

## **Supporting Online Material for Manuscript 1115273:**

### **Materials and Methods**

***Virus isolation and subtyping.*** Oropharyngeal swab, liver, spleen, lungs and brain were collected from the affected or dead birds. Tissue samples were placed in 10% w/v of PBS buffer containing antibiotics (penicillin and streptomycin, 2,000 IU), ground and centrifuged to collect supernatants as inoculates. The inoculum for each sample was propagated in 10-day old embryonated SPF chicken eggs. Allantoic fluids were harvested from those embryonated eggs when the embryo died 24-48 hours after inoculation.

Subtyping of the virus was carried out by HA and NA inhibition assay, respectively, using reference serum (H1-H15; N1-N9), a gift from Dr. Hiroshi Kida, Laboratory of Microbiology, Department of Disease Control, Hokkaido University Graduate School of Veterinary Medicine, Sapporo 060-0818, Japan. All work was performed in a biosafety level 3 (BSL-3) facility.

The four viruses used for further genomic sequencing were respectively named as: A/bar-headed goose/Qinghai/1/05 (BhGoose/QH/1/05), A/bar-headed goose/Qinghai/2/05 (BhGoose/QH/2/05), A/great black-headed gull/Qinghai/1/05 (GbhGull/QH/1/05) and A/brown-headed gull/Qinghai/1/05 (BhGull/QH/1/05).

***Detection of the antibody in the affected birds.*** The antibody titers of the infected birds were evaluated by hemagglutination inhibition (HI) assay and immunodiffusion (S1) with the reference antigens (a gift from Dr. Hiroshi Kida and a purchase from the National Avian Influenza Reference Laboratory, Harbin, China).

***Infection of the experimental animals.*** To test the pathogenicity of the virus, 6-week-old chickens (n=8) were inoculated with 0.1 ml of ten-fold diluent of the allantoic fluid intravenously. All chickens were killed by the infection within 20 hours of inoculation. 6-week-old female BALB/c SPF mice (n=8, purchased from Beijing Laboratory Animal Center, Beijing, China) were infected with 0.05 ml of  $10^6$ ELD<sub>50</sub> (half embryo lethal dose) by intranasal route under anesthesia. All experiments were conducted in a biosafety level 3 (BSL-3) facility.

***Sequencing of the genomes.*** Reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify the gene segments for sequencing analysis (1). Viral RNA extracted from 200 µl allantoic fluid using Trizol reagents (GIBCO-BRL), and reverse transcription was performed using influenza virus oligonucleotide universal primer: 5'-AGC AAA AGC AGG-3'. A series of primers were designed to amplify PB2 (4 fragments), PB1 (4 fragments), PA (4 fragments), HA (3 fragments), NP (3 fragments), NA (3 fragments), M (2 fragments) and NS (2 fragments) genes for sequencing by Amersham ET Dye terminator kit and analyzed by ABI 3730 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). The data were analyzed and phylogenetic trees were drawn using the PAUP 4.02 program.

### **Supporting References and Notes**

S1. J. H. Liu et al., *Virus Gene* **29**, 81, (2004).

S2. For more information on the latest news and some basic knowledge of AIV, esp. in China and Southeast Asia, please see our specifically-devoted webpage:

<http://www.avian-flu.info.cn> This webpage is supported by a special grant: CAS-Information-Special Grant No. INF105-SDB-3-A2. Thanks to Dr Baoping Yan from Computer Network Information Center, Chinese Academy of Sciences, for her vision to make this webpage possible.

S3. For the worldwide information on the subject and the basic fact we described in the main text, please see WHO webpage: <http://www.who.int/en/>

S4. The authors thank Hua Tao, Yingchun Zhou, Huiling Wei, Yu Bai, Si Chen, Da Chang, Ning Liu and Baoping Yan for their assistance.

### **Legends to Supporting Online Figures**

**Figure S1.** Phylogenetic trees of the 8 genomic segments of the four isolates in comparison with other known sequences. It clearly shows that the four isolates are clustered in one group (labeled in red italics) in any of the 8-gene segment analysis, indicating the same virus was involved in the outbreak. Most of the segments (5/8) are closely related to a Hong Kong peregrine falcon isolate (Pf/HK/D0028/04) but the HA and NA segments are closer to a Shantou isolate (Ck/ST/4231/03) and the NP segment closer to a Japan isolate (Ck/Yamaguchi/7/04). Abbreviation used: Tg, tiger; Ld, leopard; Ck, chicken; Dk, duck; Gs, goose; Pf, peregrine falcon.

