ORIGINAL ARTICLE

Epidemiological situation and genetic analysis of H7N9 influenza viruses in Shanghai in 2013

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Abstract The first reported human case of H7N9 influenza virus infection in Shanghai prompted a survey of local avian strains of influenza virus, involving the analysis of a large number of samples taken from poultry, wild birds, horses, pigs, dogs and mice. Seven instances of H7N9 virus infection were identified by real-time RT-PCR (1.47 % of samples), all in chickens sold in live-poultry markets. H7N9 antibody was not detected in serum samples collected from local poultry farms since 2006. The two H7N9 virus strains in the live-poultry markets and one H9N2 virus strain in the same market were genetically characterized. Resequencing of two of the seven isolates confirmed that they closely resembled H7N9 virus strains characterized elsewhere. Various strains co-exist in the same market, presenting a continuing risk of strain reassortment. The closure of live-poultry markets has been an effective short-term means of minimizing human exposure to H7N9 virus.

Introduction

A re-assorted H7N9 strain of avian influenza virus, which emerged in eastern China in February 2013, has been associated with human mortality, but there is as yet no evidence of its having any deleterious effect on the health of either poultry or wild birds. H7-subtype influenza has occurred in poultry, wild birds and horses [1, 2]. The Shanghai region lies along the East Asia–Australia bird migration route and provides numerous habitats for wild birds. In addition, the region has a high domestic pig population, providing the opportunity for the generation of new pandemic strains; influenza virus has also been isolated from the dog population [3]. The prevalence of this new H7N9 virus in these animal hosts was determined in the surveillance. H7N9 infectious virus and viral RNA were detected by real-time RT-PCR, and H7N9 influenza antibodies were detected using a hemagglutination inhibition (HI) assay. Understanding the host antibody response is crucial for predicting disease severity and for vaccine development [4]. The antibody test is complementary to pathogen detection. The H7N9 strain is believed to have arisen following a number of genetic reassortment events. Its HA gene probably arose within virus populations that had adapted to ducks, while its NA gene is thought to have evolved in migratory birds. The six other genes harbored by the virus were related to two distinct H9N2 avian influenza virus strains, both of which have been recovered from chickens [5]. The present study set out to characterize, at both the sequence and phylogenetic level, two recently isolated H7N9 virus strains (A/chicken/Shanghai/017/2013 and A/chicken/Shanghai/019/2013).

Materials and methods

Ethics statement

This study was conducted according to the animal welfare guidelines laid down by the World Organization for

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Fig. 1 Geographical distribution of wild-bird samples in the Shanghai region. The seven red dots show the sampling sites in wild-bird habitats
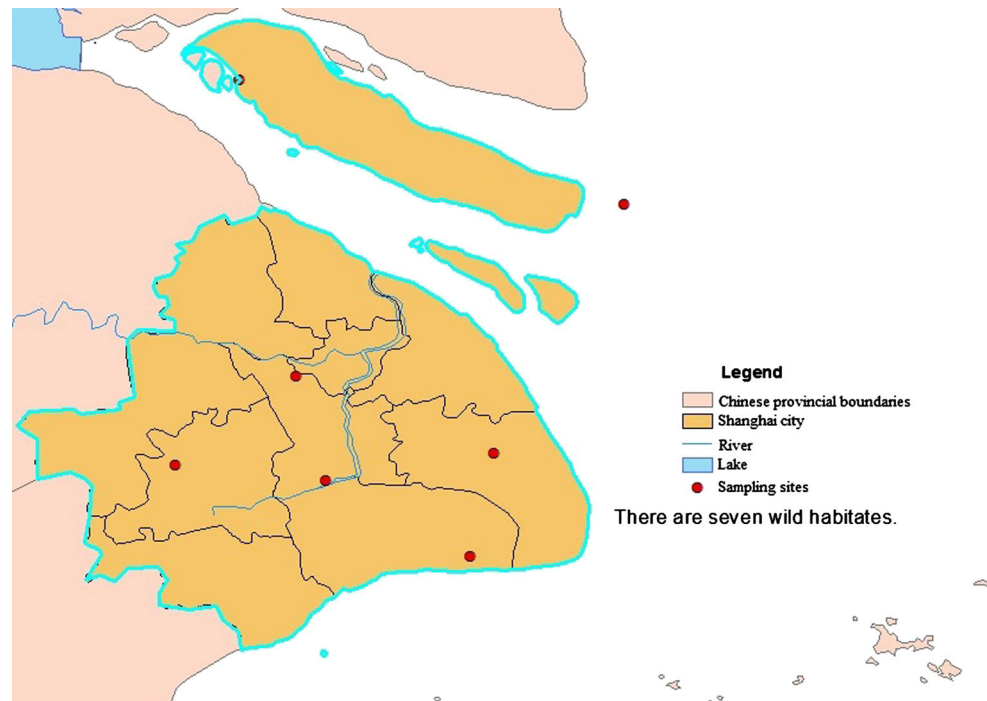

Table 1 The numbers of swab and tissue samples collected in the Shanghai region between March and the end of April 2013

| Types of samples	Number of samples									
	Local poultry farms	Live-poultry markets	Poultry slaughterhouses	Wild habitats	Horses raised in parks and some equine farms	Pig slaughterhouses	Dogs	Birds through the city	Mice captured in the live-poultry markets	Poultry samples from surroundings linked to confirmed human cases
Oropharynx and cloaca swabs	3926	476	180	N	N	N	N	N	N	365
Nasal swabs	N	N	N	N	81	769	22	N	N	N
Faeces	N	N	N	235	N	N	N	131	N	N
Tissue samples	N	N	N	N	N	N	N	N	15	N

N, no samples

Animal Health and was approved by the Shanghai Municipal Commission of Agriculture (Permit number: 2013 [18]). Permission was obtained from the owners of the domesticated animals sampled. GPS coordinates referring to the sites of collection from wild birds are given in Fig. 1. Sampling of poultry was based on the LaoDu Professional Cooperative and the DaYing YaYa slaughterhouse. The abattoirs from which pig-based samples were taken consisted of two in QingPu district (Shanghai) (FuRun Butchering Processing Co., Ltd. and QingPu slaughterhouse), two in JiaDing district (Shanghai) (WuFeng ShangShi Food Co., Ltd. and Nanxiang YuNong slaughterhouse), two in Jinshan district (Shanghai) (CaoJing slaughterhouse and FengJing slaughterhouse), two in ChongMing district (Shanghai) (Mingzhu River Meat Food Co., Ltd. and Meat Food Co., Ltd,), one in PuDong district (Shanghai) (HeLi slaughterhouse), two in FengXian district (Shanghai) (FengXian ShenLan slaughterhouse and AiSen Meat Food Co., Ltd.), and one in Songjiang district (Shanghai) (Songjiang Sijing slaughterhouse).

