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Short communication

Emergence of H3N8 equine influenza virus in donkeys in China in 2017

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ABSTRACT

Equine influenza virus is a major respiratory pathogen in horses. Although both horses and donkeys belong to the genus Equus, donkey infection with influenza viruses is rare. In March 2017, an influenza outbreak occurred in donkeys in Shandong province, China. The causative virus, A/donkey/Shandong/1/2017(H3N8), was isolated from a dead donkey. Genetic analysis indicated that the virus originated from influenza A (H3N8) clade 2 of the Florida sub-lineage that has been circulating in Asian equine populations. Comparison of the deduced amino acid sequence of the HA gene of this causative virus with that of the A/equine/Richmond/1/2007 vaccine strain showed that substitutions had occurred in the antigenic regions A, B, and C. This study provides insight into the currently circulating and newly emerging H3N8 strains in donkeys in China.

1. Introduction

Equine influenza (EI) is a major infectious respiratory disease caused by equine influenza A virus (EIV) in equids worldwide (Alves Beuttemmuller et al., 2016; Fougerolle et al., 2017; Legrand et al., 2015; Rosanowski et al., 2016). The infection damages respiratory epithelia, reducing mucociliary clearance and inducing inflammation; it can be severe and even fatal, particularly if exacerbated by other pathogens or factors. H3N8 EIV was first isolated in 1963 in Miami, Florida, and continues to circulate in most horse populations in the world. The H3N8 viruses have been subdivided into two different evolutionary lineages since 1986: the American lineage and the European lineage (Daly et al., 1996). Both lineages have been circulating together without geographic barriers. The American lineage has been further divided into three sub-lineages: the Florida, Kentucky, and South American sub-lineages (Lai et al., 2001). The Florida sub-lineage had evolved further into two genetic groups with divergent hemagglutinin (HA) sequences: clade 1 and clade 2 (Bryant et al., 2009). Influenza A viruses infect many different species, and these viruses occasionally cross the species barrier to infect other species. The H3N8 EIVs have caused infection and disease in canines, swine, camels, and donkeys (Crawford et al., 2005; Qi et al., 2010; Tu et al., 2009; Yondon et al., 2014).

China is the second largest equid-raising country in the world; however, no vaccines against influenza viruses have yet been licensed and used in equids. In March 1989, the first severe outbreak of equine influenza caused by H3N8 virus occurred in Jilin province, China, with morbidity and mortality rates reaching 81% and 20%, respectively, in certain herds (Guo et al., 1992). In 1993, a second H3N8 influenza outbreak occurred among equine species in Gansu province. Phylogenetic analysis of the HA gene of the causative virus indicated that the virus belonged to the European lineage (Guo et al., 1995). Since 2007, Florida sub-lineage clade 2 H3N8 viruses have been the predominant subtype isolated from equine species in China. A Florida sub-lineage clade 2 H3N8 virus also caused an outbreak among donkeys in Xinjiang, China in 2007 (Qi et al., 2010). In this study, we performed a detailed characterization of the influenza virus that caused a disease outbreak in donkeys in Shandong province in 2017.

2. Materials and methods

2.1. Collection of clinical material

In March 2017, an outbreak of donkey influenza occurred in a 300-head donkey farm in Shandong province, East China. The donkeys showed severe disease symptoms, including depression, anorexia, asthma, nasal discharge, and fever. The period of illness lasted for 2 weeks and resulted in a mortality of 25%. Tissues from dead donkeys were submitted to the Animal Influenza Laboratory of the Ministry of Agriculture, which belongs to Harbin Veterinary Research Institute,

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CAAS, for respiratory disease diagnostic tests. In addition, 11 serum samples were collected from donkeys on the affected farm two weeks after the onset of respiratory signs and symptoms for routine serological testing.

2.2. Histopathology, virus isolation, and virus identification

After being fixed in 10% neutral buffered formalin and embedded in paraffin, the tissue sections were processed routinely for hematoxylin/eosin (H/E) staining. For virus isolation, the lung tissues were thawed and homogenized in 10 vols of Dulbecco modified Eagle medium (DMEM) supplemented with 0.5% bovine serum albumin and penicillin and streptomycin. Samples were centrifuged at 1500*g* for 10 min at 4 °C and 0.2 ml of the supernatant was filtered through 0.22-μm syringe filters and inoculated into Madin-Darby canine kidney (MDCK) cells. The presence of virus was determined by using a hemagglutination assay. HA subtypes of influenza viruses were identified by using the hemagglutination inhibition (HI) test with a panel of antisera provided by the National Reference Laboratory for influenza, Harbin Veterinary Research Institute, CAAS, China. Neuraminidase (NA) subtypes were determined by means of direct sequencing.

2.3. Genetic and phylogenetic analyses

Viral RNA was extracted by using a Viral RNA Mini Kit (QIAGEN) according to the manufacturer's instructions, and was reverse transcribed by using the Uni12 primer (5′-AGCAAAAGCAGG-3′) with the AMV reverse transcriptase (TaKaRa, Dalian, China). Complete genome amplification was performed using specific primers (primer sequences available upon request). PCR products of the expected sizes were purified using a PCR purification kit (Qiagen), and the products were sequenced on an Applied Biosystems DNA analyzer. The nucleotide sequences were edited using the Seqman module of the DNAStar package. Sequences were aligned with representative influenza virus sequences downloaded from GenBank. Nucleotide phylogenetic trees were constructed with the MEGA program (version 7.0) using the neighbor-joining method with 1000 bootstrap replications based on the sequences of the open reading frames.

