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Morphological features and pathogenicity of mutated canine influenza viruses from China and South Korea

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Abstract

The canine influenza virus (CIV) has spread globally from East Asia to the United States and mutated and evolved to generate various CIVs. Since 2010, the mutant CIVs found in China and Korea have presented increased virulence in mice, guinea pigs and ferrets, which has raised concerns about public health and outbreak of a severe canine flu. We analysed and compared the morphology, cellular uptake and pathogenicity of CIV variants in host animals, to determine their characteristics. The Chinese mutant, A/canine/Jiangsu/06/2010[H3N2](JS10), has two amino acid insertions at the distal end of the NA stalk, and A/canine/Korea/01/2007[H3N2](KR07) presented comparable efficiency of cell uptake and a similar morphology to spherical or small ovoid particles. However, KR07M generated from swapping of M segment of the pandemic isolate, A/California/04/2009 [H1N1] (CA04) into KR07 alone accounted for morphologic change and higher efficiency of cell uptake to the wild-type CIV. This study will provide an insight into the pathogenesis, transmission and evolution of CIVs and help determine future countermeasures.

KEYWORDS

canine influenza virus, cellular uptake, morphology, pathogenicity

1 | INTRODUCTION

Influenza virus is a clinically and economically important virus that infects diverse species and phyla, including humans, pigs, horses and fowl (Wright, Webster, Knipe, & Howley, 2001). Dogs have been considered a neglected host for influenza viruses; however, epidemiological studies have revealed several outbreaks of inter-species transmission, such as the equine H3N8 (Crawford et al., 2005) and the avian H3N2 influenza virus that crossed the host barrier to dogs (Song et al., 2008). Sialic acid (SA) receptors in dogs have a distribution

Na and Xie made equal contribution.

similar to that the avian sialic acid (SA) receptor (Song et al., 2009), which enables influenza viruses to enter the respiratory epithelial cells. Dogs are also susceptible to highly pathogenic influenza viruses, and infected dogs show respiratory clinical signs (Lyoo et al., 2017). Avian influenza viruses crossed the host barrier to give rise to the canine influenza virus (CIV), which indicates that dogs co-infected with various influenza viruses may act as intermediate hosts for avian influenza virus re-assortment.

In addition to avian-to-dog transmission, evidence has been reported for transmission of seasonal human H3N2 and pandemic H1N1 (pH1N1) viruses to dogs. Serum samples collected from field dogs revealed that these dogs had experienced seasonal H3N2 infection since 2008 and pH1N1 infection alone or in combination with H3N2 CIV after 2009. The infectivity of pH1N1 and seasonal H3N2 viruses in dogs was proven when artificial inoculation of the viruses with active viral shedding caused pathological changes in the lungs (Song et al., 2015). Studies on sero-prevalence and artificial infection suggested the possibility of re-assortment between the two viruses in dogs; subsequently, H3N1 and M segment-swapped CIV between pH1N1 and wild-type H3N2 CIV were isolated (Moon et al., 2015; Song et al., 2012). The M gene of pdm H1N1 and the HA gene of H3N2 CIV were predominant in mutants (Na et al., 2015). Notably, the H3N2 CIV with the M segment of the pdm H1N1 virus showed enhanced transmission in ferrets than the classic H3N2 CIV (Moon et al., 2015).

Canine influenza virus mutants have continuously been reported in China, and an isolate from the Jiangsu province, A/canine/Jiangsu/06/2010(H3N2) (JS10), has 2-amino acid insertions at the distal end of the NA stalk. The presence of 2-aa insertions in NA was observed in many isolates from different provinces of China, such as Zhejiang (Teng et al., 2013), Beijing and Liaoning (Sun et al., 2013). The isolate showed enhanced viral replication in mice and guinea pigs (Xie et al., 2018), albeit it was low in chickens, indicating inappropriateness for replication in poultry (Lin et al., 2016).

The aim of this study was to elucidate the adaptive evolution of the CIV mutants by comparing the pathogenicity in canine hosts, morphology of viruses and extended cellular uptake in MDCK cell line. This study will provide an important insight into pathogenesis, transmission and evolution of CIVs, which emerged recently in Korea and China, and help determine future countermeasures.

2 | MATERIALS AND METHODS

2.1 | Viruses

Madin-Darby canine kidney (MDCK, ATCC CRL-2936) cells were obtained from the American Type Culture Collection (ATCC). The MDCK were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% foetal bovine serum (FBS) and antibiotics and maintained at 37°C in 5% CO_2.