Detection of samples

Swab and tissue samples were subjected to H5, H7 and H9 subtyping based on real-time RT-PCR assays. The three real-time RT-PCR detection kits were from Beijing

Fig. 2 **1** Detection and H5, H7 and H9 subtyping results for all swab and tissue samples tested by real-time RT-PCR. **2** Detection and H5, H7 and H9 subtyping results of all samples from local poultry farms and live-poultry markets tested by real-time RT-PCR
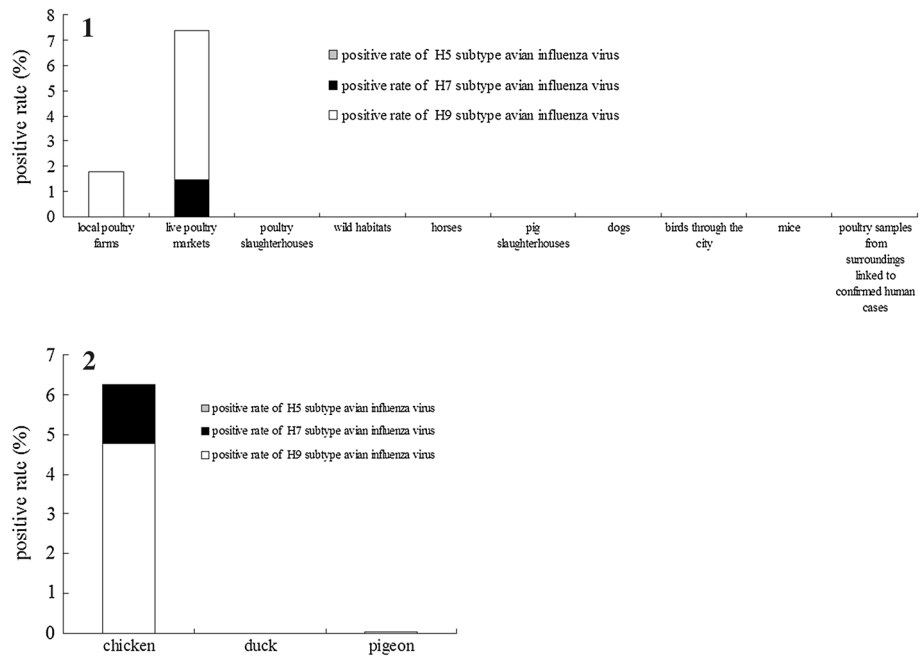
Shengkeshangyi Technology Co., Ltd. Samples testing positive for H7 were screened for H7N9 subtypes, again using a real-time RT-PCR assay kit from Shanghai Zhijiang Bio-Tech Co., Ltd. Serum samples were subjected to an H7 subtype HI test based on chicken red blood cells. H7 antigens from A/pigeon/Shanghai/S1421/2013(H7N9) derived from the emergent H7N9 avian influenza virus and H7-positive chicken sera were used as controls. These reagents were obtained from Harbin Weike Biotechnology Development Co., Ltd., the Chinese Academy of Agricultural Sciences (CAAS), Harbin Veterinary Research Institute (Harbin, China). A serum sample was considered positive when the HI titer was ≥16 according to our national standard [6].

Sequence and phylogenetic analysis

Each pair of tracheal and cloacal swabs was incubated in 2 mL of minimal essential medium containing 2000 U of penicillin and 2000 U of streptomycin per mL. Viral RNA was extracted using a QIAmp viral RNA Mini Kit (QIA-GEN, Hilden, Germany). For the H7N9 strains A/chicken/Shanghai/017/2013(H7N9) and A/chicken/Shanghai/019/2013(H7N9), reverse transcription was performed using PathAmp™ FluA Preamplification Reagents (Life Technologies, USA), and amplification was carried out using PathAmp™ FluA Reagents. Sequencing was done using an Ion PGM™ Sequencing 200 Kit v2, together with an Ion 316™ Chip. For the H9N2 strain A/chicken/Shanghai/020/

2013, reverse transcription was done by random priming. The RT-PCR reactions were directed at the eight genes HA, NA, M, NS, NP, PA, PB1 and PB2 (primer sequences available on request). The resulting amplicons were purified using a QIAquick PCR purification kit (QIAGEN), cloned into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced. Sequence data were compiled and edited using the Lasergene sequence analysis software package (DNASTAR Inc., Madison, WI, USA). Multiple sequence alignments were based on Clustal W software, and unrooted phylogenetic trees were generated by applying the distance-based neighbor-joining method, as implemented in MEGA 6.06 software. Bootstrap probabilities were based on 1,000 replicates. All of the nucleotide sequences obtained have been deposited in the GenBank database under accession numbers KF542876-91 and KF500977-84. All of the gene sequences of influenza viruses used in the phylogenetic analysis were obtained from GISAID Epiflu database (www.gisaid.org).

Results and discussion

Up to April 17, 2013, the sample collection comprised 6,200 entries, of which 3,926 had been obtained from local poultry farms, 476 from live-poultry markets, 180 from poultry slaughterhouses, 235 from wild-bird faeces, 81 from horse nasal swabs, 769 from pig nasal swabs, 22 from dog nasal

Fig. 3 Location of live-poultry markets in Shanghai
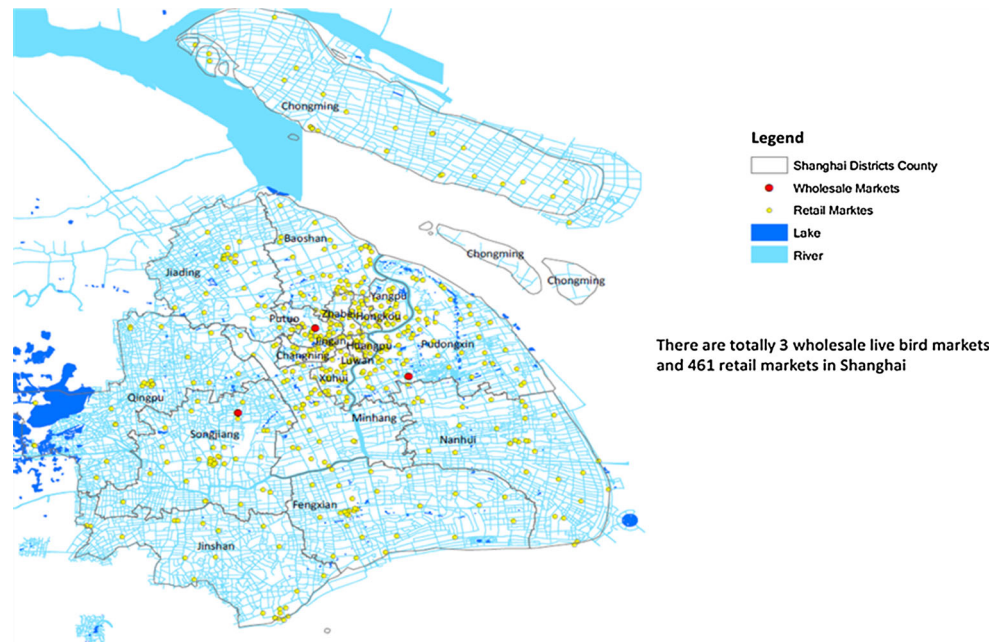
Table 2 Nucleotide sequence identity of the two novel H7N9 strain genes and their most closely related sequences

