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BRIEF REPORT

Isolation and molecular characterization of an H5N1 swine influenza virus in China in 2015

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Abstract In 2015, an H5N1 influenza virus was isolated from a pig in Zhejiang Province, Eastern China. This strain was characterized by whole-genome sequencing with subsequent phylogenetic analysis. Phylogenetic analysis showed that all segments from this strain belonged to clade 2.3.2 and that it had received its genes from poultry influenza viruses in China. A Glu627Lys mutation associated with pathogenicity was observed in the PB2 protein. This strain was moderately pathogenic in mice and was able to replicate without prior adaptation. These results suggest that active surveillance of swine influenza should be used as an early warning system for influenza outbreaks in mammals.

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The H5 subtype influenza viruses, which have been identified in humans and other species, have attracted worldwide attention. As of 16 May 2017, 859 human cases of H5N1 avian influenza virus infection had been reported to WHO; 453 (52.7%) cases were fatal [1]. In May 2014, the first H5N6 avian influenza case emerged in China [2]. As pigs are susceptible to infection by both human and avian influenza viruses, they serve as 'mixing vessels' for novel influenza strains that have pandemic potential in human populations [3]. Previous surveillance results and published data from GenBank show that H5 viruses have infected swine in China [4–6]. To date, at least 14 H5N1 and two H5N6 viruses have been isolated from swine in mainland China. However, there is limited information regarding the molecular characteristics of H5N1 swine influenza viruses (SIVs) from Zhejiang Province. In this study, we genetically evaluated the relationship between the H5N1 strain [A/swine/Zhejiang/SW57/2015(H5N1) (ZJ-SW57)] isolated from swine in Zhejiang Province, Eastern China, and strains isolated from other Asian countries, and we determined the pathogenicity of H5N1 SIV in mice.

We inoculated lung samples (n = 60) from sick pigs into embryonated chicken eggs as described previously [7], and only one influenza virus (ZJ-SW57) was isolated from these samples. We extracted RNA from HA-positive allantoic fluid samples (n = 3) using TRIzol Reagent and subjected this RNA to reverse transcription polymerase chain reaction using a One-Step RNA PCR Kit (TaKaRa, China). We amplified all segments using primers and sequenced the fragments as described elsewhere [8]. All experiments with viruses were performed in a biosafety level 3 laboratory.

We sequenced eight gene segments of the SIV isolate: polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1), polymerase acidic protein (PA), hemagglutinin (HA), nucleocapsid protein (NP), neuraminidase (NA), matrix protein (M), and nonstructural protein (NS), comparing them with reference viruses [9–11]. We analyzed the sequences using BioEdit version 7.0.9.0 DNA software. We constructed phylogenetic trees using Molecular Evolutionary Genetics Analysis (MEGA) software version 6.0, applying the maximum-likelihood method and the Tamura–Nei model with bootstrap analysis (1,000 replicates) [12]. We deposited the nucleotide sequences into the GenBank database under the accession numbers MF969269-MF969276.

Phylogenetic analysis of all eight genes showed that the H5N1 virus clustered in the Eurasian lineage (Fig. 1 and Fig. S1). In particular, the HA gene of ZJ-SW57 is very closely related to H5 viruses that circulated in China from 2008 to 2012 (clade 2.3.2). One analysis suggests that ZJ-SW57 is most closely related to viruses isolated from Chinese poultry. Previous studies have shown that, since 2007, H5 viruses from clade 2.3.2 have been circulating widely among China's poultry and wild birds [13–15]. Therefore, our results provide further evidence supporting the active evolution of H5 viruses in China.

We found that the PB2, PA, HA, NA, M and NS genes of ZJ-SW57 are closely related to those of A/goose/Zhejiang/727098/2014(H5N1), while the PB1 and NP genes are closely related to those of other H5N1 viruses: A/chicken/Jiangsu/927/2013(H5N1) and A/pigeon/Zhejiang/727097/2014(H5N1), respectively (Table S1).