2.4. Serological analysis

Serological analysis was carried out using HI assays. All viruses used as antigens were grown in the allantoic cavities of 10-day-old embryonated chicken eggs and were inactivated with 0.05% (vol/vol) β-propiolactone (Sigma, St. Louis, MO) for 12 h at 4 °C. Hemagglutination assays revealed that the HA titers of the antigens ranged from 32 to 1024. Nonspecific inhibitors were removed from the serum by overnight treatment with receptor-destroying enzyme (Denka Seiken, Tokyo, Japan). Sera were 2-fold serially diluted in 96-well V-bottom plates starting at a dilution of 1:20, and tested by using the HI assay as described in the WHO Manual on Animal Influenza Diagnosis and Surveillance. The influenza viruses tested included the donkey-origin virus isolated in this study, A/Sichuan/1/2009 (pandemic H1N1 2009), A/Guangxi/Qixing/2013 (H3N2) (a recent human H3N2 isolate), and a swine Eurasian avian like H1N1 Chinese isolate (A/swine/Guangxi/18/2011); avian H5N1, H7N9, and H9N2 influenza A strains were also used for serologic detection. H5N1 and H7N9 live viruses were handled in the enhanced-biosafety level 3facility of Harbin Veterinary Research Institute.

2.5. Mouse study and ethics statement

Six-week-old female BALB/c mice were purchased from Vital River Laboratories, Beijing, China. Eight mice were inoculated intranasally with $10^{6.0}$ 50% tissue culture infective doses ($TCID_{50}$) of the respective influenza viruses, as previously described (Yang et al., 2016). Control mice were sham infected with phosphate-buffered saline. Three mice from each group were sacrificed on day 3 post-inoculation (p.i.), and their lungs, nasal turbinates, spleens, kidneys, and brains were collected and titrated in MDCK cells. The remaining mice were monitored for 14 days for body weight loss and mortality. All surviving mice were euthanized by use of CO_2 at the end of the experiment (14 days p.i.). All animals were handled in 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. The protocol for animal study was approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences.

3. Results

3.1. H3N8 virus was isolated from a dead donkey that had severe lung lesions

Three dead donkeys (deaths occurred 7 to 10 days after initial clinical signs) were subjected to post-mortem investigation. The lungs were heavy and dark red. Microscopically, the alveoli were extremely swollen, with variably extensive formation of hyaline membranes and fibrinous exudation. Typical bronchointerstitial pneumonia, characterized by necrotizing bronchiolitis and hemorrhage, was observed (Fig. 1). Part of the lung sample of one donkey was homogenized and inoculated into MDCK cells for virus isolation. After three passages, cytopathic effects were observed and an HA titer of 64 was detected in the supernatant of the cell culture. The viral subtype was determined to be H3N8 by HI testing and sequencing. The isolate was designated as A/donkey/Shandong/1/2017(DK/SD/1/17).

3.2. The donkey H3N8 virus DK/SD/1/17 belongs to Florida sub-lineage clade 2

Different lineages or clades of H3N8 viruses have been detected from different animals around the world. To investigate the genetic origin of the DK/SD/1/17 virus, we performed full genome sequence and phylogenetic analyses as previously described (Yang et al., 2016) (GenBank accession nos. MG132044-MG132051).

DK/SD/1/17, like some Florida sub-lineage clade 2 viruses (Bryant et al., 2009), had a two-amino-acid insertion that created a 'FIF' motif within the predicted signal peptide at positions −11 and −10 (negative numbers represent the predicted signal peptide sequence) in its HA protein. The HA of the H3N8 viruses isolated in China from 2007 to 2017 shared 97.6%–100% homology at the amino acid level, and shared 98.2%–99.7% homology at the amino acid level with the OIE-recommended vaccine strain for Florida sub-lineage clade 2 (A/equine/Richmond/1/2007). Sequence alignment revealed that the putative N-glycosylation sites located at positions 8, 22, 38, 53, 63, 165, and 285 of HA1 are conserved in all of the H3N8 viruses. There were six amino acid differences (A144T, E198G, I276 V, N290T, E291D, and K307N) between the HA1 of DK/SD/1/17 and that of the A/equine/Richmond/1/2007 vaccine strain (Table 1). Of note, the amino acid substitutions A144T, E198G, and I276 V are located in the antigenic sites A, B, and C, respectively. We also found the same amino acid substitutions in the strain EQ/Xuzhou/01/2013. PB2 701N is present in all of the H3N8 viruses that have been isolated in China since 1994, and is considered to be an important molecular marker for the adaptation of avian influenza viruses to mammals (Alves Beuttemmuller et al., 2016; Baz et al., 2013; Li et al., 2005). Moreover, the S31N amantadine-resistance mutation in the influenza A M2 sequence was not present in any of the viruses mentioned above.

To investigate the origin of the DK/SD/1/17 virus, we performed a phylogenetic analysis of 67 H3N8 viruses isolated globally from 1963 through 2017, obtained from the NCBI and GISAID databases, including all 17 viruses isolated from China from 1994 to 2013. Distinct clusters