Viral gene	Closest influenza virus relative	nt identity
A/chicken/shanghai/017/2013-HA7	A/duck/Zhejiang/12/2011(H7N3)	96 %
A/chicken/shanghai/019/2013-HA7	A/duck/Zhejiang/12/2011(H7N3))	96 %
A/chicken/shanghai/017/2013-NA9	A/mallard/Czech Republic/13438-29 K/2010(H11N9)	96 %
A/chicken/shanghai/019/2013-NA9	A/mallard/Czech Republic/13438-29 K/2010(H11N9)	96 %
A/chicken/shanghai/017/2013-M	A/chicken/Zhejiang/607/2011(H9N2)	98 %
A/chicken/shanghai/019/2013-M	A/chicken/Zhejiang/607/2011(H9N2)	98 %
A/chicken/shanghai/017/2013-NP	A/chicken/Zhejiang/611/2011(H9N2)	99 %
A/chicken/shanghai/019/2013-NP	A/chicken/Zhejiang/611/2011(H9N2)	99 %
A/chicken/shanghai/017/2013-NS	A/chicken/Dawang/1/2011(H9N2)	99 %
A/chicken/shanghai/019/2013-NS	A/chicken/Dawang/1/2011(H9N2)	99 %
A/chicken/shanghai/017/2013-PA	A/brambling/Beijing/16/2012(H9N2)	99 %
A/chicken/shanghai/019/2013-PA	A/brambling/Beijing/16/2012(H9N2)	99 %
A/chicken/shanghai/017/2013-PB1	A/chicken/Jiangsu/Q3/2010(H9N2)	99 %
A/chicken/shanghai/019/2013-PB1	A/chicken/Hebei/1102/2010(H5N2)	99 %
A/chicken/shanghai/017/2013-PB2	A/brambling/Beijing/16/2012(H9N2)	99 %
A/chicken/shanghai/019/2013-PB2	A/brambling/Beijing/16/2012(H9N2)	99 %

swabs, 131 from bird faeces collected within the city, 15 from mice captured in live-poultry markets and 365 from poultry located in areas associated with outbreaks of human influenza (Table 1). The chicken, duck and pigeon samples were taken from both the oropharynx and the cloaca. An additional 1,177 poultry serum samples were collected over the period 2006-2012 from local poultry farms (1,073 chicken and 104 duck, of which 50 dated to 2006, 35 to 2007, 50 to 2008, 50 to 2009, 50 to 2010, 166 to 2011 and 776 to 2012). During March and April 2013, serum was sampled from 2,609 chickens, 50 ducks, 70 pigeons and 20 rare birds from a number of local poultry farms.

Fig. 4 Phylogenetic analysis of the HA (a) and NA (b) genes of the novel H7N9 influenza viruses isolated from chickens in Shanghai, China. A neighbor-joining tree was constructed using MEGA version 6.06. Bootstrap values obtained from 1000 replicates are shown (≥60 %). Viruses characterized in the present study are indicated by a filled triangle. The HA and NA sequences for the recent novel H7N9 viruses were downloaded from the database of the Global Initiative on Sharing All Influenza Data (GISAID). HA, haemagglutinin; NA, neuraminidase
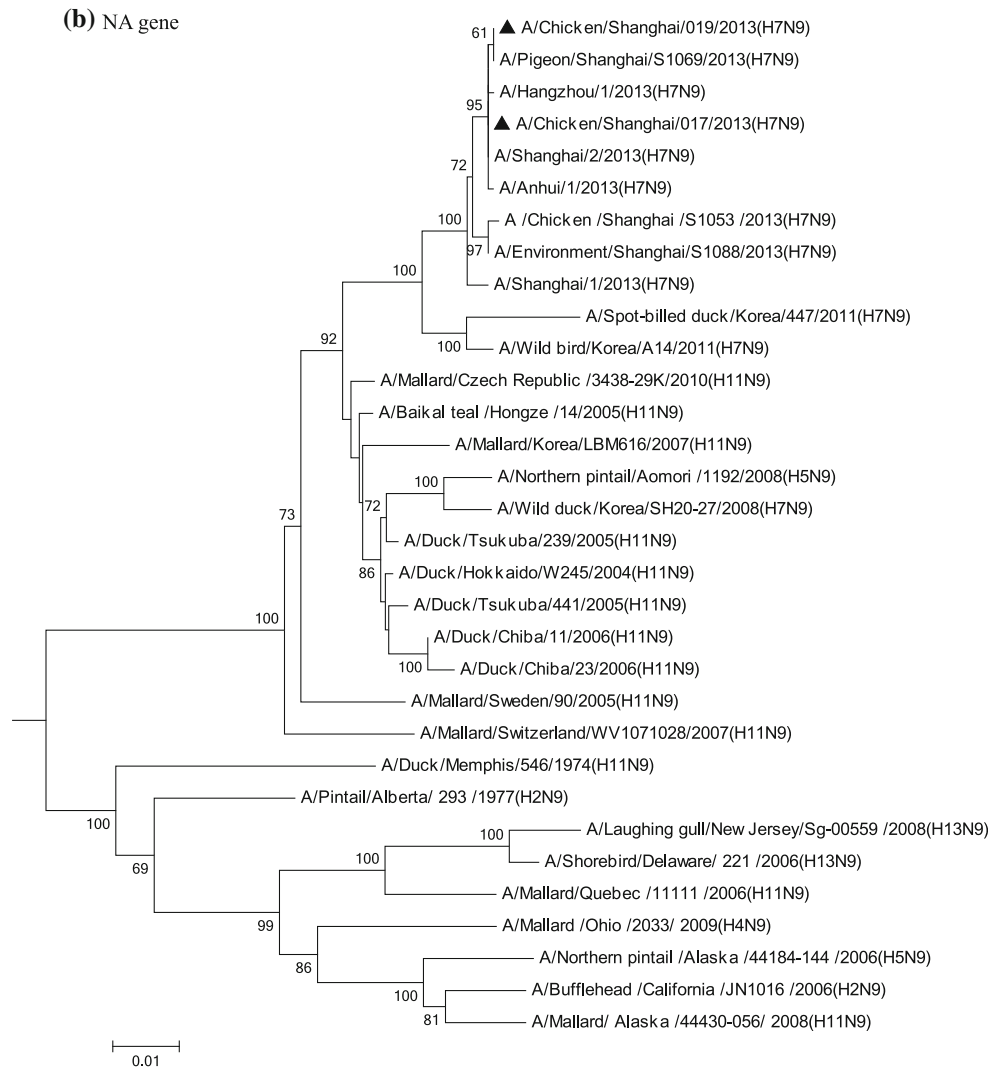
(a) HA gene

