

## **Characterization of a highly pathogenic H5N1 influenza virus derived from bar-headed geese in China**

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The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are DQ137873, DQ137874 and DQ237951–DQ237956.

Influenza A viruses are usually non-pathogenic in wild aquatic birds, their natural reservoir. However, from May to July 2005, at Qinghai Lake in China, an unprecedented outbreak of highly pathogenic H5N1 avian influenza virus caused the death of thousands of wild migratory waterbirds. Herein, H5N1 influenza virus from bar-headed geese collected during the outbreak was characterized. Genomic analysis showed that A/Bar-headed Goose/Qinghai/0510/05 (Bh H5N1 virus) is a reassortant virus. Amino acid residue (lysine) at position 627 in the PB2 gene of the Bh H5N1 virus was the same as that of the human H5N1 virus (A/HK/483/97) and different from that of H5N1 avian influenza viruses deposited in GenBank. Antigenic analysis showed that significant antigenic variation has occurred in the Bh H5N1 virus. The Bh H5N1 virus induced systemic infections and caused 100 % mortality in chickens and mice, and 80 % mortality in ducks and geese. Bh H5N1 virus titres were higher in multiple organs of chickens, ducks and geese than in mice, and caused more severe histological lesions in chickens, ducks and mice than in geese. These results support the need to pay close attention and create control programmes to prevent the transmission of highly pathogenic avian influenza virus from wild migratory waterbirds into domestic chickens, ducks, geese and mammalian hosts.

## INTRODUCTION

Highly pathogenic avian influenza (HPAI) of the H5N1 subtype is an epidemic disease and has produced infections that resulted in grand-scale economic losses. Until recently, HPAI transmission to humans who work or live in close contact with poultry or other birds occurred rarely. Now, however, highly virulent zoonotic strains are arising from HPAI and transmission to humans has become a grave concern. The problem was highlighted by the observation of H5N1, an avian influenza A virus (AIV) that was transmitted from birds to humans in Hong Kong and caused the death of 6 of 18 people infected in 1997 (Claas *et al.*, 1998; Subbarao *et al.*, 1998). Following surveillance other strains have also been shown to infect humans, including the H9N2 AIV subtype, which was isolated from two infected children in Hong Kong (Peiris *et al.*, 1999) and from six patients in mainland China (Guan *et al.*, 2000), and H7N7, which was isolated from a woman with conjunctivitis in the UK (Kurtz *et al.*, 1996) and caused deaths in Holland (Fouchier *et al.*, 2004). Of these, H5N1 remains the most pathogenic. This strain has been responsible for epidemic outbreaks of avian influenza in many countries in Southeast Asia since mid-December 2003, and has resulted in at least 56 human fatalities (of 115 infected cases) in Vietnam and Thailand (<http://www.cdc.gov/flu/avian/outbreaks/asia.htm>). The AIV is a persistent potential health threat to both veterinary and human species as well as wild species.

The first isolation of AIV from wild birds occurred in South Africa from common terns (*Sterna hirundo*) in 1961 (Becker, 1966). Since then, many AIV subtypes have been isolated from waterfowl and migratory wild waterbirds, including ducks, geese, charadriiformes, ruddy turnstones and anatinae (Hinshaw *et al.*, 1980; Kawaoka *et al.*, 1988; Webster *et al.*, 1992). In general, influenza viruses appear to be genetically stable in these birds, particularly among waterfowl, where the influenza virus replicates in the gastrointestinal tract, generating large viral loads, usually without producing clinical signs (Hinshaw *et al.*, 1980; Sharp *et al.*, 1997; Webster *et al.*, 1978). Therefore, wild aquatic birds provide a natural reservoir for AIV and play an important role in their ecology and propagation. The fact that wild birds do not show clinical signs increases the potential of cross-species contagion to infect domestic poultry and humans (Webster *et al.*, 1973). In 2004, Sturm-Ramirez *et al.* isolated H5N1 viruses from dead wild migratory birds, including little egrets, grey herons and black-headed gulls found in Hong Kong parks, confirming that some of the H5N1 isolates were shown to be highly pathogenic in ducks. In this study, we compared H5N1 virus isolated from bar-headed geese (*Anser indicus*) collected during the Qinghai Lake outbreak to previously isolated H5N1 viruses, and found that the virus is evolving antigenically and genetically. We also assessed the pathogenicity, replication and transmissible potential of the H5N1 virus isolated from bar-headed geese in four species. To the best of our knowledge, this is the first report on the complete analysis of H5N1 virus isolated from migratory waterfowl during an endemic H5N1 outbreak. The information presented in this study will be valuable for further understanding of the roles of HPAI isolates from migratory wild birds in epidemiology of HPAI outbreak.

## METHODS

**Virus isolation and identification.** We collected oropharyngeal and cloacal swabs from bar-headed geese, great cormorants, great black-headed gulls, brown-headed gulls and ruddy shelducks that died during the 2005 HPAI outbreak at Qinghai Lake in China. The samples were thawed, and the inoculum for each sample (0.1 ml per egg) was propagated in 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs as described previously (Swayne *et al.*, 1998). After incubation at 37 °C, the allantoic fluid was harvested and tested by haemagglutination (HA) assay. To remove bacterial contamination, the allantoic fluid was passed through a 0.2 µm pore-size syringe filter (Millipore). The filtered HA-positive allantoic fluids were inoculated further into new embryonated eggs to isolate the pathogenic agent. After the third passage, the subtypes of the virus isolates were determined by conventional HA inhibition (HI) and neuramidinase inhibition (NI) assays (Office International des Epizootics, 1996), according to the Office International des Epizootics (OIE) manual. The A/Bar-headed Goose/Qinghai/0510/05 (Bh H5N1 virus) was selected for further analysis and its 50 % egg infectious dose (EID<sub>50</sub>) titre was determined. Virus titration end point was calculated by the method of Reed & Muench (1938). All experiments with the H5N1 isolates were performed in a Biosafety Level 3 laboratory and animal experiments were conducted in high-efficiency particulate air-filtered (HEPA-filtered) isolators.