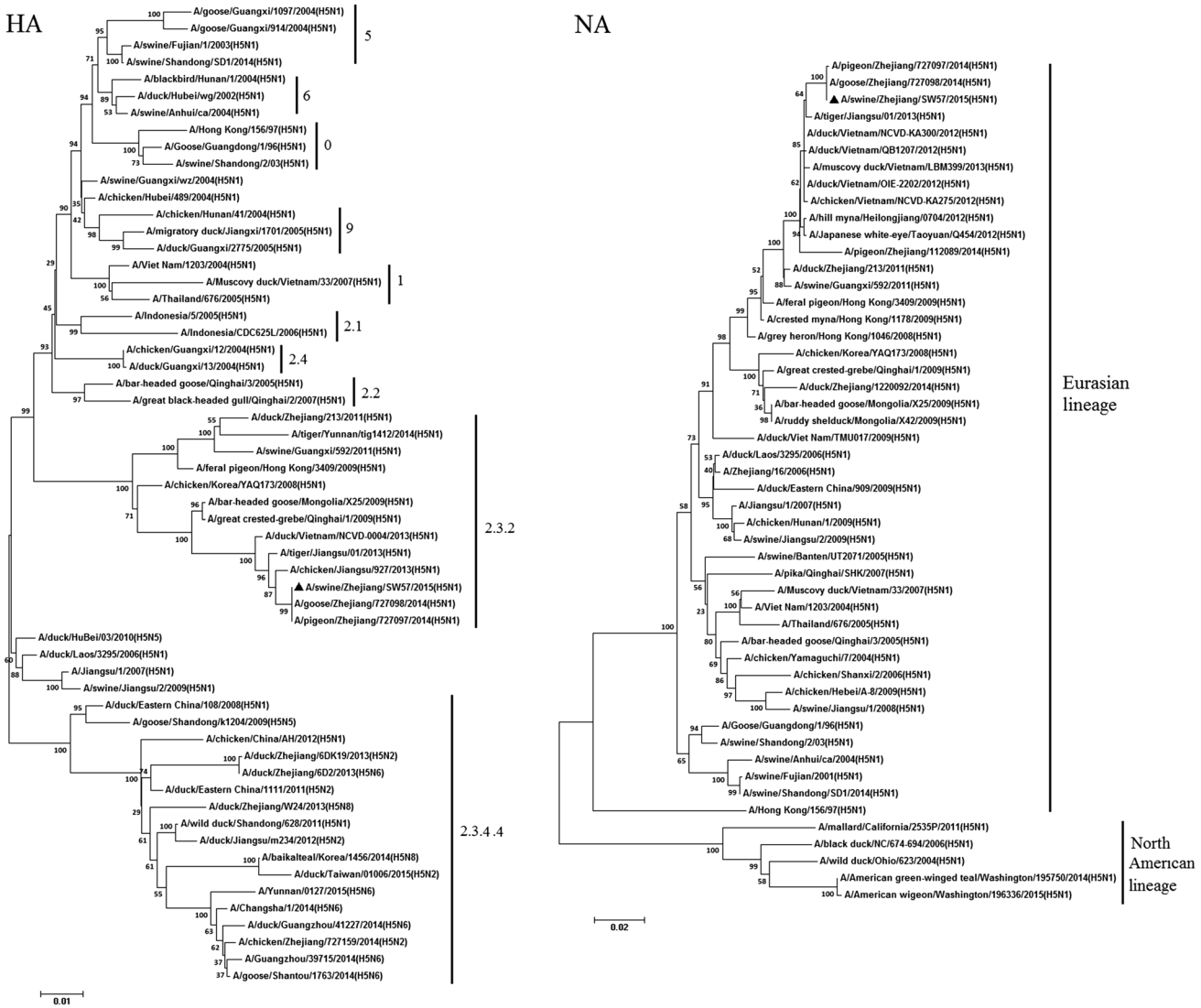

Fig. 1 Phylogenetic trees of HA (positions 1-1704) and NA (positions 1-1350) genes from H5N1 SIV. We created the tree using the neighbor-joining method with 1,000 bootstrap replicates using MEGA software version 6.0. The H5N1 virus from swine in this study is indicated by a triangle. The scale bar represents the distance unit between sequence pairs

Previous reports have shown that H5N1 viruses, which have been circulating widely among poultry in Eastern China, have all undergone frequent and extensive reassortment with other H5N1 viruses [14, 15]. In our study, we found that ZJ-SW57 has undergone reassortment and has received genes from various H5N1 viruses from poultry in Eastern China.

