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Short communication

Phylogenetic analysis of a novel H6N6 avian influenza virus isolated from a green peafowl in China and its pathogenic potential in mice

Zhaobin Fan [a,b,c], Yanpeng Ci [b], Yixin Ma [a], Liling Liu [b], Deli Wang [b], Jianzhang Ma [a,*], Yanbing Li [b,*], Hualan Chen [b]

[a] College of Wildlife Resources, Northeast Forestry University, Harbin 150040, People's Republic of China
[b] Harbin Veterinary Research Institute, Harbin 150001, People's Republic of China
[c] College of Animal Science and Veterinary Medicine, Liaoning Medical University, Jinzhou 121001, People's Republic of China

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ABSTRACT

To explore the ecology of the H6 subtype avian influenza viruses in Hebei Province, China, a long-term surveillance was conducted in 2012 among domestic poultry and birds in a wildlife park. In this study, we report the characterization of a novel H6N6 avian influenza virus isolated from a healthy green peafowl in Qinghuangdao Wildlife Park in 2012. A phylogenetic analysis indicated that the isolated H6N6 strain has the same gene constellation as the ST3367-like strains, which are mainly distributed in southern and eastern China. A mouse experiment showed that the isolate replicated efficiently in the lungs and turbinates of infected mice without previous adaptation, resulting in locally thickened alveolar septa and interstitial pneumonia. Further studies of the H6 subtype viruses are required to clarify their evolutionary pattern in north China, which will benefit disease control and pandemic preparedness for novel viruses.

1. Introduction

The avian influenza viruses (AIVs) are classified into 16 hemagglutinin (HA) and nine neuraminidase (NA) subtypes, based on the antigenicity of these two surface glycoproteins. Worldwide AIV control and surveillance efforts have focused for a long time on the H5, H7, and H9 subtypes because of the high mortality or the significant economic losses they cause in affected flocks. However, increasing evidence demonstrates that low-pathogenic avian influenza viruses are also important, primarily because they can potentially infect domestic poultry and humans if they undergo reassortment to produce pathogenic forms (Gao et al., 2013; Shi et al., 2013; Chen et al., 2014). Therefore, the preparation of vaccines against viruses in each of the HA subtypes is necessary to ensure that we are prepared for future pandemics.

The first H6 subtype influenza virus was isolated from a turkey in 1965 (Lupiani and Reddy, 2009), and since then, the H6 viruses have been isolated from many countries (Choi et al., 2005; Jonassen and Handeland, 2007; Jahangir et al., 2008; Wahlgren et al., 2008). At present, the H6 subtype influenza viruses are some of the most abundantly detected viruses in wild birds and poultry, and this subtype has a broader host range than any other subtype. The H6 subtype viruses had attracted little public attention until the first documented infection of humans with the highly pathogenic H5N1 AIV (A/Hong Kong/156/97, 156-like) in Hong Kong in 1997, which led to an outbreak among chickens, with sporadic human cases and deaths (Leneva et al., 2000). During this period, an H6N1 AIV (A/Teal/Hong Kong/W312/97, W312-like) was isolated from a live bird market. The subsequent genetic characterization of this isolate revealed that its seven gene segments were highly homologous to those of 156-like, except for the HA gene, suggesting that the W312-like H6N1 virus might have been involved in the generation of the 156-like H5N1 virus (Chin et al., 2002). It is noteworthy that, in recent years, the H6 subtype reassortant viruses may have crossed the species barrier and infected mammals, even humans, without adaptation. For example, in southern and eastern China, H6N6 AIVs were isolated from swine with clinical signs including coughing and nasal discharge (Zhang et al., 2011; Zhao et al., 2013). Recent seroprevalence research has shown that United States veterinarians who had been exposed to birds showed significantly elevated antibody titers against H5, H6, and H7 AIVs (Myers et al., 2007). In May 2013, an H6N1 virus was isolated in Taiwan from a 20-year-old woman with symptoms including fever, cough, headache, and muscle ache (Shi et al., 2013). These data indicate that H6 subtype AIVs may pose a potential public health threat.

* Corresponding authors. Tel.: +86 18724591358 (J. Ma). Tel.: +86 13946015508 (Y. Li).
E-mail addresses: 18724591358@163.com (J. Ma), liyanbing@caas.cn (Y. Li).

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In this study, we isolated a strain of H6N6 AIV from a healthy green peafowl in Qinghuangdao Wildlife Park during the AIV surveillance of Hebei Province, China. To better understand the molecular and biological properties of this strain, we used a phylogenetic analysis combined with a complete genomic sequence analysis of the isolate, and assessed its replication and pathogenicity in mice. This study extends our understanding of the potential evolutionary and transmission features of the H6 subtype viruses and should benefit future disease control and pandemic preparedness.

2. Materials and methods

2.1. Ethics statement

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All animal procedures were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. All experiments were performed in a biosafety level 3 laboratory at Harbin Veterinary Research Institute (Harbin, China).