**Antigenic analysis.** The antigenic characteristics of the Bh H5N1 virus were analysed with the HI test as described previously (Swayne *et al.*, 1998). Post-infected antiserum to the Bh H5N1 virus was prepared in chickens. Chicken antiserum against the A/Duck/Yulin/Guangxi/0203/2001(H5N1, Dk0203) and monoclonal antibody (mAb 4A3) to H5 haemagglutinin of the A/Goose/SZG/0306/2001(H5N1, Gs0306) were kindly provided by Professor Yuehuan Liu (Institute of Animal Science and Veterinary Medicine, Beijing Academy of Agriculture and Forestry, Beijing, China) and Professor Aijian Qin (College of Veterinary Medicine, Yangzhou University, Yangzhou, China), respectively.

**Genomic sequencing and phylogenetic analysis of influenza virus genes.** The H5N1 influenza virus from the bar-headed geese was used for genomic sequencing in this study. Total viral RNA was extracted from the infectious allantoic fluid using TRIzol LS reagent (Invitrogen). RT-PCR was conducted using a One-Step RT-PCR kit (Qiagen) with specific primers for influenza virus. The primer sequences and amplification conditions used are available upon request. The PCR products were separated by agarose gel electrophoresis and purified with the QIAquick PCR purification kit (Qiagen) according to the manufacturer's protocols, then sequenced using the CEQ DTCS-Quick Start kit on a CEO 8000 DNA sequencer (Beckman Coulter). H5N1 virus genomes from GenBank were used as references for comparative analysis of the Bh H5N1 virus isolated in our laboratory. Sequence data were compiled with the SeqMan program 4.03 (DNASTAR). The nucleotide sequences were compared and the phylogenetic trees

were generated with the MEGALIGN program 4.03 (DNASTAR) by using the CLUSTAL alignment algorithm.

**Chicken experiments.** Pathogenicity tests were performed in accordance with the instructions provided in the OIE manual. Briefly, eight 10-week-old SPF white leghorn chickens, (obtained from the Beijing Merial Vital Laboratory Animal Technology, Beijing, China) housed in negative-pressure isolator cages with HEPA-filtered air, were inoculated intravenously (i.v.) with 0.2 ml of a 1:10 dilution of bacteria-free allantoic fluid containing the Bh H5N1 virus ( $10^{8.45}$  EID<sub>50</sub> per 0.1 ml) to determine the i.v. pathogenicity index (IVPI). Eight additional SPF chickens were inoculated intranasally (i.n.) with  $10^6$  EID<sub>50</sub> per 0.1 ml of the Bh H5N1 virus in a 0.1 ml volume. Day 3 after i.n. inoculation, three inoculated birds were killed and were evaluated for virus replication, gross lesions, histopathology and immunohistochemistry (IHC). Procedures for histopathology and IHC followed those described previously (Perkins & Swayne, 2002). Briefly, tissue samples were fixed in 10 % neutral buffered formalin solution, sectioned and stained with haematoxylin and eosin. Duplicate sections were stained by IHC methods to screen virus antigen in individual tissues. In IHC, chicken antiserum against the Bh H5N1 virus was used as the primary antibody and diaminobenzidine tetrahydrochloride was used as the enzyme substrate. Portions of the tissue samples plus oropharyngeal and cloacal swabs were stored at  $-70$  °C and titres of infectious virus were subsequently determined in chicken embryos as described above. Similar tests were performed on birds that had died during the experiment.

**Pathogenicity and transmission studies in ducks and geese.** Groups of five 4-week-old shaoxing ducks (SX duck, a chinese local breed, obtained from the Yuyao Shennong Poultry, Zhejiang, China) and five 3-week-old Eastern Zhejiang white geese (EZW goose, a chinese local breed obtained from the Xiangshan Institute of Eastern Zhejiang White Goose, Ningbo, China) were inoculated i.n. with  $10^{8.45}$  EID<sub>50</sub> per 0.1 ml of the Bh H5N1 virus in a volume of 0.1 ml. One day later, three uninfected ducks and three uninfected geese were, respectively, introduced into each group. Ducks and geese were observed daily for signs of disease. Oropharyngeal and cloacal swabs were collected from all ducks and geese at 3–11 days after inoculation for determining the presence of infectious virus by growth in embryonated chicken eggs. In addition, tissues were collected from each duck and goose for virus isolation, histopathology and IHC as described above. Ducks and geese that died during the experimental period were evaluated for gross lesions. The collected samples from the infected birds were titrated for infectivity by determining the EID<sub>50</sub>.

**Mouse experiments.** Eight 6–8-week-old SPF BALB/c mice (purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences, Shanghai, China) were infected i.n. with 0.05 ml of  $10^6$  EID<sub>50</sub> per 0.1 ml under anaesthesia. One day later, three uninfected mice were placed in direct contact with the inoculated mice. On day 3, three of eight inoculated mice were sacrificed for virus titration of the lung, kidney, spleen, heart, liver and brain. Tissue samples were homogenized in 1 ml cold PBS and centrifuged at 16000 *g* for 10 min before homogenates were

titrated for virus infectivity in eggs with initial dilutions of 1:10 (lung) and 1:2 (other organs). The remaining five inoculated mice were monitored daily for weight loss and mortality. Tissue samples were collected from dead mice for histopathology and IHC. The mouse 50 % minimal lethal dose (MLD<sub>50</sub>) was determined for the Bh virus that caused lethal infection in mice by i.n. inoculation. The MLD<sub>50</sub> was calculated by the method of Reed & Muench (1938).

## RESULTS

### Epidemiology, clinical signs and gross lesions

On 3 May 2005, visitors to Bird Islet on Qinghai Lake in China found a dead bar-headed goose. Subsequently, many sick and dead bar-headed geese were found and on 15 May 2005, Qinghai Lake was closed to control the spread of the disease. On 16 May 2005, a large number of great cormorants died on Luci Islet (3 km from Bird Islet) and numerous wild birds of various species were found dead on Sankuaishi Islet (25 km from Bird Islet). In all, more than 6000 wild waterbirds died during the outbreak, including approximately 50.3 % bar-headed geese, 22.16 % great cormorants, 15.99 % great black-headed gulls, 9.05 % brown-headed gulls, 2.3 % ruddy shelducks and 0.21 % tufted ducks.

Clinical findings of the sick migratory wild waterbirds (bar-headed goose, great cormorants, great black-headed gulls, brown-headed gulls, ruddy shelducks and tufted ducks) were similar to the features of H5N1 disease in waterfowl (Ellis *et al.*, 2004) and included paralysis, unusual head opisthotic tilt, staggering and tremors. At necropsy, as shown in Fig. 1(A–C), 12 samples from dead bar-headed geese showed that the key gross lesions harboured mainly pancreatic haemorrhage and necrosis (8/12), proventricular haemorrhage and ulcerations (8/12), haemorrhagic dots of cardiac coronary groove and endocardiac membrane (8/12), renal swelling and congestion (7/12), hepatic haemorrhagic mass (8/12) and haemorrhagic spots of the digestive tracts (7/12) and ovaries (4/6).