(b) NA gene
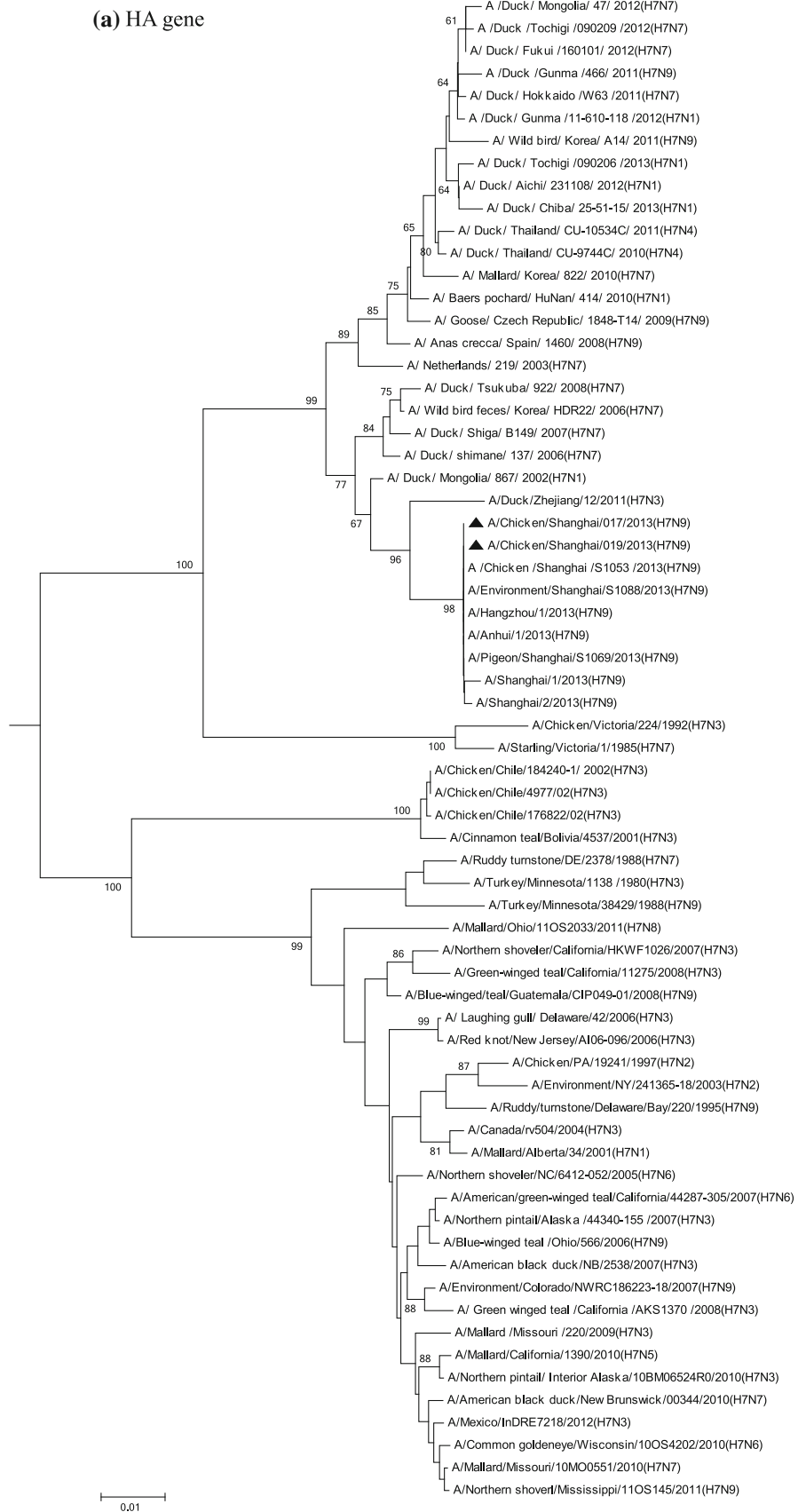
Fig. 4 continued

Seven (1.47 %) of the chicken samples (all obtained from live-poultry markets) proved to be positive for H7N9 virus by real-time RT-PCR. We tested 4402 samples from local poultry farms and live-poultry markets, including 2029 chicken samples, 151 duck samples and 2222 pigeon samples. There were 98 positive samples (97 chicken samples and one pigeon sample) of H9 subtype influenza virus, including 70 samples from local poultry farms and 28 samples from live-poultry markets, as determined by real-time RT-PCR. It was noted that there were three mixed infections of H7- and H9-subtype influenza viruses. We did not detect any positive samples of H5-subtype influenza virus. The detection results are shown in Figures 2-1 and 2-2.

The Shanghai region operates three wholesale live-poultry markets and 461 retail markets, which are geographically widely distributed (Fig. 3). It is presumed that H7N9 virus arose in some of these live-poultry markets. Following the closure of all of these markets between the end of April and the end of June 2013, no human cases of H7N9 virus were reported. After their reopening, the first reported human case, according to the Shanghai Municipal Health & Family Planning Commission, was in January 2014. Although closure of such markets appears to have been an effective short-term means of minimizing human exposure to H7N9 virus, a multisectoral approach is clearly required to reduce the risk of infection of the human population in the medium to long term.
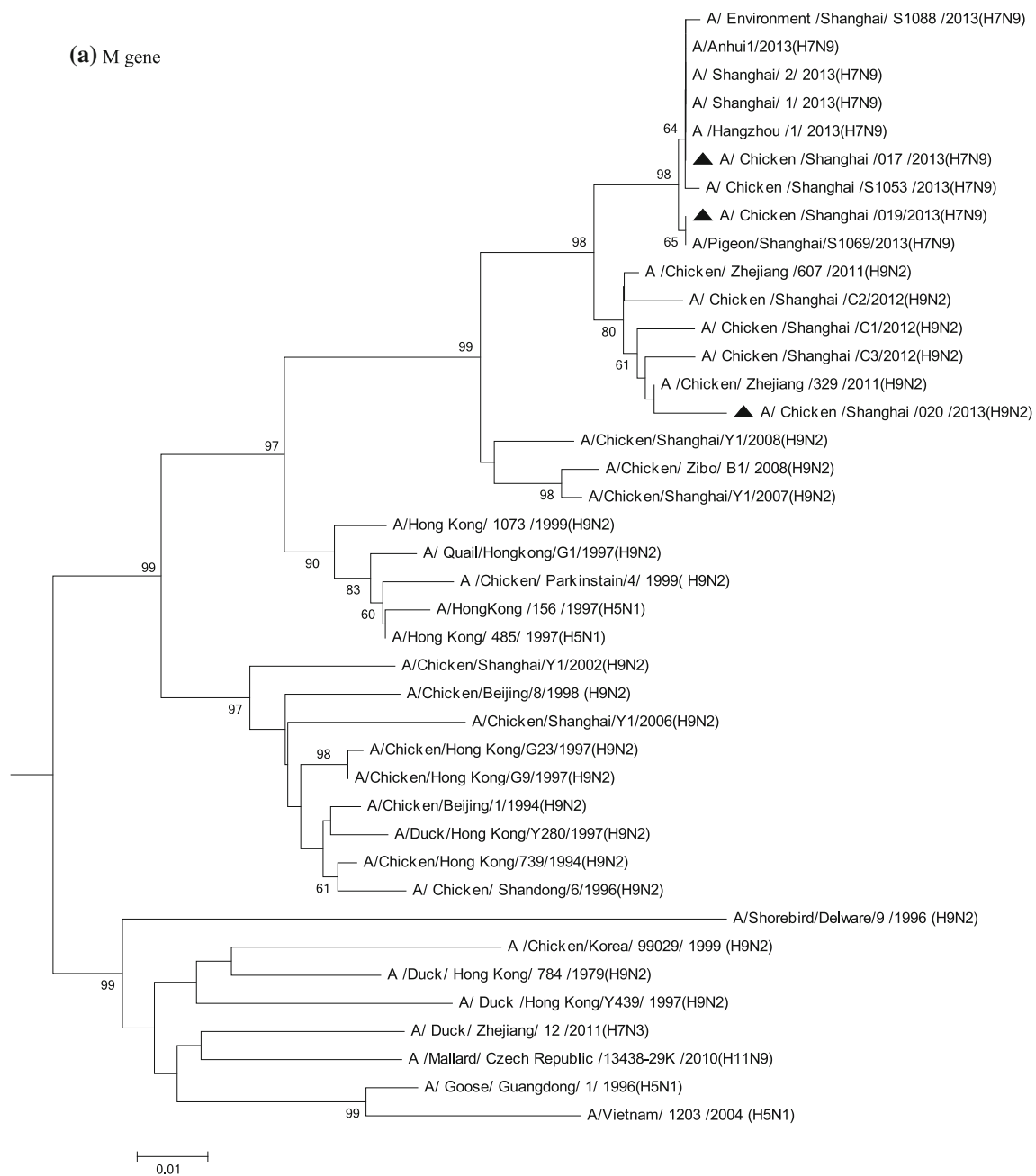
(a) M gene

