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PB2 E627K or D701N substitution does not change the virulence of canine influenza virus H3N2 in mice and dogs

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ABSTRACT

Recently, canine influenza virus H3N2 (CIV H3N2) has circulated continuously in the dog populations of Asia and the United States (US). As humans have close contact with pet dogs, the circulation of CIV H3N2 is a cause for concern. Previous studies have reported that the E627K and D701N substitutions in the PB2 subunit enhanced viral pathogenicity to mammals in various influenza viruses. However, how the E627K and D701N substitutions in the PB2 subunit might affect the virulence of CIV H3N2 is unclear. Here, we constructed recombinant viruses by introducing E627K or D701N into the PB2 gene in the genetic background of A/Canine/Guangdong/02/2011H3N2 using a reverse-genetic system. The results showed that the E627K or D701N substitutions in the PB2 subunit of CIV H3N2 enhanced polymerase activity, but these substitutions did not impact viral pathogenicity in mice or beagles.

1. Introduction

The influenza A virus (IAV) can infect various animals, including humans, birds, pigs, horses, and dogs (Yoon et al., 2014). The influenza viruses of animals and humans all evolved directly or indirectly from viruses in aquatic birds, which are their natural reservoirs (Yoon et al., 2014). Among dogs, the equine-origin canine influenza virus H3N8 (CIV H3N8) and the avian-origin canine influenza virus H3N2 (CIV H3N2) are the main subtypes of canine influenza virus (CIV). CIV H3N8 was the first influenza virus identified among dogs in the United States (US) in 2004, and the virus has become enzootic mainly in the US (Crawford et al., 2005; Payungporn et al., 2008). CIV H3N2 was first isolated in South Korea (Song et al., 2008) and was then traced back to 2006 when a closely related virus was reported in China in 2010 (Li et al., 2010). Since then, CIV H3N2 has mainly circulated in Asia. Nevertheless, an H3N2-subtype CIV outbreak occurred in Chicago and then spread to various states in the US in 2015 (CDC, 2015).

RNA genome viruses have no proofreading mechanism during replication, so the negative sense RNA genome of the influenza virus is extremely error prone, giving rise to a high rate of mutation. Pathogenicity and determinants of host range can change with certain amino acid (aa) substitutions in influenza virus. Among these substitutions, the best characterized positions are 627 and 701 in the PB2 subunit. PB2 627 and 701 are determinants of viral pathogenicity and mammalian inter-host transmission in diverse viral backgrounds (Manzoor et al., 2009; Quynh et al., 2009; Steel et al., 2009; Subbarao et al., 1993). PB2 E627K, which was first identified in 1993, is a significant determinant position for the host range of the influenza virus (Subbarao et al., 1993). In 1997, the H5N1 IAV infected humans in Hong Kong (Chan, 2002), and a PB2 E627K substitution influenced its efficient replication (Shinya et al., 2004) and increased the virulence of this H5N1 variant in mice (Hatta et al., 2001). In addition, the aa at the PB2 627 position influenced the growth of an H5N1 virus in pigs (Manzoor et al., 2009). Moreover, the viruses involved in the human H7N9 outbreaks in China in 2014 were characterized by PB2 E627K, while the viruses isolated from poultry were not (Gao et al., 2013; Jonges et al., 2013; Kageyama et al., 2013). PB2 E627K has also been detected in a human H7N7 case (Jonges et al., 2014). The second substitution, PB2-D701N, has also been shown to enhance the replication of some strains of IAV in mammalian hosts, including H5N1, H1N1,

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and H7N1 (de Jong et al., 2006; Gabriel et al., 2005; Le et al., 2009; Zhou et al., 2013).

Other aa mutations also influence pathogenesis in CIV H3N2. A two-aa insertion in the neuraminidase (NA) stalk of CIV H3N2 enhanced replication in eggs, cell cultures and mice (Lin et al., 2016). A mutation of tryptophan (W) to leucine (L) at position 222 in hemagglutinin (HA) could facilitate H3N2 IAV infection in dogs (Yang et al., 2013). Notably, the best characterized substitutions in influenza viruses, PB2 E627K and D701N, have not yet been explored in CIV H3N2. To address this lack, in this study, the recombinant viruses rH3N2-wt (wild-type), rH3N2-627K (with PB2 mutation E627K), rH3N2-701N (with PB2 mutation D701N), and rH3N2-627K 701N (with PB2 mutations E627K and D701N) were generated using reverse genetics to evaluate the significance of CIV H3N2 E627K and D701N substitutions on pathogenesis in mice and dogs.

2. Materials and methods

2.1. Viruses, cells and animals

CIV H3N2, A/canine/Guangdong/02/2011 (GD02), is preserved in our laboratory. Madin-Darby canine kidney (MDCK) and human embryonic kidney (HEK) 293 T cells were obtained from the American Type Culture Collection (ATCC) and propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Viral titers were evaluated by EID50/ml.