### Virus identification and genomic analysis of H5N1 AIV from bar-headed geese

Twelve swab samples from the dead bar-headed geese, great cormorants, great black-headed gulls, brown-headed gulls and ruddy shelducks were tested for AIV by embryo inoculation in SPF eggs. The inoculated chicken embryos died within 30 h post-infection, and 12 haemagglutinating virus isolates were isolated. Subtype analysis of 12 virus isolates from the migratory waterfowl showed they belong to the H5N1 subtype of AIV. No bacteria were isolated from the blood, liver or heart of the dead waterfowl. Subsequently, one of 12 virus isolates [the A/Bar-headed Goose/Qinghai/0510/05 (Bh H5N1 virus)] was selected to determine its replication in chicken embryo, and the result revealed that the virus titre of the Bh H5N1 virus was  $10^{8.45}$  EID<sub>50</sub> per 0.1 ml. Therefore, these data indicate that the isolated H5N1 virus might be the cause of death of migratory wild waterfowl.

We analysed the genomic sequence of the H5N1 virus to identify the origin of the virus that infected the bar-headed geese. Sequencing results showed that the amplified genomic sequence (GenBank accession nos DQ137873, DQ137874, DQ237951–DQ237956) was approximately 13.6 kb in length, which was 99.8–100 % identical to those of the Qinghai virus strains isolated by Liu *et al.* (2005) and Chen *et al.* (2005). The data revealed that the Bh H5N1 virus isolated by us was identical to the Qinghai H5N1 virus strains identified by Liu *et al.* (2005) and Chen *et al.* (2005). Furthermore, compared to other H5N1 virus sequences obtained from GenBank, the Bh H5N1 virus shared the highest nucleic sequence similarity with other H5N1 viruses (data not shown), including HA fragment (98.4 %, A/Ck/ST/4231/2003), NA fragment (98.8 %, A/Ck/ST/4231/2003), HA fragment (98.4 %, A/Ck/ST/4231/2003), NA fragment (98.8 %, A/Ck/ST/4231/2003).

A/Ck/ST/4231/2003), NS fragment (99.1 %, A/Ck/Kyoto/3/04), MA fragment (99.6 %, A/Peregrine Falcon/HK/D0028/04), NP fragment (99.4 %, A/Ck/Kyoto/3/04), PA fragment (98.6 %, A/Peregrine Falcon/HK/D0028/04), PB1 segment (98.9 %, A/Peregrine Falcon/HK/D0028/04) and PB2 segment (98.3 %, A/Peregrine Falcon/HK/D0028/04). Similarly, the phylogenetic analysis of the eight gene segments (Fig. 2) showed that the Qinghai HA and NA gene fragments were most closely related to the A/Ck/ST/4231/2003, and that fragments M, PA, PB1 and PB2 of the Bh H5N1 virus were closely related to the A/Peregrine falcon/HK/D0028/04, and that fragments NS and NP of the Bh H5N1 virus were correlative with the A/Goose/Shantou/1621/05 and the A/Crow/Osaka/102/04, respectively. These results imply that the Bh H5N1 virus is a reassortant virus.

Based on the deduced amino acid sequence comparisons at the HA cleavage site that characterized HPAs as described previously (Guan *et al.*, 2002), the basic amino acid motif 'GERRRKKR' of the Bh H5N1 virus differs from the other H5N1 strains isolated from human, chicken, duck, crow and teal (Table 1). Amino acid residues encoded by the HA gene of the Bh H5N1 virus had an Arg (R) to Gly (G) substitution at position 323. In neuraminidase of the Bh H5N1 virus, we discovered a deletion of 20 aa 'CNQSIITYENNTWVNQTYVN' at sites 49–68. However, this sequence is closely related to the high virulence characteristic of the H5N1 virus (Puthavathana *et al.*, 2005). In addition, the Bh H5N1 virus had a Glu (E) to Lys (K) substitution at position 627 of the PB2 gene, similar to human influenza viruses (A/HK/483/97), which also have Lys at position 627 of PB2 (Hatta *et al.*, 2001).

### **Antigenic characteristics of the Bh H5N1 virus**

To detect antigenic characteristics of the Bh H5N1 virus, we compared cross-reactivity between the Bh H5N1 virus and other H5N1 viruses by HI assay. As shown in Table 2, chicken antiserum against DK0203 (H5N1) from duck and mAb 4A3 to Gs0306 (H5N1) from goose cross-reacted to lower titres with the Bh H5N1 virus than the A/Chicken/Jiande/1218/2001 and the A/Duck/Jinhua/0226/2002. Correspondingly, the post-infection chicken antiserum raised against the Bh H5N1 virus cross-reacted preferably with the A/Chicken/Jiande/1218/2001 virus and the A/Duck/Jinhua/0226/2002 virus. The three H5N1 viruses we tested were assigned to one distinguishable group based on HI reactivity patterns with antiserum to the Bh H5N1 virus. This suggested that the Bh H5N1 virus is antigenically similar to the A/Chicken/Jiande/1218/2001 virus and the A/Duck/Jinhua/0226/2002 virus.

### **Pathogenicity of the Qinghai virus in chickens**

We evaluated the pathogenicity of the Bh H5N1 virus in 10-week-old SPF white leghorn chickens by i.v. and i.n. inoculations. According to the OIE criteria, the IVPI of the Bh H5N1 virus was 2.9 and mean death time (MDT) was 1 day. Chickens inoculated i.n. with  $10^6$  EID<sub>50</sub> of the Bh H5N1 virus revealed 100 % mortality (8/8), and disease signs were typical of those seen in chickens infected with H5N1 Hong Kong/97 viruses (Suarez *et al.*, 1998). MDT of the infected chickens was 3 days (2.6–3.9 days) following i.n. inoculation. Data (see Tables 3 and 4) showed that the Bh virus replicated to high titres ( $7.03$ – $7.6 \log_{10}$  EID<sub>50</sub> g<sup><MIN>1</sup> tissue) in brain, lung and

heart, with lower titres ( $5.56\text{--}5.93 \log_{10} \text{EID}_{50} \text{ ml}^{-1}$ ) in oropharynx and cloaca. Tissue analysis for gross lesions, histopathology and virus antigen expression in chickens that died showed that the Bh virus produced similar systemic lesions seen in chickens infected with H5N1 Hong Kong/97 viruses (Suarez *et al.*, 1998). Histologically, microscopic lesions comprised severe necrosis of the pancreatic and cerebral tissues (Fig. 1D and E), severe nephrosis (Fig. 1F), congestion of the pulmonary and hepatic tissues, and mild to moderate proventriculitis. The H5N1 virus antigen was identified in pancreatic acinar epithelium, necrotic neurons of the brain, kidney tubular epithelial cells and histiocytes of the lungs (Fig. 1d, e and f).