A/ Environment /Shanghai/ S1088 /2013(H7N9)
A/Anhui1/2013(H7N9)
A/ Shanghai/ 2/ 2013(H7N9)
A/ Shanghai/ 1/ 2013(H7N9)
64 ⎡ A /Hangzhou /1/ 2013(H7N9)
98 ⎣ ▲ A/ Chicken /Shanghai /017 /2013(H7N9)
A/ Chicken /Shanghai /S1053 /2013(H7N9)
▲ A/ Chicken /Shanghai /019/2013(H7N9)
65 A/Pigeon/Shanghai/S1069/2013(H7N9)
98 A /Chicken/ Zhejiang /607 /2011(H9N2)
A/ Chicken /Shanghai /C2/2012(H9N2)
80 A/ Chicken /Shanghai /C1/2012(H9N2)
A/ Chicken /Shanghai /C3/2012(H9N2)
61 A /Chicken/ Zhejiang /329 /2011(H9N2)
▲ A/ Chicken /Shanghai /020 /2013(H9N2)
99 A/Chicken/Shanghai/Y1/2008(H9N2)
A/Chicken/ Zibo/ B1/ 2008(H9N2)
98 A/Chicken/Shanghai/Y1/2007(H9N2)
97 A/Hong Kong/ 1073 /1999(H9N2)
90 A/ Quail/Hongkong/G1/1997(H9N2)
83 A /Chicken/ Parkinstain/4/ 1999(H9N2)
60 A/HongKong /156 /1997(H5N1)
A/Hong Kong/ 485/ 1997(H5N1)
99 A/Chicken/Shanghai/Y1/2002(H9N2)
A/Chicken/Beijing/8/1998 (H9N2)
97 A/Chicken/Shanghai/Y1/2006(H9N2)
98 A/Chicken/Hong Kong/G23/1997(H9N2)
A/Chicken/Hong Kong/G9/1997(H9N2)
A/Chicken/Beijing/1/1994(H9N2)
A/Duck/Hong Kong/Y280/1997(H9N2)
A/Chicken/Hong Kong/739/1994(H9N2)
61 A/ Chicken/ Shandong/6/1996(H9N2)
A/Shorebird/Delware/9 /1996 (H9N2)
A /Chicken/Korea/ 99029/ 1999 (H9N2)
A /Duck/ Hong Kong/ 784 /1979(H9N2)
A/ Duck /Hong Kong/Y439/ 1997(H9N2)
99 A/ Duck/ Zhejiang/ 12 /2011(H7N3)
A /Mallard/ Czech Republic /13438-29K /2010(H11N9)
A/ Goose/ Guangdong/ 1/ 1996(H5N1)
99 A/Vietnam/ 1203 /2004 (H5N1)

0.01

Fig. 5 Phylogenetic analysis of the M(a), NP(b), NS(c), PA(d), PB1(e) and PB2(f) genes of the novel H7N9 influenza viruses. A neighbor-joining tree was constructed using MEGA version 6.06. Bootstrap values obtained from 1000 replicates are shown (≥60 %). Viruses characterized in the present study are indicated by a filled triangle

As the H7N9 virus has not been detected in any wild-bird samples, the likelihood of H7N9 virus transmission to humans from the wild bird population was concluded to be very small. The HI test showed that all of the serum samples lacked the H7 subtype antibody, so there remains no firm evidence for any H7N9 virus infection in the local poultry industry in Shanghai.

Two of the H7N9 virus samples recovered from chickens were sequenced, along with an H9N2 virus strain isolated from the same live-poultry market. Various strains co-exist
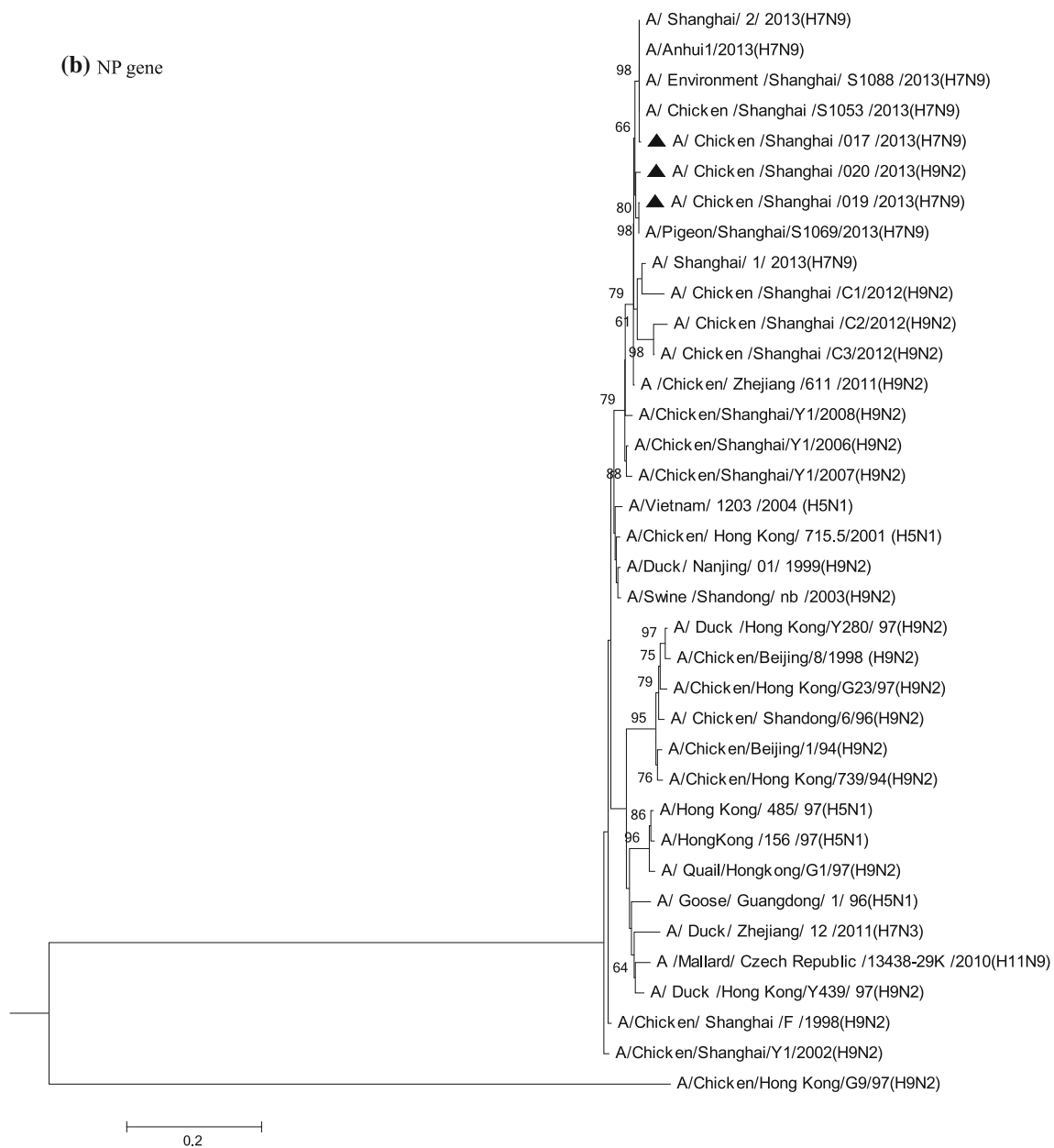
(b) NP gene

