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Inefficient Transmissibility of NS-Truncated H3N8 Equine Influenza Virus in Dogs

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H3N8 equine influenza virus (EIV) causes respiratory diseases in the horse population, and it has been demonstrated that EIV can transmit into dogs owing to its availability on receptors of canine respiratory epithelial cells. Recently, we isolated H3N8 EIV from an EIV-vaccinated horse that showed symptoms of respiratory disease, and which has a partially truncated nonstructural gene (NS). However, it is not clear that the NS-truncated EIV has an ability to cross the host species barrier from horses to dogs as well. Here, we experimentally infected the NS-truncated H3N8 EIV into dogs, and monitored their clinical signs and viral load in respiratory organs to determine the virus's transmissibility.

Keywords: NS truncation, transmission, H3N8 EIV

Introduction

Influenza viruses cause significant respiratory diseases in a variety of animals, including land and sea animals and birds [15]. Historically, an epizootic outbreak of respiratory illness in horse populations has occurred in North America, recorded as "The Great Epizootic of 1872," which was probably caused by influenza A virus [6]. The causative agent of equine respiratory diseases was first substantialized by isolating influenza A virus from horses in 1956 [11]. To date, two immunologically distinct influenza A viruses have been isolated from horses: H7N7 and H3N8 subtypes [12]. Of those, H7N7 equine influenza virus (EIV) has not been isolated since 1980 [14], whereas H3N8 EIV has subsequently become endemic in many countries (*e.g.*, China, Japan, and Australia) since it was recognized in Florida in the United States of America in 1963 [13]. In fact, the H3N8 EIV was responsible for the emergence of H3N8 canine influenza A virus (CIV) in North America in 2004. A genetic characterization revealed that H3N8 CIV originated from a racing grey hound that was infected with H3N8 EIV [1], and the newly emerged H3N8 CIV is still circulating in pet dogs, being a representative subtype of CIV in North America [8]. Thus, H3N8 EIV seems to have the ability to cross the host species barrier from horses to dogs. Recently, we isolated H3N8 EIV (A/equine/Kyonggi/SA1/2011, (KYG11)) from a horse showing typical symptoms of respiratory disease, especially severe runny nose, in South

Korea. Our genetic and phylogenetic analyses revealed that KYG11 belonged to the Florida sublineage clade 1, but it was unique in possessing a naturally truncated nonstructural (NS) gene segment [7], which could be the main reason for its deduced viral replication in an *in vitro* kinetic study of growth [9]. We speculate that the NS-truncated KYG11 may affect its interspecies transmissibility, even if placed in the same clade with the American sublineage EIVs that are infectious to dogs. If the KYG11 is transmissible, there are opportunities of a newly reassorted influenza virus emerging in dogs co-infected with H3N2 CIV or a novel H3N1 CIV that contains the HA gene of CIV and the rest of pandemic H1N1 [10], and this raises concern about recombination events that could bring a triple-reassorted CIV in dogs; equine, canine, and human. We inoculated the Korean EIV nasally into Beagle dogs and monitored the viral loads in nasal swabs and respiratory organs, and conducted histopathological analysis. It was revealed that the NS-gene-truncated EIV had inefficient infectivity and transmission, although the virus had high homology with transmissible EIVs. This study is the first trial of experimental infection with the NS-truncated EIV in a dog model and an important reference for further investigation on the transmission of NS-truncated influenza viruses.

Materials and Methods

Animal Study

Three beagle dogs of 7 weeks of age (Bridge, Seoul, South Korea) were used in this study, and this animal experiment was performed in biosafety level 2 facilities at Green Cross Veterinary Products (Yong-in, South Korea). Two dogs were inoculated nasally with $10^{6.75}$ 50% egg infectious dose (EID_{50}) of KYG11 and paired with a naive dog in direct contact. Clinical signs and behavioral changes were monitored during the period of the experiment. Body temperature was measured using a microchip (SSEN Biochip, South Korea) and nasal swabs were collected daily during the experiment to confirm viral shedding. General animal care was provided as required by the Institutional Animal Care and Use Committee of Green Cross Veterinary Products.