### **Pathogenicity and shedding of the Bh H5N1 virus in infected ducks**

To determine the susceptibility and transmissibility of the Bh H5N1 virus, we inoculated five juvenile ducks and housed three contact ducks in the same cage. Signs of sickness (e.g. lethargy, diarrhoea, ataxia and paralysis) were observed in one of five inoculated ducks 2 days after inoculation and in one of three contact ducks 4 days after housing with infected ducks. All ducks infected by the Bh H5N1 virus showed signs of severe central nervous system (CNS) dysfunction, including violent tremors, uncontrollable shaking, marked loss of balance and lack of coordination. Four of five inoculated ducks died 2.9–4.7 days (MDT is 3.7 days) post-inoculation (p.i.), and two of three contact ducks died 5.4–6.1 days p.i. (MDT is 5.7 days), indicating the contact ducks were highly susceptible to infection with naturally transmitted Bh H5N1 virus. The virus was shed from oropharynx and cloaca of the inoculated ducks 3 days p.i., and from oropharynx and cloaca of the contact ducks 5 days p.i. (Table 3). Furthermore, the Bh H5N1 virus replicated to high titres in lung, brain and heart of ducks (Table 4), and lower levels of virus replication presented in the oropharynx and the cloaca of ducks (Table 3).

At necropsy, gross lesions of the infected ducks were similar to lesions of sick bar-headed geese, and showed pancreatic necrosis, haemorrhagic dots of endocardiac membrane and hepatic swelling. Histological lesions observed in the infected ducks included severe pulmonary haemorrhage with infiltration of heterophils in the bronchi (Fig. 1I), moderate pancreatic necrosis (Fig. 1G), and small focal necrosis characterized by an infiltration of lymphocytes in cardiac muscles. In addition, we noted haemorrhagic splenitis, mild hepatic fatty degeneration, particularly haemorrhagic meningitis and perivascular cuffings with an infiltration of lymphocytes as well as small foci of gliosis in the brain (Fig. 1H and h2). The corresponding viral antigen was demonstrated by IHC assay in the pancreatic glands, brains, lungs and other tissues (Fig. 1g, h1 and i).

### **Pathogenicity of the Bh H5N1 virus in geese**

Two of five geese experimentally infected with the Bh H5N1 virus began to show lethargy and depression 8 days p.i., and another two geese showed severe neurological signs (ataxia) 10 days p.i. Four of the inoculated geese died from 8.7 to 12.9 days p.i. (MDT was 10.4 days), and two of three contact geese died 20 days p.i., showing that the contact geese were susceptible to infection with naturally transmitted Bh H5N1 virus. Virus was detected in the oropharynx and cloaca of the inoculated geese 8 days p.i. (Table 3), and in the oropharynx and cloaca of the

contact geese 18 days p.i. At necropsy, gross lesions were not found in the juvenile geese used in the infection and transmission study, except for in the pancreas and endocardium. Tissue samples were collected for virus titre determination. Tables 3 and 4 show that the virus replicated to high titres in brain ( $5.93 \pm 0.94 \log_{10} \text{EID}_{50} \text{g}^{<MIN>1}$ ), heart ( $3.59 \pm 0.38 \log_{10} \text{EID}_{50} \text{g}^{<MIN>1}$ ), lung ( $5.02 \pm 0.49 \log_{10} \text{EID}_{50} \text{g}^{<MIN>1}$ ), oropharynx ( $> 6.00 \log_{10} \text{EID}_{50} \text{ml}^{<MIN>1}$ ) and cloaca ( $4.46 \pm 0.32 \log_{10} \text{EID}_{50} \text{ml}^{<MIN>1}$ ). Histologically, there was mild to moderate focal necrosis of the pancreatic glands (Fig. 1J), neuronal atrophy and severe haemorrhagic meningitis with heterophil infiltration and oedema in brain (Fig. 1K, k2), mild haemorrhage in cardiac muscles (Fig. 1L), mild pulmonary haemorrhage, moderate splenitis and mild nephrosis with haemorrhage. The corresponding AIV antigen appeared in brain, pancreas, lung, spleen and kidney (Fig. 1j and k1). However, no AIV antigen was detected by IHC in cardiac muscle.

### **Pathogenicity of the Bh H5N1 virus in mice**

To examine the pathogenicity of the Bh H5N1 virus in a mammalian host, 6–8-week-old SPF BALB/c mice were inoculated i.n. The virus replication, morbidity and mortality were determined. The Bh H5N1 virus-infected mice began to lose weight 4 days p.i. (Fig. 3a) and showed signs of illness, such as ruffled fur and hunched posture. The mortality of the infected mice reached 100 % within 8 days after i.n. inoculation (Fig. 3b) and the MDT of the inoculated mice was 7.3 days (6.7–7.7). The Bh H5N1 virus was detected in the lung (positive), brain ( $3.63 \log_{10} \text{EID}_{50} \text{ml}^{<MIN>1}$ ), spleen ( $4.97 \log_{10} \text{EID}_{50} \text{ml}^{<MIN>1}$ ) and heart (positive) of mice euthanized 3 days after i.n. inoculation. The virus replicated to high titres in the lungs and brains of mice that died on day 7.7 p.i, but was not detected in the liver, spleen, heart or kidney of the dead mice (Table 4). The  $\text{MLD}_{50}$  of the Bh H5N1 virus was  $2.3 \log_{10} \text{EID}_{50}$ . Necropsy examination revealed that the infected mice were more severely affected, displaying haemorrhage in 100 % of the lung area and swollen, yellowed livers. Histologically, microscopic lesions showed severe haemorrhagic and histiocytic pneumonia with the infiltration of neutrophils, including acute pyogenic bronchitis (Fig. 1O), severe meningeal and parenchymal haemorrhage with an infiltration of lymphocytes in brain (Fig. 1N), severe pancreatic necrosis (Fig. 1M), severe nephrosis and moderately hepatic fatty degeneration. AIV antigen was present in pancreatic acinar cells, neurons, pneumocytes and necrotic epithelium, and luminal debris of the bronchi and bronchioles (Fig. 1m, n and o). Contact mice were still alive without clinical signs of disease 15 days after inoculation, and no virus was isolated from contact mice.