2.2. Virus rescue

First, the reverse-genetic plasmids were generated as follows: the viral RNA of GD02 was extracted for cDNA generation with the Uni12 primer using a cDNA Synthesis Kit (Tiangen, China). All eight viral genes of GD02 were amplified using the Fast HiFidelity PCR Kit (NEB, UK) and cloned into the pHW2000 plasmid. Mutation primers were designed for the generation of the E627K and D701N substitutions in the PB2 subunit. All plasmids were sequenced to ensure the absence of unwanted mutations. Second, the viruses were rescued as follows: 293 T cells were co-cultured with MDCK cells in six-well plates. Cell monolayers (approximate 70%–80% confluent) were used for co-transfection with eight reverse-genetic plasmids (0.8 μg each plasmid) using Lipofectamine 3000 Reagent (Life Technologies, USA). At 72 h post-transfection, the culture supernatant was inoculated to 10-day-old specific pathogen-free (SPF) hens' eggs for 48 h at 37 °C to prepare virus stocks. Then, the viruses were plaque-purified and confirmed by hemagglutination (HA) and sequencing.

2.3. Luciferase reporter assay

Fifty nanograms (ng) each of NP, PA, PB1, and PB2 (wild-type or with mutations) viral protein expression plasmids; 100 ng of pPolI-NP-luc; and 10 ng of pPL-TK (Promega) were co-transfected into 297 T cells using Lipofectamine 3000 (Invitrogen). At 24 h after transfection, the cells were harvested to perform a dual-luciferase reporter assay (Promega). The firefly and *Renilla* luciferase activities were measured using a GloMax® Discover System (Promega) according to the manufacturer's protocol. The ratio of firefly luciferase activity to *Renilla* luciferase activity was calculated to represent the efficiency of the transcription/replication of the viral-like reporter RNA.

2.4. Mouse infection

Six-week-old female BALB/c mice were purchased from the Laboratory Animal Center of South China in Guangzhou, China. Each group of mice (n = 13) was challenged with 10^5 EID50 of virus in a volume of 50 μl via the intranasal route under light anesthesia. Control animals were inoculated intranasally with an equal volume of PBS. Mice were monitored daily for survival and body weight for up to 14 dpi. On day 5 post-inoculation (dpi), three mice were euthanized, and the lungs, hearts, livers, spleens, kidneys and brains were collected for titration of viral replication in the organs. The lungs and tracheas were sectioned for histopathological and immunohistochemical examinations. Tissue sections were fixed in 10% phosphate-buffered formalin. Twenty-four hours later, these tissues were dehydrated, embedded in paraffin, and cut into 5-μm-thick sections before being stained with hematoxylin and eosin (HE, Sigma). Standard immunohistochemistry (IHC) assays were performed by incubating the sections with a mouse monoclonal antibody raised against the IAV nucleoprotein (Sigma), then with horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (H + L) antibody (Abbkine), and finally stained with diaminobenzidine (DAB).

2.5. Beagle infection

Nine- to twelve-week-old beagles were used for this study. Prior to the experiment, nasal and rectal swabs and serum samples were collected and tested by virus isolation and serological assays to ensure that the animals were negative for infection with CIV H3N2.

Five groups of three beagles were inoculated with 10^6 EID50 of rH3N2-wt, rH3N2-627K, rH3N2-701N, or rH3N2-627K 701N via the intranasal route. As a control, three beagles were inoculated with PBS. Body temperatures were recorded daily from two days before the challenge to 14 dpi. Nasal and rectal swabs were collected daily, while sera were collected from 5 dpi until no antibody response was found. At 5 dpi, one animal from each challenge or control group was euthanized by an intravenous injection of pentobarbital, and necropsies were performed. The intestines, kidneys, spleens, livers, hearts, lung lobes, tracheas and nasal turbinates were collected to evaluate viral replication, and the lungs and tracheas were sectioned for histopathological and immunochemical examination, using the protocols described above.

2.6. Statistical analysis

Statistical significance was determined using the conventional Student's *t*-test All experiments were performed in triplicate. The results are presented as the mean ± SD, and a p value < 0.05 was considered statistically significant (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

2.7. Ethics statement

All procedures in animal experiments met the requirements and were approved by the Experimental Animal Welfare Ethics Committee of the South China Agricultural University. Euthanasia was performed using an intravenous injection of pentorbarbital. All experimental animals were monitored by university-licensed veterinarians.

3. Results

3.1. E627K and D701N substitutions significantly enhance polymerase activity but do not change viral replication in MDCK cells

To assess the potential effects of the PB2 E627K and D701N substitutions on CIV H3N2, we constructed recombinant viruses by introducing E627K or D701N to the PB2 gene in the background of A/Canine/Guangdong/02/2011H3N2. The substitution viruses were rescued successfully; the wild-type virus was designated CIV-wt, and the substitution viruses were designated CIV-E627K, CIV-D701N and CIV-E627K/D701N.

PB2, PB1, PA, and NP constitute the viral replication complex of IAV; therefore, we compared viral polymerase activity in the CIV-wt and CIV-E627K, CIV-D701N and CIV-E627K/D701N substitution minigenomes in MDCK cells by using a luciferase assay(Yamayoshi et al.,