Based on the deduced amino acid sequence of HA, the cleavage site pattern, PQRERRRKR, of ZJ-SW57 displayed features of a multi-basic cleavage site, suggesting that ZJ-SW57 is a highly pathogenic virus. It is known that the addition of multiple amino acids at the cleavage site, such as arginine (R) and lysine (K), may turn a low-pathogenic avian influenza virus of subtype H5 or H7 into a highly pathogenic strain [16]. In this study, the amino acids at positions 236-241 and 146-150 of ZJ-SW57were NGQSGR and GVSAA, respectively. It has been noted that the receptor-binding sites Gln226 and Gly228 of ZJ-SW57 would preferentially bind to avian SAα2,3Gal receptors [14]. HA glycosylation affects virulence and the affinity of influenza virus for specific receptors [17]. We detected seven potential N-linked glycosylation sites in HA of ZJ-SW57 at positions 26, 27, 39, 181, 302, 499 and 559 (Asn-X-Ser/Thr, where X is any amino acid except Pro or Asp [18]) (Fig. S2).

The Glu627Lys mutation of PB2 influences the host range and virulence of H5N1 virus in mammals [19]. We observed this mutation in PB2 of ZJ-SW57, suggesting high levels of pathogenicity in mice (Table S2). Moreover, ZJ-SW57 contains the NS1 Pro42Ser mutation, which is associated with increased virulence in mice [20].

We analyzed receptor binding preferences of ZJ-SW57 using hemagglutination receptor-specific red blood cells (RBCs), including normal chicken RBCs (containing both α-2,6 and α-2,3 receptor), α-2,3-specific-neuraminidase-treated chicken RBCs (containing only α-2,6 receptor after treatment), and sheep RBCs (mainly expressing α-2,3 receptor). We prepared α-2,6 receptor-specific RBCs by treating chicken RBCs with α-2,3-specific neuraminidase as described previously [21, 22]. The results showed that ZJ-SW57 fails to agglutinate α-2,3-specific-neuraminidase-treated chicken RBCs, indicating that ZJ-SW57 possesses avian receptor specificity (Table S3).

To explore the pathogenicity of ZJ-SW57 in mice, we inoculated 15 six-week-old female BALB/c mice intranasally with 10^6 times the 50% egg infective dose (EID_{50}) of virus and sacrificed three mice after three, six and nine days post-inoculation as described previously [14]. We conducted animal studies according to the recommendations of the Office International des Epizooties [23], and they were

Table 1 The results of animal experiments with the H5N1 swine influenza virus isolate from China

No. of survivors/ no. tested	HI titer (log_2)	Virus replication in experimentally infected mice Virus titers in organs of mice (log10 EID_{50}/ml)			
		Tissue	3 days	6 days	9 days
3/6	6.3 ± 0.6	Lung	2.5 ± 0.7	3.5 ± 0.7	3.0 ± 0
		Brain	1.0 ± 0	1.5 ± 0.7	1.0 ± 0
		Heart	1.0 ± 0	2.0 ± 0	1.0 ± 0
		Liver	1.0 ± 0	2.0 0	0

We intranasally inoculated 15 six-week-old female BALB/c mice with 10^6 EID_{50} of virus and sacrificed three mice at three, six and nine days post-inoculation. We collected lung, brain, heart and liver samples and titrated the virus in embryonated chicken eggs. We observed the remaining six mice for 14 days after inoculation. Values are presented as the mean ± SD. We harvested sera at 14 days after infection from three mice that had survived. We confirmed seroconversion using the HI test. Results are expressed as the mean ± SD of the values obtained from three infected mice