## DISCUSSION

Thus far most pathogenic H5N1 infection outbreaks have occurred in domestic poultry (Chen *et al.*, 2004; Lee *et al.*, 2005; Tumpey *et al.*, 2002; Wan *et al.*, 2005). No H5 subtype virus has been identified outside poultry raising areas; although there have been reported cases of pathogenic AIV in wild birds in parks or in areas associated with HPAI outbreaks in poultry (Becker, 1966; Sturm-Ramirez *et al.*, 2004). However, during the period from May to July 2005, an epidemic resulted in the deaths of more than 6000 migratory waterbirds of various species at Qinghai Lake in China. The only pathogenic agent isolated from oropharyngeal and cloacal swabs of dead bar-headed geese from this outbreak was HPAI H5N1 virus, suggesting that the H5N1 virus was the cause of death in these birds. This is the first time HPAI has been observed in a large-scale outbreak in migratory wild waterbirds in China.

Previous studies have revealed considerable variations in the antigenic epitopes presented on the surface of H5 viruses (Guan *et al.*, 2002; Sturm-Ramirez *et al.*, 2004). Some researchers believe AIV transfer between species resulted in increased antigenic variation, particularly in the surface glycoproteins, due to strong immune selective pressure (Ludwig *et al.*, 1995; Matrosovich *et al.*, 1999). In this study, the Bh H5N1 virus has a lower antigenic cross-reactivity with the H5N1 viruses isolated from duck and domestic geese in 2001 and 2002 in China but whether this represents a specific change in adaptation to circulation in wild fowl will need further epidemiological investigation.

Earlier reports showed that HPAI viruses that were lethal in domestic poultry could replicate in the internal organs of ducks, but caused no overt signs of disease (Chen *et al.*, 2004; Kawaoka *et al.*, 1988; Tumpey *et al.*, 2002). The H5N1 viruses (Gs/HK/739.2/02) isolated recently from dead wild migratory birds found in Hong Kong parks resulted in severe disease and significant virus replication in experimentally infected ducks (Sturm-Ramirez *et al.*, 2004). By comparing the pathogenicity of the reemerging H5N1 influenza viruses in Hong Kong in 2002 to duck, Sturm-Ramirez *et al.* (2004) also described that approximately 80 % of ducks survived after infection with A/Ph/HK/FY155/01 and A/Teal/HK/2978.1/02, respectively. Previously described H5N1 infection in ducks tended to be pneumotropic with mild lesions localized to the respiratory tract and some virus detected in the spleen and bursa (Perkins & Swayne, 2002). However, in the present study, chickens, SX ducks and EZW geese developed systemic infection with high mortality after inoculation with the Bh H5N1 virus (100 % in the inoculated chickens 2.6–3.9 days p.i., 80 % in the inoculated ducks 2.9–4.7 days p.i. and 80 % in the inoculated geese 8.7–12.9 days p.i.), and significant titres of virus were detected from sampled tissues. These results imply that this newly isolated H5N1 virus isolated from the bar-headed geese is more virulent in domestic poultry and wild birds than previously reported H5N1 viruses (Sturm-Ramirez *et al.*, 2004).

In our experiments, infectious virus was recovered from oropharyngeal and cloacal swabs taken from infected ducks 3 days p.i., consistent with a previous report on H5N1 virus-infected ducks (Sturm-Ramirez *et al.*, 2004). In contrast, detectable virus was isolated in oropharyngeal and cloacal swabs from the inoculated geese up to 8 days p.i. This suggests virus is shed earlier in

ducks and chickens than in geese. H5N1 viruses have been reported to infect the CNS and cause histopathological changes in the brain in different species of ducks (Perkins & Swayne, 2001, 2002; Shortridge *et al.*, 1998; Sturm-Ramirez *et al.*, 2004). Severe clinical signs and gross lesions were observed in infected ducks and geese (i.e., ataxia), similar with our findings in the bar-headed geese and other wild birds at Qinghai Lake. Furthermore, we observed that microscopic lesions of the infected geese were smaller than those of the infected ducks (Fig. 1), suggesting geese have a stronger resistance to the Bh H5N1 virus. High titres of virus were titrated from brains, lungs and hearts of both ducks and geese. These results show that the newly isolated Bh H5N1 virus has acquired characteristics of systemic infection in domestic waterfowls and wild birds. Therefore, we hypothesize that this new ability to result in broader tissue tropisms to the infected waterfowl is central to the increasing pathogenicity of this virus. To our knowledge, this is the first detailed pathological description of an influenza infection in laboratory waterfowl caused by an H5N1 virus isolate derived from migratory wild waterbirds.

Contact ducks and geese developed similar clinical symptoms, systemic infection and death, as those of the inoculated ducks and geese. Correspondingly, the high titres of virus isolated post-mortem from the brains and visceral organs of contact birds were consistent with those of the experimentally infected birds. As mentioned above, we confirmed that the naturally transmitted virus could cause systemic infection and death. Previous reports showed that influenza viruses replicate preferentially in the intestinal tracts of wild ducks and are excreted at high titres in faeces, and are thought to spread to other wild birds and domestic poultry via contamination of water (Webster *et al.*, 1978). However, a recent report suggested that ducks may shed the virus primarily from the upper respiratory tract (Perkins & Swayne, 2002; Sturm-Ramirez *et al.*, 2004). Our findings indicate that the Bh H5N1 virus causing systemic and respiratory infection in ducks and geese was detected at higher virus titres in oropharynx than in cloaca. In this study, ducks and geese were prevented from swimming in the water pans or contaminating them with faeces. In addition, in the fields, we found that sick and dead wild birds of various species were distributed on three non-geographical inhabitable islets on Qinghai Lake. Possibly, airflow could explain the transmission of H5N1 virus from Bird Islet to the other islets, and, based on the data, we hypothesize that the possible transmission route is by air. Future studies are needed to confirm this hypothesis.