**Figure S2.** Multiple alignment of the amino acid region covering the amino acid 627 in PB2. The 627K was first seen from 1997 Hong Kong human isolates (Green,

HK/483/97 and HK/485/97) and became popular in the 2004 mammalian isolates (blue). Four isolates in this study are labeled in red and the most closely-related strain to our isolates PF/HK/D0028/04 (E at position 627) is labeled in brown. The viruses used for virulence analysis (K627E swap experiments) by Kawaoka and colleagues (main text reference 5) are labeled in green. The common reference strain (Gs/GD/1/96) in recent studies (main text reference 3, 6) is labeled in cyan.

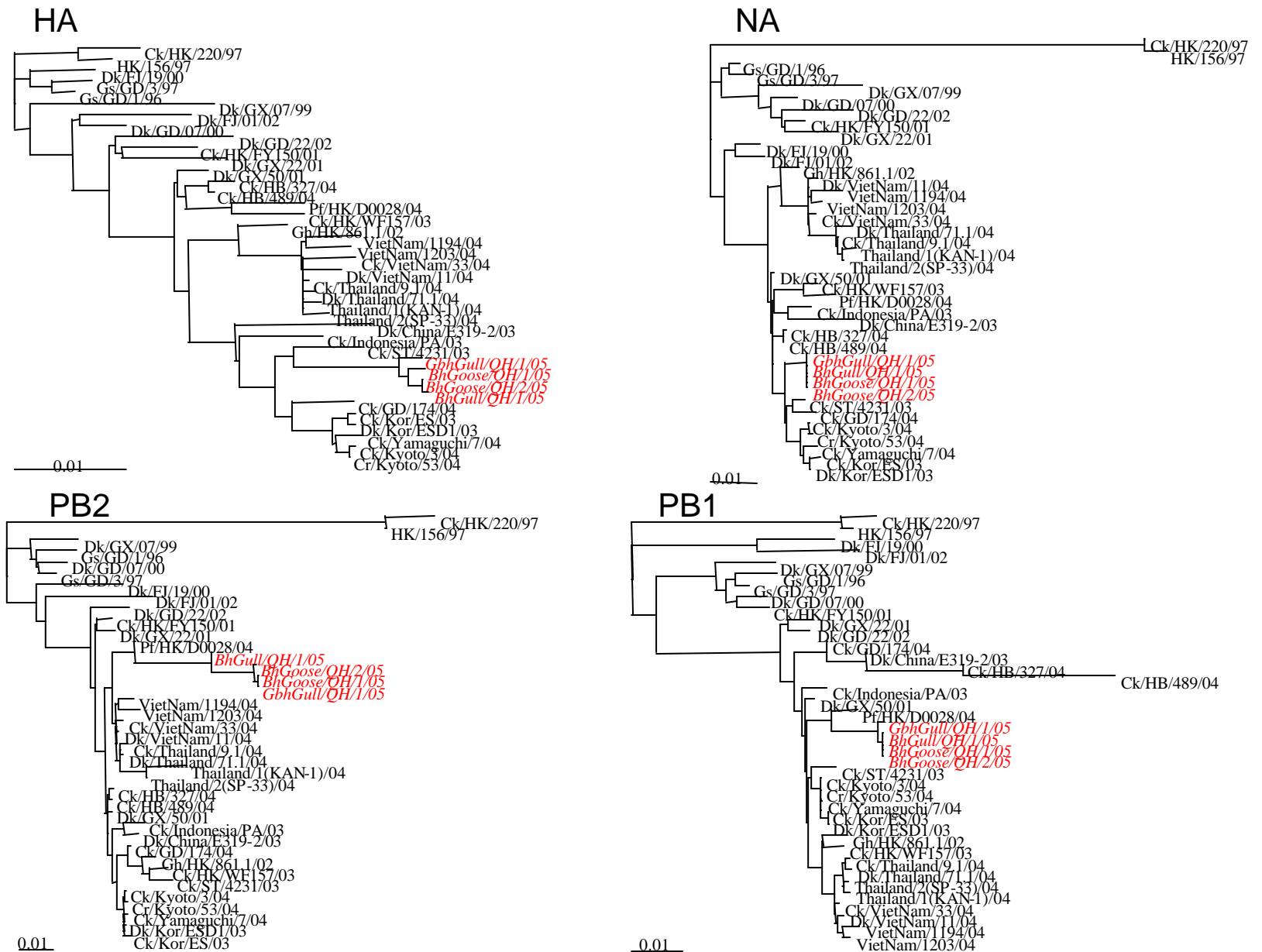


Figure S1-MS1115327

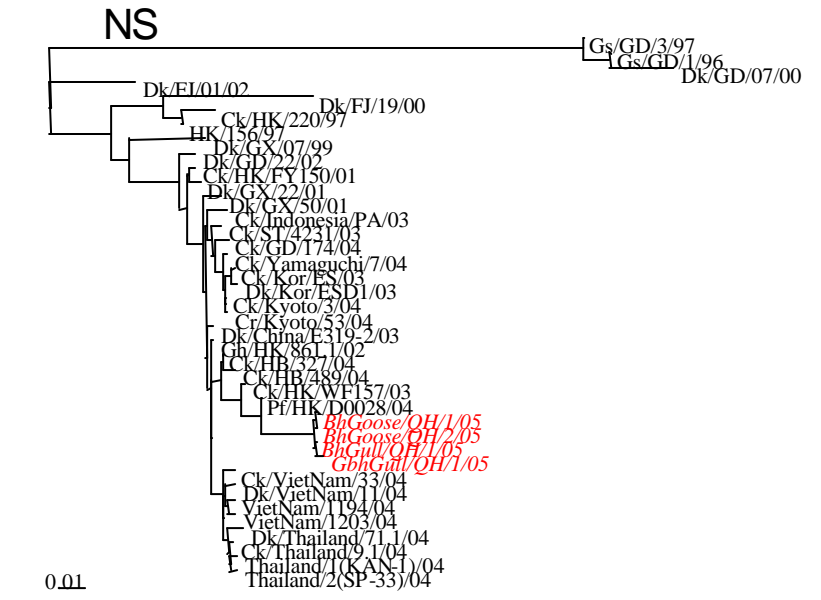
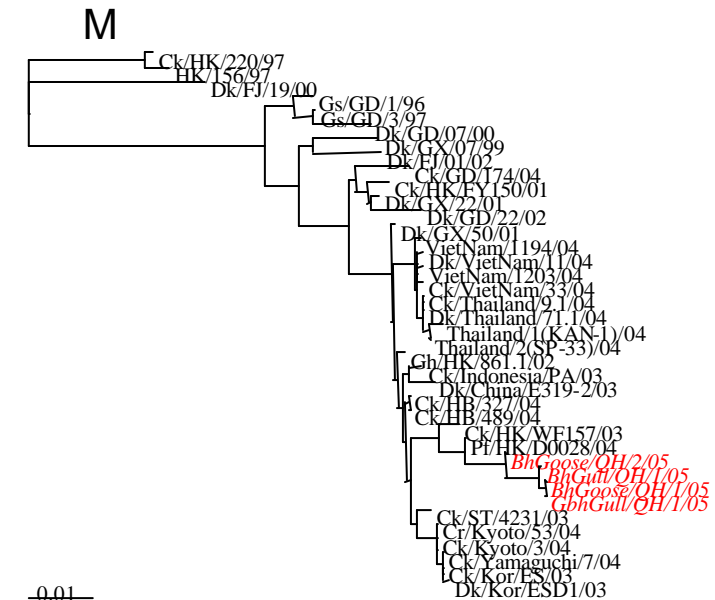
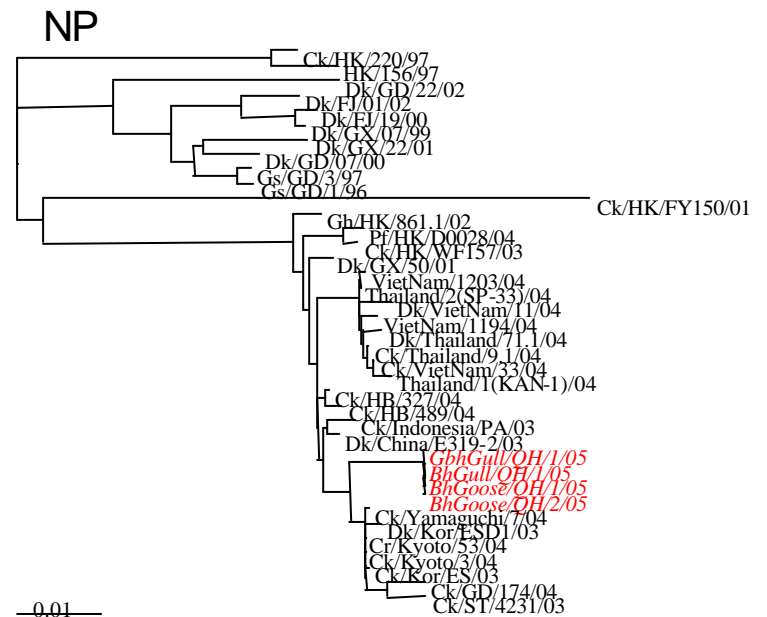
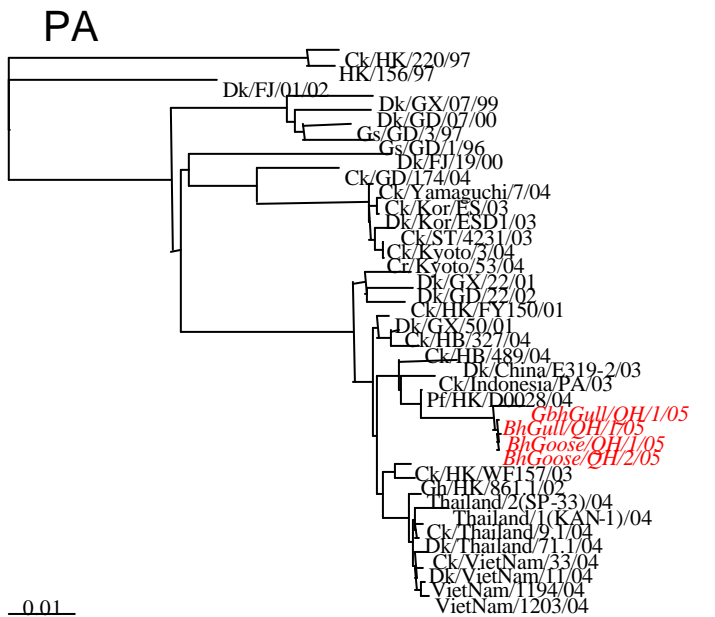