2.2. Virus isolation

In April 2012, a novel H6N6 virus was isolated from a green peafowl in Qinghuangdao Wildlife Park in Hebei Province, China, and designated A/Green peafowl/Qinghuangdao/56/2012(H6N6) (GP/QHD/56/12). Oropharyngeal and cloacal swabs or excrement samples were suspended in antibiotic-treated phosphate-buffered saline (PBS; pH 7.2) and centrifuged at 5000 rpm for 10 min 4 °C. The allantoic cavities of 10-day-old embryonated specific-pathogen-free (SPF) chicken eggs were inoculated with the supernatant. The presence of AIV was detected with a hemagglutination–hemagglutination inhibition assay and confirmed with RT-PCR. All the isolated viruses were purified with three rounds of limiting dilution in 10-day-old SPF embryonated chicken eggs. The 50% egg infection dose (EID_{50}) was calculated with the method of Reed and Muench (Reed and Muench, 1938). The purified virus allantoic fluid was stored at −80 °C for future analysis. All procedures were performed under aseptic conditions.

2.3. Genome sequencing and phylogenetic analysis

Viral RNA was extracted from the allantoic fluid using TRIzol Reagent (Invitrogen Carlsbad, CA, USA) and then reverse transcribed with a 12-bp primer. Reverse transcription (RT)-PCR was performed with a set of sequence-specific primers (primer sequences available on request) to generate eight full-length gene fragments of the influenza virus. The RT-PCR products were purified with the Watson PCR Purification Kit (Watson Biotechnologies Inc., Shanghai, China) and sequenced directly with the CEQ™ DTCS Quick Start Kit on a CEQ™ 8800 Genetic Analysis System (Beckman Coulter, Inc., Brea, CA, USA). The MEGA 5 software was used to generate a multiple sequence alignment with the ClustalW algorithm, and the phylogenetic tree was constructed with the neighbor-joining method and the Kimura two-parameter model, with 1000 bootstrap replications. Potential glycosylation sites were analyzed with the NetNGlyc 1.0 online software (www.cbs.dtu.dk/services/NetNGlyc/).

2.4. Mouse study

To investigate the virulence of the isolate in mice, eight six-week-old female BALB/c mice were lightly anesthetized with ketamine at doses of 25 mg/kg and inoculated intranasally with the virus at $10^{6.0}$ EID_{50} in 50 μL. Three additional mice were placed into the same isolation units to assess the extent of contact infection. The three inoculated and three contacted mice were euthanized with a peritoneal injection of sodium pentobarbital at a dose of 200 mg/kg at 3 days postinfection (dpi), and their tissues (lung, kidney, spleen, turbinate, and brain) were harvested for viral titration and histopathological evaluation. The viral titers in the tissues were calculated with the method of Reed and Muench (Reed and Muench, 1938). The control group (five mice) was mock infected with PBS. Each group was monitored daily for 14 days for weight loss and mortality.

3. Results and discussion

3.1. Phylogenetic analysis

To clarify the genetic evolution of GP/QHD/56/12, the whole genome of this H6N6 virus was sequenced and eight gene segments were compared with the sequences of representative influenza virus sequences retrieved from the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/).

As was reported in a previous study of the HA gene, the H6 subtype AIVs in the influenza virus library have been phylogenetically divided into five groups: the early group, group II, group III, W312-like group, and the North America lineage (Huang et al., 2010). As shown in Fig. 1A, the phylogenetic tree of the HA gene revealed that the isolated H6N6 strain had the same gene constellation as the ST3367-like strains, which are distributed mainly in southern and eastern China, and were mainly isolated from waterfowl in 2003–2011 (except for A/chicken/Hunan/989/05[H6N2] and A/swine/Guangdong/K6/10[H6N6]). The NA gene of the H6N6 virus isolated in this study originated from the H6N6 viruses circulating among waterfowl in southern and eastern China (Fig. 1B). In southern and eastern China, the H6 AIVs have established stable lineages, which are difficult to eradicate and probably contribute to the genetic diversity of the influenza viruses in the local domestic poultry. However, the H6 subtype viruses have not been reported previously in Hebei Province. The results of our study indicate that at least two subtypes, H6N2 and H6N6, are circulating in this region. This invasion of southern and eastern China-like H6 subtype viruses may be attributable simply to the many waterfowl moving from these regions to north China by migration or to the live poultry trade. The internal genes of this H6N6 virus show significant diversity (Table 1), suggesting that reassortment among the multiple subtypes of the influenza viruses has occurred in this region.

3.2. Molecular characterization

The HA gene of this H6N6 isolate has an open reading frame of 1701 bp, encoding 567 amino acids, with a signal peptide (16 amino acids). The H6N6 isolate contains the motif PQIETR*GLF at the cleavage site between HA1 and HA2 (Table 2), but has no consecutive basic amino acids in the motif, and thus meets this criterion for the low-pathogenic AIVs (Steinhauer, 1999). Mutations Q226S and G228S were not detected in the HA protein (Table 2), suggesting that this H6N6 virus retains the characteristic of preferentially binding to the α-2,3-linked sialic acid receptor, a predominant characteristic of avian influenza virus species (Yamada et al., 2006). GP/QHD/56/12 has six potential N-glycosylation sites in HA1 (positions 27, 39, 293, and 306) and HA2 (positions 498 and 557), and the T160A substitution in HA leads to the loss of an N-glycosylation site at residue 158, which has been found in recently circulating viruses (Wang et al., 2010).