To understand better the virulence and pathogenesis of the Bh H5N1 virus in mammals, we included an infection model in BALB/c mice. Previous reports have observed conflicting pathogenicity of the H5N1 virus in mice isolated from humans, chickens and ducks. The fatal human H5N1 virus (A/HK/156/97) showed intermediate lethality in mice (MLD<sub>50</sub> values of 10<sup>5.9</sup> EID<sub>50</sub>), and a lethal chicken H5N1 virus (DK/Anyang/AVL-1/01) from healthy ducks resulted in only 22–33 % mortality in mice (Lu *et al.*, 1999; Tumpey *et al.*, 2002). However, Chen *et al.* (2004), only four of 21 H5N1 viruses isolated from healthy ducks between 1999 and 2002 were highly pathogenic to mice and caused death. Furthermore, Lee *et al.* (2005) demonstrated that the chicken HPAI H5N1 virus (A/Chicken/korea/ES/03) did not cause mortality in mice. Our results indicate that mice inoculated i.n. with the Bh H5N1 virus (MLD value of 10<sup>2.3</sup> EID<sub>50</sub>) showed higher titres of virus replication and 100 % mortality within 8 days p.i. More widespread and severe

lesions were detected in lungs, kidneys and liver than that of other H5N1 viruses described in previous reports (Chen *et al.*, 2004; Lee *et al.*, 2005; Tumpey *et al.*, 2002). Particularly, severe cerebral microscopic lesions were observed for the first time in mice infected with the Bh H5N1 virus. Taken together, these results indicate that the Bh H5N1 virus was able to replicate efficiently in mice without prior adaptation and was more pathogenic for BALB/c mice than previous H5N1 viruses (Chen *et al.*, 2004; Lee *et al.*, 2005; Lu *et al.*, 1999; Tumpey *et al.*, 2002). Based on MLD<sub>50</sub> values of  $<10^{3.0}$  EID<sub>50</sub> the Bh H5N1 virus was considered highly pathogenic in the mouse model (Chen *et al.*, 2004; Katz *et al.*, 2000). The molecular basis of the transmissibility and pathogenesis of AIVs to mammals is not completely clear. Previous reports showed that residue 627 of the PB2 protein is critical for H5N1 pathogenicity in mice, as well as a series of basic amino acids at the HA cleavage site (Hatta *et al.*, 2001). Interestingly, the Lys at position 627 of PB2 has been observed only in influenza viruses isolated from humans and in those adapted to mammalian cell lines (Hatta *et al.*, 2001; Subbarao *et al.*, 1993), whereas all previous AIVs isolated from avian species had a Glu acid residue at this position (Li *et al.*, 2005). The Bh H5N1 virus substituted Glu to Lys at position 627 of the PB2 gene, and possesses a basic amino acid motif at the HA cleavage site, indicating that the Bh H5N1 virus has the potential for interspecies transmission to mammals. Recently, an experiment confirmed that amino acid substitution of Asp to Asn at position 701 of PB2 was an important determinant for enabling AIV to cross host species barriers and infect mice (Li *et al.*, 2005), but the molecular correlations of other proteins (NA, M1, PB1) with pathogenicity in mice have also been reported (Katz *et al.*, 2000). Therefore, elucidating the true mechanism of pathogenesis for interspecies transmission of AIV needs further evaluation.

## **ACKNOWLEDGEMENTS**

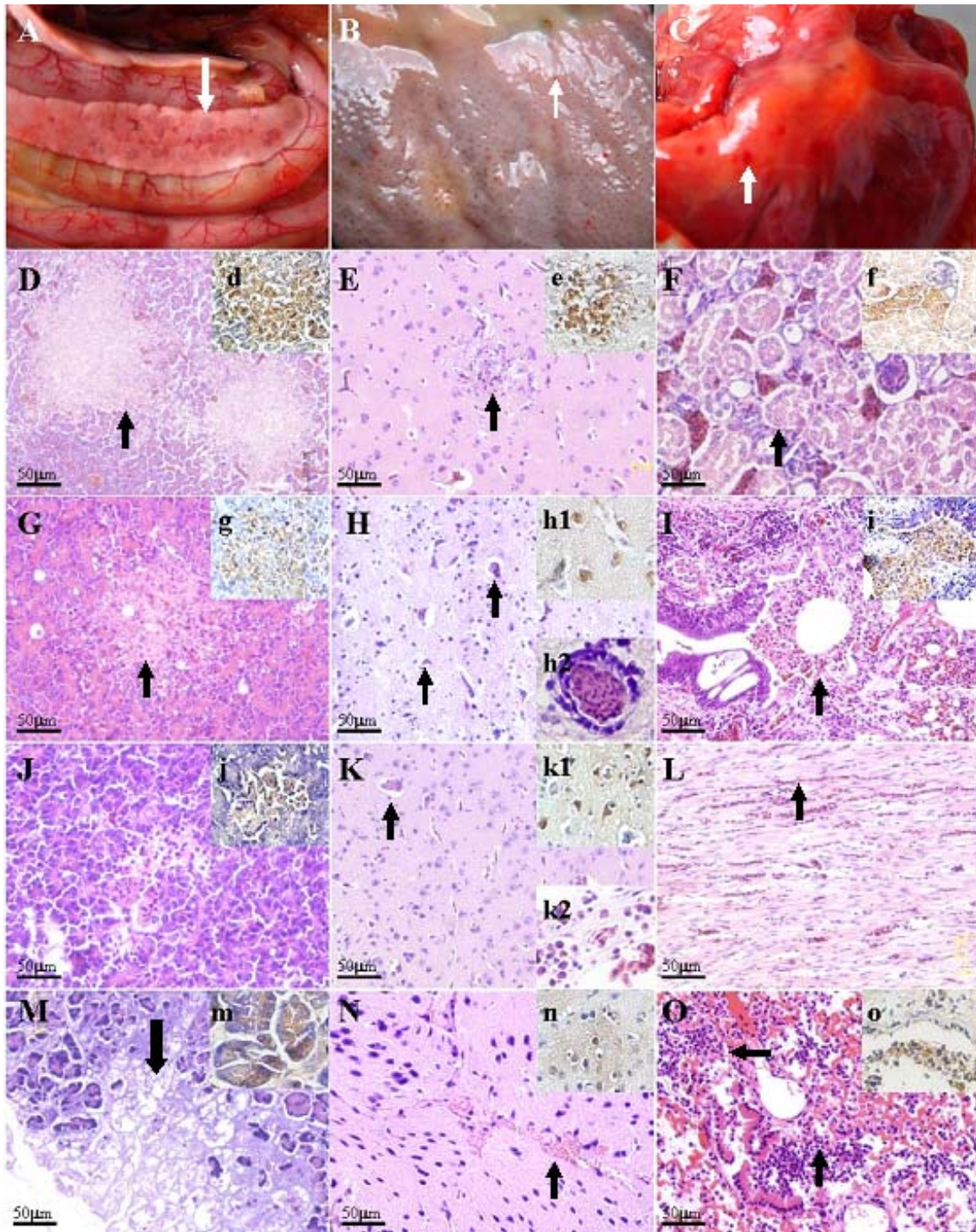
This work was supported by grants from National Key Basic Research Program of China (Grant no. 2005CB523203) and National Science and Technology Development Program of China (2004BA519A33, 2004BA519A46).