Determination of Virus Titers

Nasal mucus was collected from both nostrils of experimental dogs with conventional, short, sterile, and cotton-tipped swabs in transport medium containing penicillin G (2×10^6 U/l), polymyxin B (2×10^6 U/l), gentamicin (250 mg/l), nystatin (0.5×10^6 U/l), ofloxacin HCl (60 mg/l), and sulfamethoxazole (0.2 g/l). The swab samples were centrifuged and stored at −80°C. After thawing, viral RNA (vRNA) was extracted from the swab mixture with the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). The real-time reverse transcriptase-polymerase chain reaction (RT-PCR) was employed to quantify a viral load in nasal shedding with the QuantiTect Probe RT-PCR Kit (Qiagen Inc.) and the Roche Lightcycler 96 system. The 50 µl final reaction volume contained 0.4 µM of matrix (M) gene specific primer (forward: GACCRATCCTGTCAC CTCTGAC; reverse: AGGGCATTYTGGACAAAKCGTCTA) and 0.2 µM of specific probe (FAM- TGCAGTCCTCGCTCACTGGGC ACG- BHQ-1); thermal cycling was reverse transcription at 50°C for 30 min, initial denaturation at 95°C for 5 min, followed by 40 cycles of 94°C for 15 sec, 60°C for 60 sec, according to the manufacturer's protocol.

Lung and trachea were homogenized and the viral load was determined by EID_{50} by inoculating into 11-day-old embryonated chicken eggs and 50% of tissue culture infectious dose ($TCID_{50}$) in Madin-Darby canine kidney cells grown in minimum essential medium with Eagle salts containing 5% fetal bovine serum.

Genetic Analysis

A/equine/Newmarket/5/2003 (NM03), A/equine/Sydney/6085/2007 (SDN07), and A/equine/Ibaraki/1/07 (IBK07) were used as reference sequences, and they were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The full-length gene sequences of the virus were edited with the Lasergene sequence analysis software package ver. 5.0 (DNASTAR, USA) and aligned using Clustal V [4].

Serological Analysis

Serum specimens were collected from experimental dogs at 10 days post-infection, and seroreactivity was analyzed using the hemagglutination inhibition (HI) assay, which was performed using chicken erythrocytes according to standard methods [16].

Histopathological Examination of Tissues

A total of three dogs were sacrificed at 10 days post-infection, and lung and trachea tissue samples were harvested from the three dogs. The tissue samples were collected in 10% buffered formalin to fix the tissues and were embedded in paraffin wax, sectioned (4 to 5 µm thick), and placed on glass slides. Histological examination of the tissues was conducted using hematoxylin and eosin staining to detect lesions consistent with viral infection.

Nucleotide Sequence Accession Numbers

Gene sequences of the EIVs used in this study were deposited in GenBank under the following accession numbers: A/equine/Newmarket/5/2003: FJ375209, FJ375213, FJ375216, FJ375221, FJ375224, FJ375228, FJ375233, and FJ375236; A/equine/Sydney/6085/2007: GU045763, GU045766, and GU045769; A/equine/Ibaraki/1/07: AB360549 and AB360608; and A/equine/Kyonggi/SA1/2011: JX844143 to JX844150.

Results

We found that inoculated dogs showed no noticeable clinical signs of respiratory disease and presented a normal

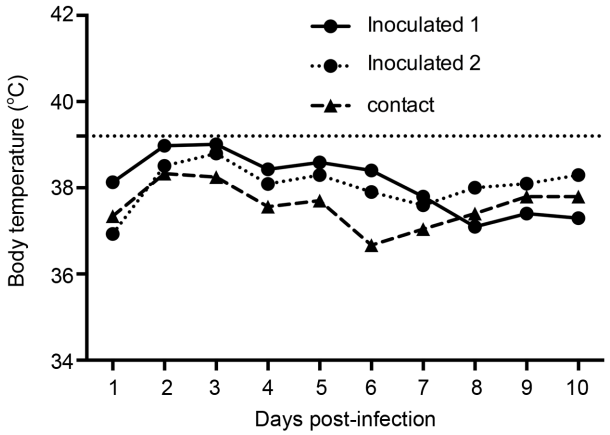

Fig. 1. Change of body temperature after infection.
Body temperature was measured for 10 days after KYG11 infection. The dotted line indicates the upper limit of the normal body temperature range of dogs.

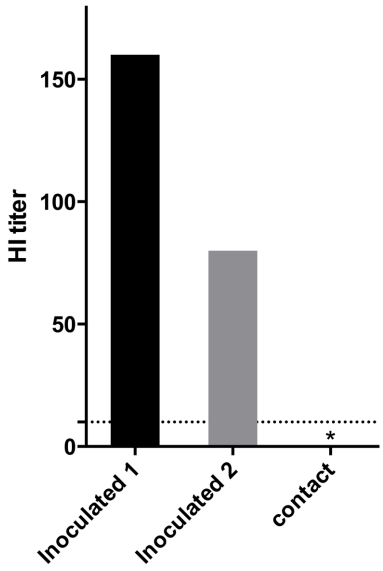

Fig. 3. Specific antibody levels after infection.
Antibody levels of experimentally infected dogs and contact dog at 10 days after KYG11 infection, using hemagglutinin inhibition assay of serums. The detection limit (<10) is indicated by the dotted line.