Fig. 5 continued

in the same market, presenting a continuing risk of strain reassortment. The sequences of the two H7N9 virus samples (A/chicken/Shanghai/017/2013(H7N9) and A/chicken/Shanghai/019/2013(H7N9)) were very similar to those of other characterized H7N9 virus strains [7]. Nucleotide identities of the eight re-sequenced genes (HA, NA, M, NP, NS, PA, PB1 and PB2) are summarized in Table 2. The HA and NA sequences of the two H7N9 virus isolates were 99.2 %–100.0 % homologous to those of H7N9 virus strains isolated recently from human patients (A/Anhui/1/2013, A/Shanghai/1/2013 and A/Shanghai/2/2013) and were also very similar to those of three isolates sampled from live poultry markets in Shanghai (A/chicken/Shanghai/S1053/2013, A/environment/Shanghai/S1088/2013 and A/pigeon/Shanghai/ S1069/2013). A phylogenetic analysis showed that all of the HA sequences clustered within a single clade, as did all of the NA sequences (Fig. 4). A similar analysis directed at five of the other six gene
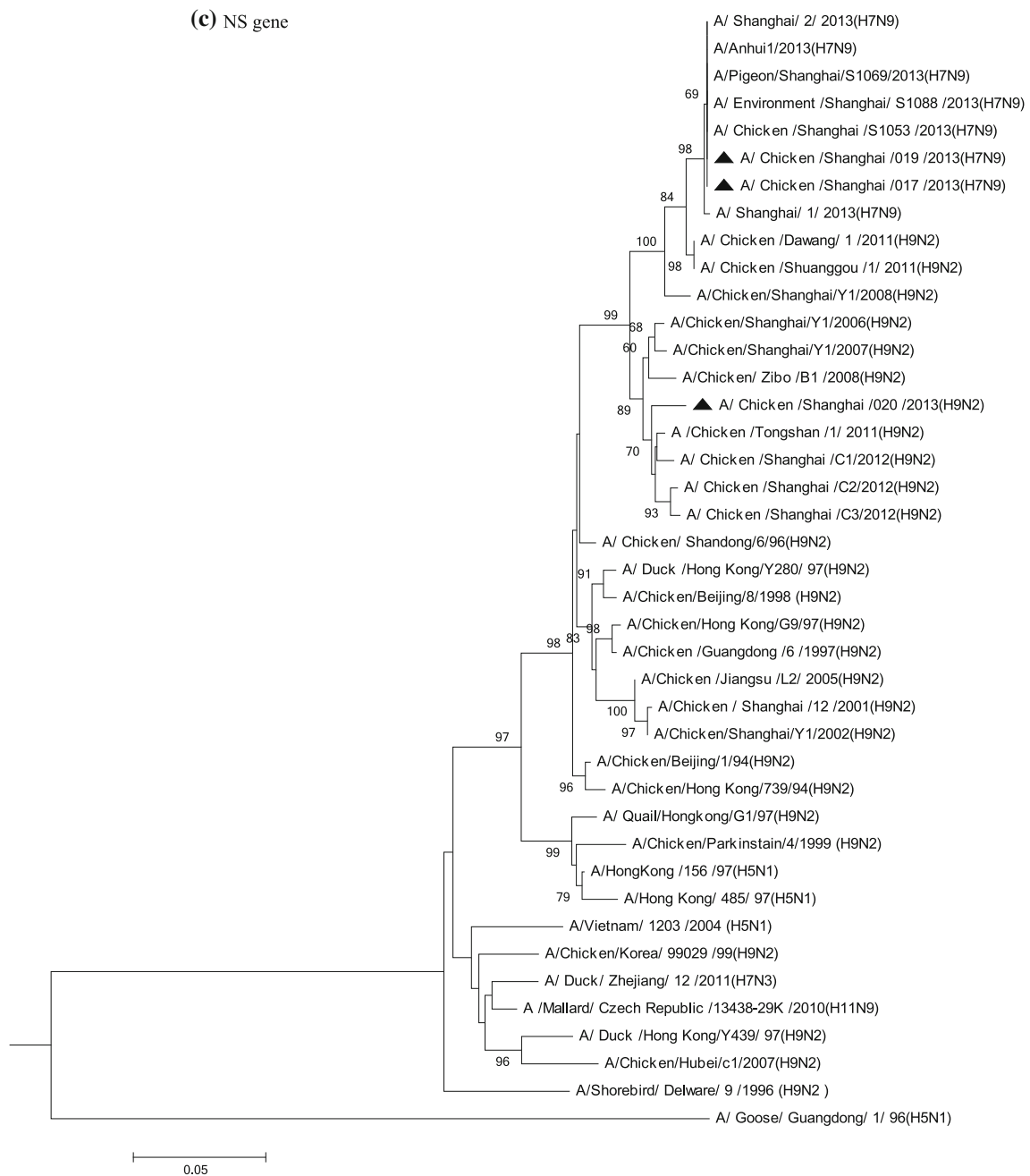
(c) NS gene

Fig. 5 continued

sequences (M, NP, NS, PA, PB2) revealed that the two new H7N9 virus strains belonged to the same lineage as other H7N9 virus strains, but variation in the PB1 gene sequence suggested an independent origin (Fig. 5).