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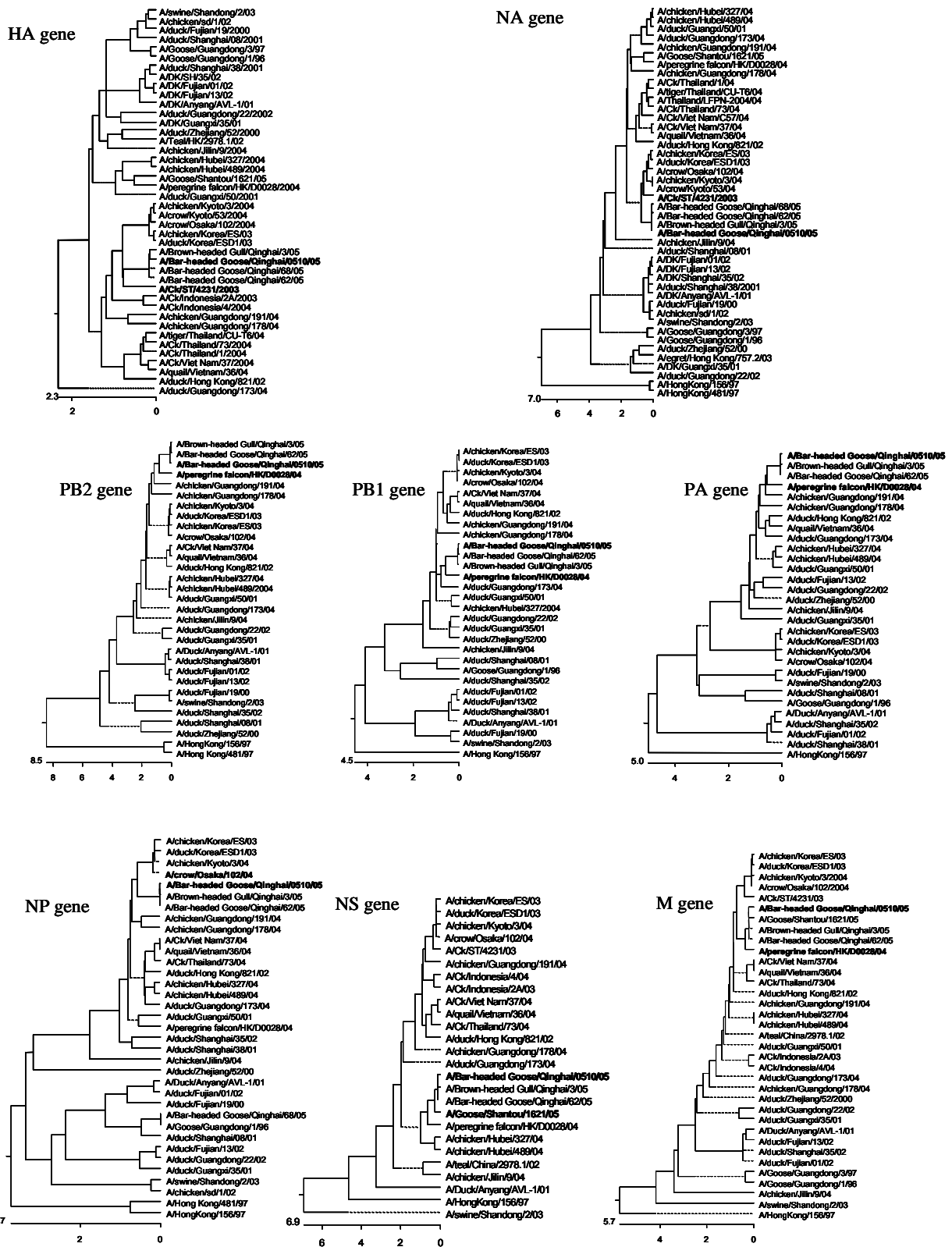
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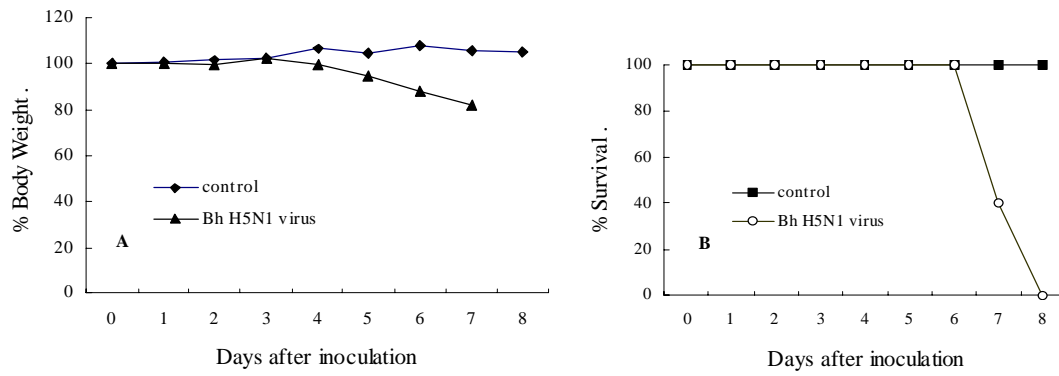
**Fig. 1.** Gross lesions characterizing dead bar-headed geese (A–C), and microscopic observations of chickens, ducks, geese and mice that were intranasally inoculated with the A/Bar-headed Goose/Qinghai/0510/05 virus (D–O and d–o). (A) Necrotic foci of the pancreatic gland (arrow). (B) Haemorrhagic foci of the proventriculus (arrow). (C) Haemorrhagic spots of the cardiac coronary groove (arrow). Photomicrographs of haematoxylin and eosin stained tissue sections (D–O, h2 and k2) and sections stained by IHC to demonstrate AIV (d–g, h1, i–j, k1, l–o). (D–F) Necrotic foci of pancreas and brain, and nephrosis in a 10-week-old chicken that died 3 days p.i. (d–f) AIV antigen of necrotic pancreatic cells, necrotic neurons and degenerative renal

