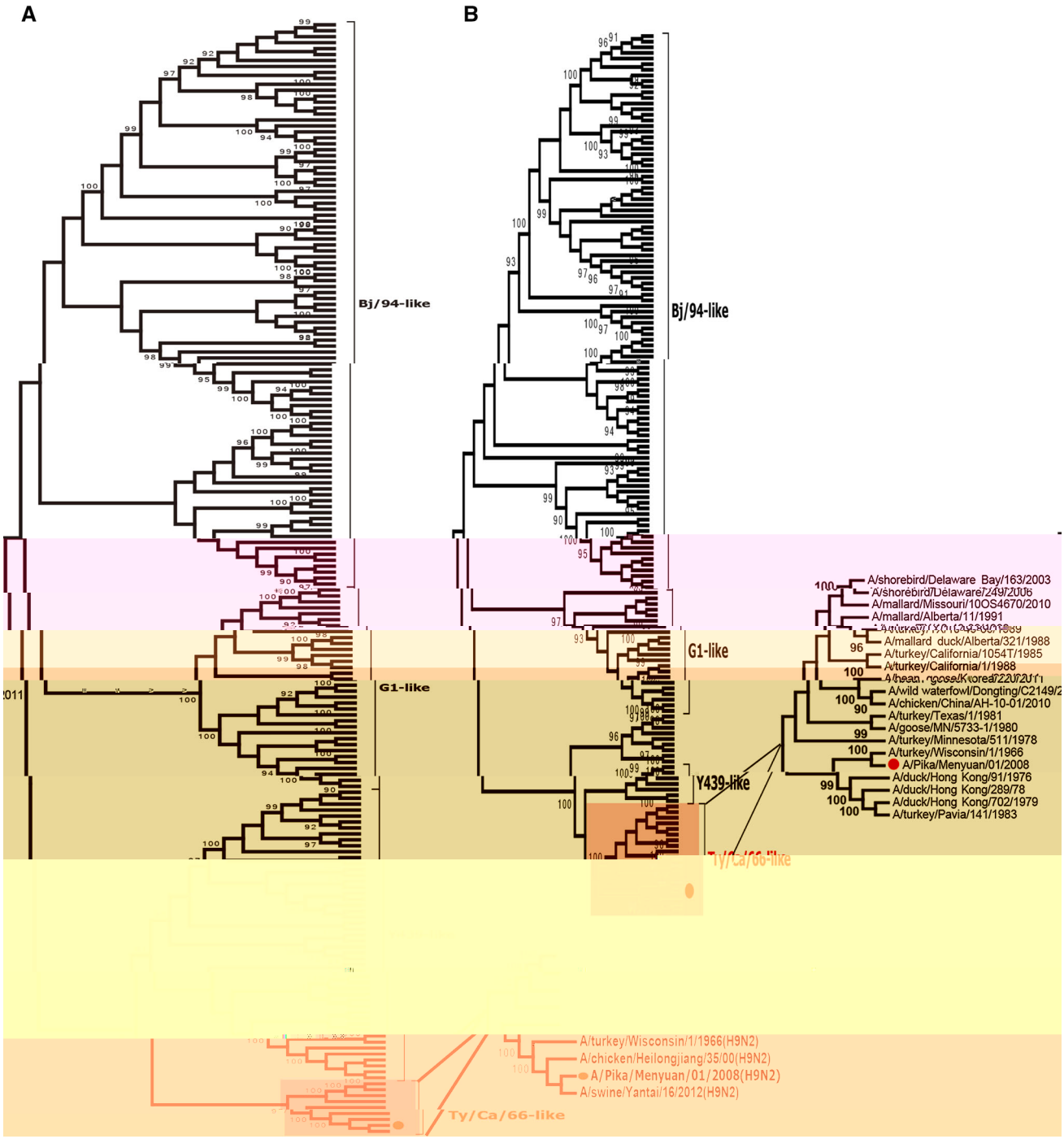

Fig. 2 Phylogenetic trees of the HA and NA genes of H9N2 avian influenza viruses. The phylogenetic trees show that HA (A) and NA (B) of the A/Pika/Menyuan/01/2008 strain belong to the Ty/Ca/1/66-like lineage. The phylogenetic trees were generated in MEGA 4 using the neighbor-joining (NJ) algorithm with 1,000 bootstrap replicates, and the NJ bootstrap values (>70%) for each node are shown in each tree. The virus characterized in this study is indicated by a red circle (color figure online)

which the A/CK/Heilongjiang/35/00 (H9N2) strain, which contains HA and NP genes of the North American lineage, was isolated [12]. Interestingly, the Pk/MY/1/08 virus was isolated from kidney tissue. Therefore, pikas with asymptomatic AIV infections may provide potential opportunities for the adaptation of avian influenza viruses to mammals and may also be involved in the epidemiology of the viruses in the Qinghai Lake region. Additional studies are needed to evaluate the potential role of plateau pikas in the transmission of AIVs in this region.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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