The HA cleavage sequence of each of the two H7N9 virus strains features a single basic amino acid (EIPKGR*GL, where * indicates the cleavage site), which suggested that the two strains are unlikely to be highly pathogenic in an avian host [8, 9]. The two mutations harbored by the strains (Gly186Val and Gln226Leu) are predicted to increase the virus' binding affinity to human 2,6 sialylated glycans [10]. Both strains have retained the
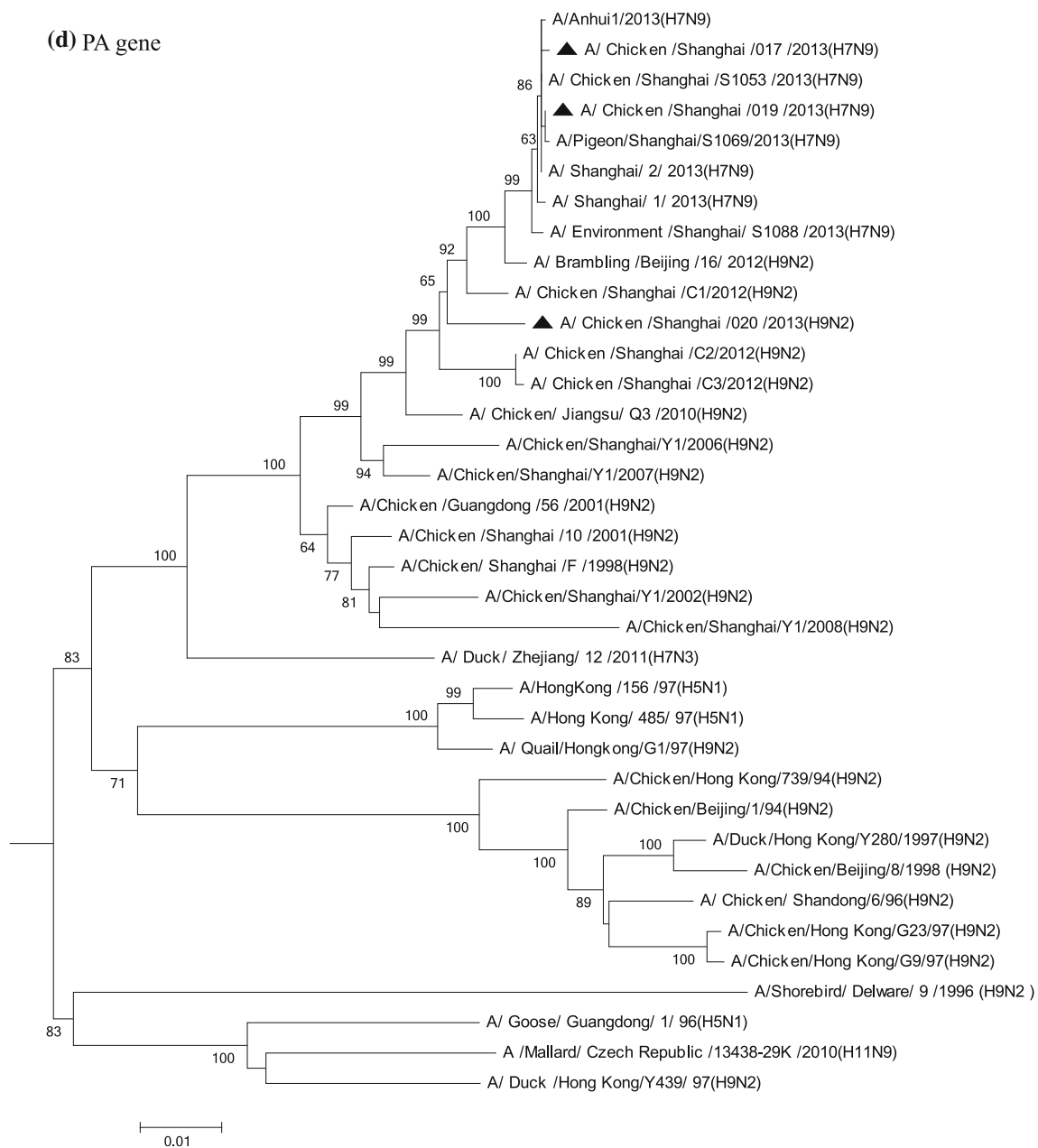
(d) PA gene

A/Anhui1/2013(H7N9)
▲ A/ Chicken /Shanghai /017 /2013(H7N9)
A/ Chicken /Shanghai /S1053 /2013(H7N9)
▲ A/ Chicken /Shanghai /019 /2013(H7N9)
A/Pigeon/Shanghai/S1069/2013(H7N9)
A/ Shanghai/ 2/ 2013(H7N9)
A/ Shanghai/ 1/ 2013(H7N9)
A/ Environment /Shanghai/ S1088 /2013(H7N9)
A/ Brambling /Beijing /16/ 2012(H9N2)
A/ Chicken /Shanghai /C1/2012(H9N2)
▲ A/ Chicken /Shanghai /020 /2013(H9N2)
A/ Chicken /Shanghai /C2/2012(H9N2)
A/ Chicken /Shanghai /C3/2012(H9N2)
A/ Chicken/ Jiangsu/ Q3 /2010(H9N2)
A/Chicken/Shanghai/Y1/2006(H9N2)
A/Chicken/Shanghai/Y1/2007(H9N2)
A/Chicken /Guangdong /56 /2001(H9N2)
A/Chicken /Shanghai /10 /2001(H9N2)
A/Chicken/ Shanghai /F /1998(H9N2)
A/Chicken/Shanghai/Y1/2002(H9N2)
A/Chicken/Shanghai/Y1/2008(H9N2)
A/ Duck/ Zhejiang/ 12 /2011(H7N3)
A/HongKong /156 /97(H5N1)
A/Hong Kong/ 485/ 97(H5N1)
A/ Quail/Hongkong/G1/97(H9N2)
A/Chicken/Hong Kong/739/94(H9N2)
A/Chicken/Beijing/1/94(H9N2)
A/Duck/Hong Kong/Y280/1997(H9N2)
A/Chicken/Beijing/8/1998 (H9N2)
A/ Chicken/ Shandong/6/96(H9N2)
A/Chicken/Hong Kong/G23/97(H9N2)
A/Chicken/Hong Kong/G9/97(H9N2)
A/Shorebird/ Delware/ 9 /1996 (H9N2)
A/ Goose/ Guangdong/ 1/ 96(H5N1)
A /Mallard/ Czech Republic /13438-29K /2010(H11N9)
A/ Duck /Hong Kong/Y439/ 97(H9N2)