epithelia in the same chicken. (G–I and h2) Necrotic foci of pancreas (G, arrow), atrophic neurons (H, arrows) and perivascular cuffing (h2) of brain, histiocytic and haemorrhagic pneumonia with pyogenic bronchitis (I, arrow) in a 4-week-old duck that died 3 days p.i. (g, h1 and i) AIV antigen in necrotic pancreatic acinar cells, degenerative neuronal cells, degenerative and necrotic heterophils within bronchus of the same duck. (J–L and k2) Pancreatic necrotic focus (J), atrophic neuron (K, arrow) and haemorrhagic meningitis (k2) with infiltration of heterophils in brain, haemorrhage of cardiac muscle (L, arrow) in a 3-week-old goose that died 10 days p.i. (j and k1) AIV antigen in necrotic focus of pancreatic acinar cells and degenerative neuronal cells of the same goose. (M–O) Pancreatic necrosis (M, arrow), parenchymal haemorrhage (N, arrow) of brain, and histiocytic and haemorrhagic pneumonia with pyogenic bronchitis (O, arrows) in an inoculated mouse 7 days p.i. (m–o) AIV antigen in pancreatic acinar cells and neuronal cells, degenerative and necrotic heterophils within bronchus in the same mouse.



**Fig. 2.** Phylogenetic trees for the HA, NA, PB2, PB1, PA, NP, NS and M genes of the H5N1 influenza A viruses were analysed, including the A/Bar-headed Goose/Qinghai/0510/05 and other H5N1 viruses obtained from GenBank. Trees were generated by using MEGALIGN software (DNASTAR) on the basis of the following gene sequences: nt 29–1733 (1705 bp) of HA, 21–1370

(1350 bp) of NA, 28–2307 (2280 bp) of PB2, 25–2198 (2174 bp) of PB1, 46–1530 (1484 bp) of NP, 26–785 (760 bp) of M and 49–871 (823 bp) of NS. The length of each pair of branches represents the distance between sequence pairs, and the units at the bottom of the tree indicate the number of substitution events. The bold type reveals the closest matches.



**Fig. 3.** Weight change (a) and lethality (b) of mice inoculated with  $10^6$  EID<sub>50</sub> (50  $\mu$ l) of A/Bar-headed Goose/Qinghai/0510/05 or mock infected with vehicle.

**Table 1.** Comparison of amino acid sequences of different gene segments of H5N1 viruses

Strain	HA aa sequence	NS sequence	PB2 sequence at aa 627
	Cleavage site 323–330	Deletion of aa 80–84	
A/Bar-headed Goose/Qinghai/0510/05	GERRRKKR	Yes	K
A/HK/156/97	RERRRKKR	No	E
A/HK/481/97	RERRRKKR	–	E
A/HK/483/97	RERRRKKR	No	K
A/CK/Shantou/4231/03	RERRRKKR	Yes	E
A/CK/Indonesia/4/04	RERRRKKR	Yes	E
A/DK/Anyang/AVL-1/01	RERRRKKR	No	E
A/CK/Gd/191/04	RERRRK-R	Yes	E
A/CK/Korea/ES/03	RE-KRKKR	Yes	E
A/crow/Osaka/102/04	RE-RRKKR	Yes	E
A/teal/China/2978.1/02	REIRRKKR	Yes	E

**Table 2.** Antigenic analysis of H5N1 influenza virus by HI test

Virus	Polyclonal antisera titres to:		mAb HI titre to Gs0306 (4A3)
	Anti-Dk 0203 (chicken)	Anti-Bh H5N1 virus (chicken)	
A/Chicken/Jiande/1218/2001	128	32	2048
A/Duck/Jinhua/0226/2002	64	32	256
A/Bar-headed Goose/Qinghai/0510/05	16	64*	32

\*Titre of homologous virus.

**Table 3.** Replication and transmission of the A/Bar-headed Goose/Qinghai/0510/05 virus in different birds

Species	Detection of A/Bar-headed Goose/Qinghai/0510/05 virus at different times p.i.											
	Virus shedding on day 3 p.i.				Virus shedding on day 5 p.i.				Virus shedding on day 8 p.i.			
	Inoculated birds*		Contact birds*		Inoculated birds		Contact birds		Inoculated birds		Contact birds	
	Oropharynx	Cloaca	Oropharynx	Cloaca	Oropharynx	Cloaca	Oropharynx	Cloaca	Oropharynx	Cloaca	Oropharynx	Cloaca
Chicken	3/8 (5.56)	4/8 (5.93)	-†	-†	0/0‡	0/0‡	-†	-†	-†	-†	-†	-†
Duck	2/5 (4.1)	1/5 (3)	0/3	0/3	2/2‡ (4.13)	2/2‡ (3.6)	2/2‡ (<2)	1/2‡ (<2)	-†	-†	-†	-†
Goose	0/5	0/5	0/3	0/3	0/5	0/5	0/3	0/3	3/5 (>6.0)	3/5 (4.46)	0/3	0/3

\*Number shedding/number sampled [virus titre ( $\log_{10}EID_{50} ml^{-1}$ )]. The virus titre is the mean of positive samples.

†-, No samples.

‡Birds died.

**Table 4.** A/Bar-headed Goose/Qinghai/0510/05 virus titres in organs of different animals

Species	Animal no.	Virus titres ( $\log_{10}\text{EID}_{50} \text{g}^{-1}$ tissue) in					
		Brain	Heart	Lung	Liver	Spleen	Kidney
Chicken*	8	7.03±1.08	7.60±0.46	7.03±0.45	-†	-†	-†
Duck*	4	6.51±0.87	5.86±0.84	7.76±0.43	-†	-†	-†
Goose*	4	5.93±0.94	3.59±0.38	5.02±0.49	-†	-†	-†
Mouse*	8	4.23±0.20	ND‡	5.04±0.44	ND‡	ND‡	ND‡

\*Samples were collected from dead birds.

†-, Not done.

‡ND, No detectable virus.