Fig. S1-MS1115273

# Amino Acid 627



BhGoose/QH/1/05	592	YSGFVRTLFQQMRDVLGTFDTVQIIKLLPFAAAPPKQSRMQFSSSLTVNVRGSGMRILIRG	651
BhGoose/QH/2/05	592	.....	651
BhGull/QH/1/05	592	.....	651
GbhGull/QH/1/05	592	.....	651
Tg/Thailand/CU-T3/04	592	.....V..	651
Tg/Thailand/CU-T4/04	592	.....V..	651
Tg/Thailand/CU-T5/04	592	.....V..	651
Tg/Thailand/CU-T6/04	592	.....V..	651
Tg/Thailand/CU-T7/04	592	.....V..	651
Tg/Thailand/CU-T8/04	592	.....V..	651
Tg/Thailand/Ti-1/04	592	.....E.....V..	651
Ld/Thailand/Leo-1/04	592	.....L.....V..	651
Thailand/2(SP-33)/04	592	.....V..	651
Thailand/5(KK-494)/04	592	.....V..	651
Thailand/1(KAN-1)/04	592	.....E.....V..	651
Viet Nam/1194/04	592	.....V..	651
Viet Nam/1203/04	592	.....V..	651
Viet Nam/3046/04	592	.....E.....V.V..	651
Viet Nam/3062/04	592	.....V..	651
Pf/HK/D0028/04	592	.....E.....	651
HK/212/03	592	.....E.....V..	651
HK/213/03	592	.....E.....V..	651
HK/97/98	592	.....E.....V..	651
HK/483/97	592	.....V..	651
HK/485/97	592	.....V..	651
HK/486/97	592	.....E.....V..	651
Gs/GD/1/96	592	.....EP.....V..	651

Fig. S2-MS1115273