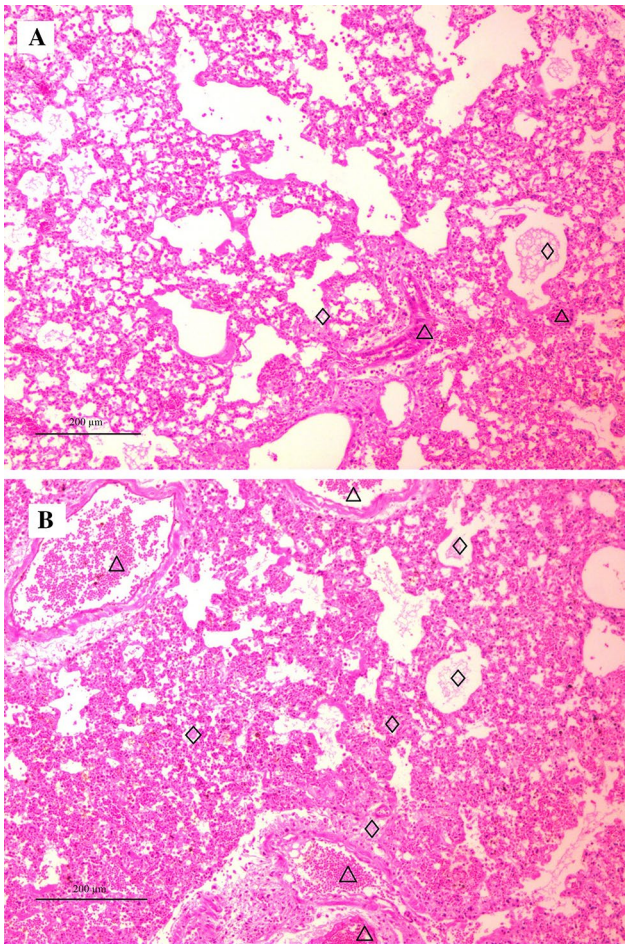

Fig. 2 Histology of lung sections from mice infected with the H5N1 virus at three days (A) and six days (B) postinfection, using hematoxylin and eosin staining. Mice infected with H5N1 exhibited severe interstitial pneumonia in the lung tissues, shown by the alveolar lumen flooded with dropout from alveolar cells, erythrocytes and inflammatory cells (diamond), and congestion in the blood vessels (triangle)

approved by The First Affiliated Hospital, School of Medicine, Zhejiang University. We collected lung, brain, heart and liver samples and titrated the virus using embryonated chicken eggs, applying the Reed and Muench method [24]. ZJ-SW57 was able to replicate without prior adaptation. On day six post-inoculation, we detected high viral titers in the lung, which spread to the heart. We also detected ZJ-SW57 in the liver and brain of mice. The mice displayed signs of illness and had survival rates of 50% (3/6), at 14 days post-inoculation (Table 1).

We fixed lung tissues from virus-inoculated mice in 10% neutral buffered formalin and embedded the tissues in paraffin using standard tissue processing procedures. We cut 4-μm-thick sections and fixed them onto glass slides and performed hematoxylin and eosin (H&E) staining as described previously [15]. Histopathological analysis showed that at six days post-inoculation, lung tissues of mice infected with ZJ-SW57 had severe multifocal interstitial inflammatory hyperaemia and exudative pathological changes, including large lesions in the lung tissue and fusion of multiple patchy lesions (Fig. 2).

In recent years, there have been many reports that at least two different clades (2.3.2 and 2.3.4.4) of H5 viruses (H5N1, H5N2, H5N6 and H5N8) are circulating in poultry in Zhejiang Province [18, 29]. In 2013, He et al. isolated two H5N1 SIVs (clade 2.3.4 and 7) from Jiangsu (a province near Zhejiang Province), and these SIVs shared high levels of sequence identity with H5N1 poultry isolates from China [4]. In this study, we isolated H5N1 virus from pigs in Zhejiang Province, which belonged to clade 2.3.2 and received genes from poultry viruses in Eastern China. These results suggest that pigs can be naturally infected with H5N1 avian virus, highlighting its potential threat to public health. Therefore, active surveillance of influenza in pigs should be used as an early warning system for influenza outbreaks in mammals.

Compliance with ethical standards

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Conflict of interest The authors declare that they had no conflict of interest.

Ethical approval The animal experiment was approved by the First Affiliated Hospital, School of Medicine, Zhejiang University (No. 2015-15).

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