body temperature (Fig. 1). No detectable viral load was recorded in the respiratory organs and nasal swabs (data not shown), and the histological result revealed a well-conserved ciliated tracheal epithelium and submucosal structure containing loose connective tissue and no noticeable viral inflammatory lesion (Fig. 2). However, the HI antibody titers of inoculated dogs were above 80 (Fig. 3), which implied that a KYG11 specific antibody response had developed in the dogs. In the genetic analysis of transmissible EIVs with KYG11, the amino acids of all 8 gene segments [polymerase basic 2 (PB2), polymerase basic 1 (PB1), polymerase acidic (PA), hemagglutinin (HA), nucleoprotein (NP), neuraminidase (NA), matrix (M) and, nonstructural (NS)] of KYG11 shared the following amino acid identity with NM03; 99% of homology in PA, PB1, PB2, HA, and NP, and >97% in NA and M. The IBK07 and SDN07 presented an amino acid identity of 99% and 100% with KYG11 in HA and NA, respectively.

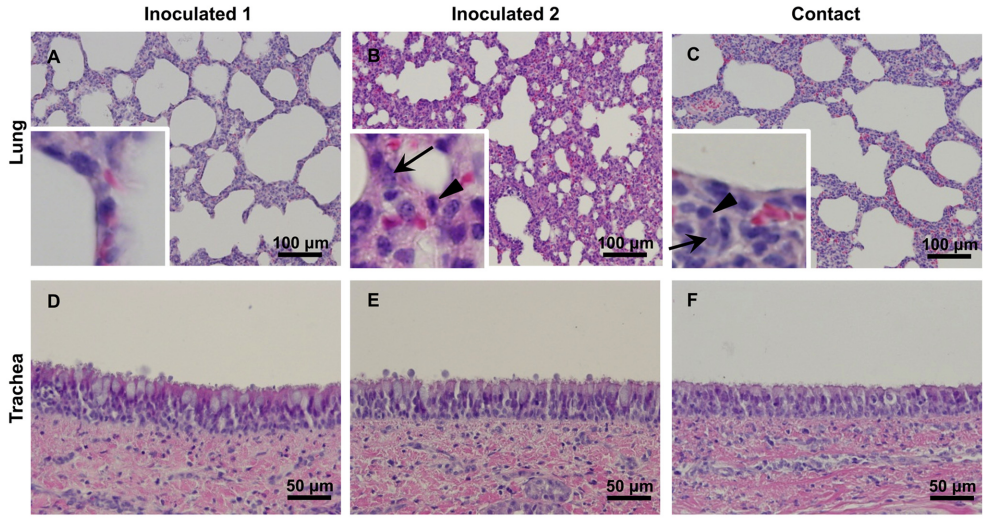

Fig. 2. The histopathological appearance after KYG11 infection.
Histopathology of lungs from dogs infected with KYG11. Dogs were inoculated intranasally with of $10^{6.75}$/ml EID_{50} in a 1 ml volume of the virus. Lungs were harvested on day 10 after virus inoculation. (**A**) Well-preserved pulmonary alveoli and alveolar wall; (**B**, **C**) Mild interstitial pneumonia characterized. (**D**-**F**) Well-conserved ciliated tracheal epithelium and submucosal structure containing loose connective tissue.

Discussion

In this study, the NS-truncated KYG11 seemed not to have efficient infectivity in dogs, even in the conditions of intranasal inoculation with maximum titer ($10^{6.75}$ EID_{50}) of KYG11, and no viral shedding in the inoculated dogs was consistent with the observation that there was no crucial evidence of complete viral transmission. However, previous studies have shown that EIVs are available on receptors of canine respiratory epithelial cells [2], and H3N8 EIVs of American lineage (*e.g.*, NM03, SDN07) could indeed transmit into dogs in which mutated H3N8 CIVs were created [1, 2, 5]. Moreover, this was experimentally confirmed that dogs exhibited seroconversion and viral shedding by close contact with EIV (IBK07)-infected horses [17]. However, KYG11 failed to transmit into dogs even though KYG11 had high identities to those transmissible EIVs except for the NS gene. It is well known that malfunction of NS1 protein leads to attenuation, low viral replication, and altered inflammation responses [3]. We thought that the truncated NS gene might be one of the reasons for the different transmissibility of KYG11 in dogs. This evokes further study to address the truncated NS gene's effect on transmissibility for other species, and naturally NS-truncated KYG11 still raises concern about being transmitted silently into dogs, which could bring potential recombination events between equine and canine influenza viruses in dogs. Therefore, continuous surveillance studies are needed to monitor the recombination in case of NS-truncated H3N8 EIV transmission into dogs.

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