0.01

Fig. 5 continued

avian-like PB2 627Glu and 701Asp residues, while the three human viruses A/Anhui/1/2013, A/Shanghai/1/2013 and A/Shanghai/2/2013 [11] each carry 627Lys, a property thought to contribute significantly to their pathogenicity and lethality in a human host [12, 13]. No resistance mutations associated with neuraminidase inhibition were present in the NA sequence of either strain [14], but both feature a deletion at positions 69–73 of the NA stalk region, which has been proposed as a marker for adaptation to terrestrial birds [15]. The peptide sequence of their M2 protein contains the Ser31Asn substitution, a diagnostic marker for resistance to the drug amantadine [16, 17]. The sequence of the PB1-F2 protein has been related to the high pathogenicity of both the 1918 Spanish flu and the H5N1
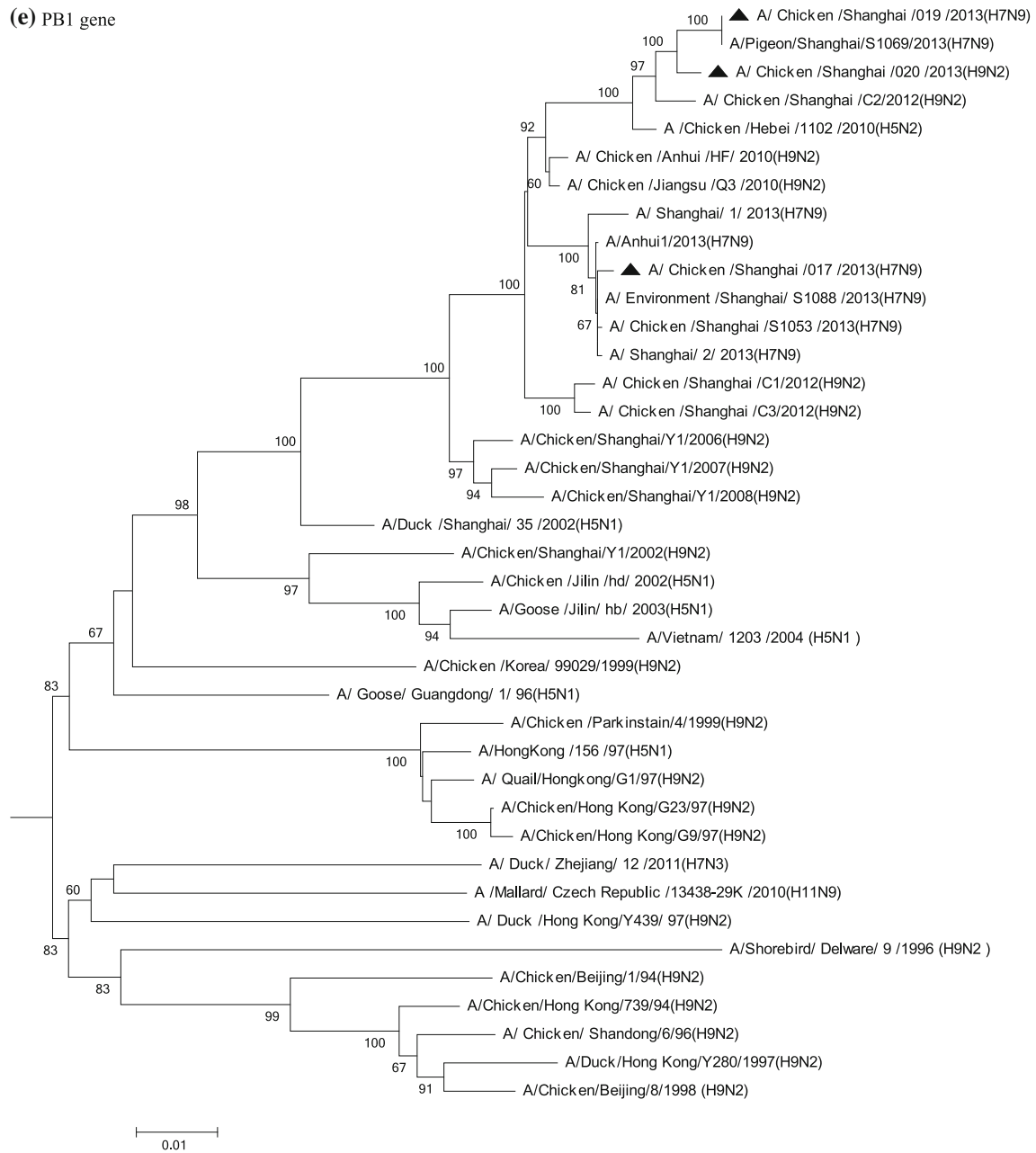
(e) PB1 gene

Fig. 5 continued

viruses [18]. A/chicken/Shanghai/019/2013 features a stop codon at position 26, whereas A/chicken/ hanghai/017/2013 encodes the full-length PB1-F2 protein. An early stop codon in PB1-F2 (as found in A/chicken/Shanghai/019/2013) [11]. These viruses were from the market of Shanghai and occurred in the same period. This suggests a possible epidemiological linkage of these two strains.

The sequences of the six internal genes of the H9N2 virus strain A/chicken/Shanghai/020/2013 shared an average identity of 95.3 %-99.3 % with their equivalents in the two new H7N9 virus strains (Fig. 5). This high level of identity can be interpreted as evidence that the internal genes in the H7N9 virus strains originated from recent H9N2 subtype influenza virus strains. The presence of mammalian adaptation mutations in the receptor-binding site of the novel H7N9 virus strains remains a cause for concern.
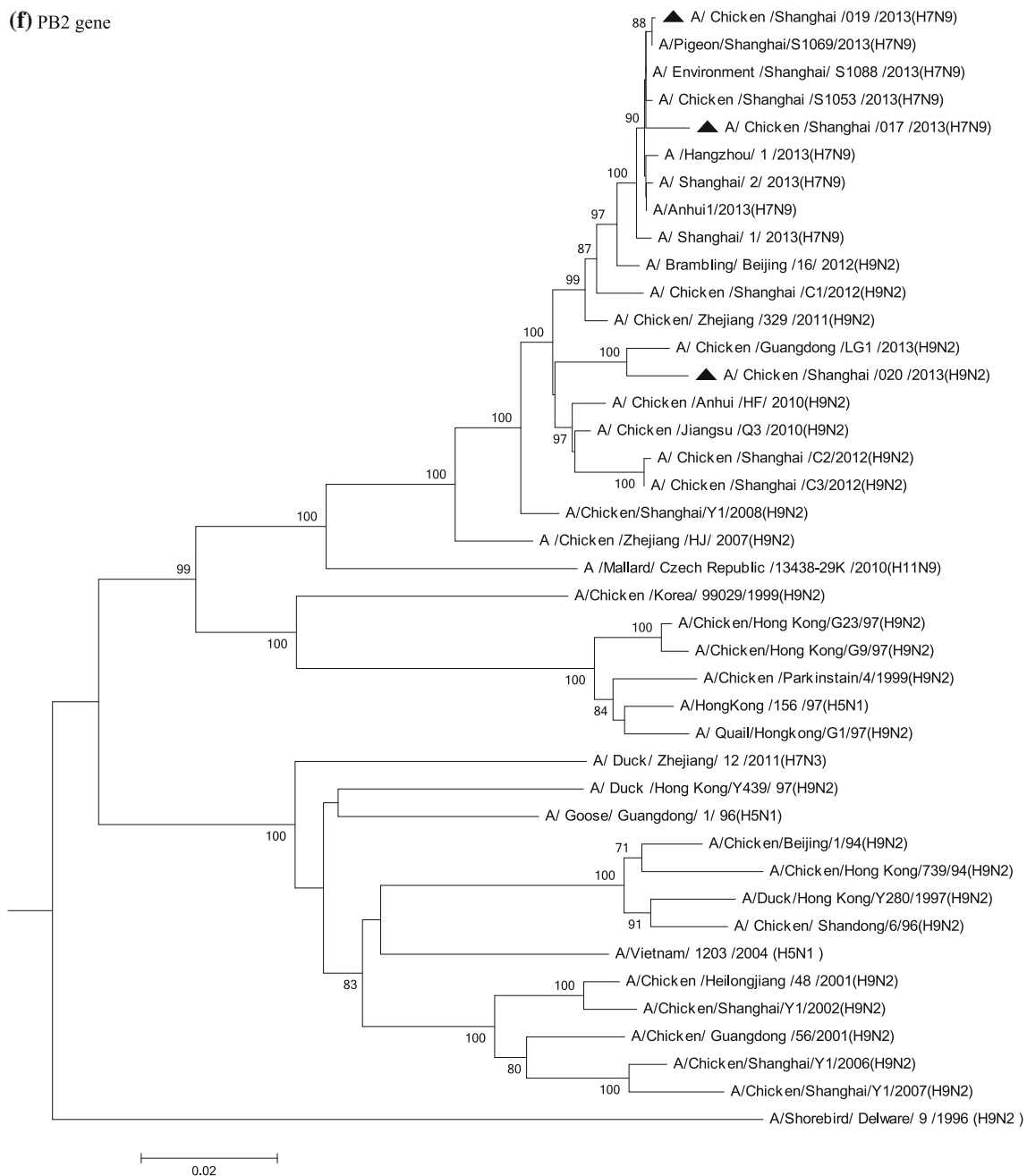
(f) PB2 gene

Fig. 5 continued

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