KR07 (No. JX163256), JS10 (No. JN247619), CA04 (No. MN371616) and KR07M (No. KF155145) were propagated in MDCK cells with 1 μg/ml of TPCK-trypsin (Thermo Fisher Scientific) and supplemented with 1% antibiotic–anti-mycotic in DMEM at 37°C and 5% CO_2.

2.2 | Animal study

Fifteen 7-week-old beagle dogs (Yangzhou University, China) were used in this study. Animal experiments were performed in biosafety level 2 (BSL2) facilities at Nanjing Agricultural University (Nanjing, China). Three dogs from each group were nasally inoculated with $10^{6.75}$ of 50% egg infectious dose (EID50) of viruses except negative control group. Clinical signs and behavioural changes were monitored during the period of experiment. During the experiment, nasal swabs were collected daily, to confirm viral shedding. The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to, and the appropriate ethical review committee approval has been received. The guidelines of Institutional Animal Care and Use Committee of Nanjing Agricultural University were followed (approval no. SYXK 2017–0027).

2.3 | Determination of virus titres

The swab samples were centrifuged and stored at −80°C. After thawing, viral RNA (vRNA) was extracted from the swab mixture, using the RNeasy Mini Kit (QIAGEN Inc.). Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was employed to quantify the viral load in nasal shedding, using the QuantiTect Probe RT-PCR Kit (QIAGEN Inc.) and the Roche Lightcycler 96 system. The reaction mixture contained 0.4 μM of matrix (M) gene-specific primer (forward: GACCRATCCTGTCACCTCTGAC; reverse: AGGGCATTYTGGACAAAKCGTCTA) and 0.2 μM of specific probe (FAM- TGCAGTCCTCGCTCACTGGGCACG-BHQ-1) in a final reaction volume of 50 μl. The conditions for thermal cycling were as follows: reverse transcription at 50°C for 30 min and initial denaturation at 95°C for 5 min, followed by 40 cycles of 94°C for 15 s and 60°C for 60 s, according to the manufacturer's protocol.

2.4 | Histopathological examination of lung tissues

Totally, fifteen dogs were killed at 10 days post-infection, and lung tissue samples were extracted from dogs. The tissue samples were fixed in 10% buffered formalin, embedded in paraffin wax, sectioned (4 to 5 μm thick) and placed on glass slides. Histological examination of tissues was performed using haematoxylin and eosin staining to detect lesions consistent with viral infection.

2.5 | Electron microscopy

Conventional TEM imaging was performed using a TEM microscope at 80 kV. Influenza viruses were stained using a PTA solution at

0.75% (w/v), supplied by Sigma-Aldrich. The solution was prepared by dissolving 37.5 mg of PTA in boiling distilled water (5 ml). The PTA solution was then filtered through a 0.2-mm filter. Further, 1 ml of virus solution (concentration, 1.7 mg/ml) was deposited onto the grids for 1 min. The grids were then blotted with filter paper and immersed in PTA staining solution for 5 s for negative staining. The grids were blotted again and dried under vacuum for 1 min.

2.6 | Analysis of cellular uptake of labelled viruses

All viruses were labelled with DiD (Sigma-Aldrich). To obtain DiD-labelled influenza viruses, 0.1 multiplicity of infection (MOI) of purified influenza virus was incubated with 10 μl of 10 mM DiD at room temperature for 6 hr. The unincorporated DiD molecules were removed by using a gel-filtration column (GE Healthcare illustra™ NAP™ Columns, NAP-5) in a total volume of 1 ml. For confocal laser scanning microscopy (CLSM) imaging, the MDCK cells were cultured in 35 × 10 mm grass-bottomed confocal dishes for 24 hr. The cells were then incubated with the prepared viruses for 1 hr at 37°C to allow virus binding. The unbound viruses were removed by washing with PBS, following which the MDCK cells were imaged by CLSM. DiD was excited with a 633 nm laser, emitting 665 nm fluorescence. To examine viral infectivity, MDCK cells were incubated with DiD-labelled influenza viruses and analysed by flow cytometry.

3 | RESULTS

3.1 | Animal experiments

The replication and pathogenicity of CIVs were examined in naïve hosts. Experimental infection of dogs was associated with typical symptoms of respiratory disease, including sneezing and nasal discharge. Each group infected with KR07, KR07M and JS10 presented high fever above normal temperature limit (Figure 1a) and showed virus shedding after challenge throughout the experiment (peak titres, 6.46, 6.67 and 6.4 log EID_{50}/mL in KR07, KR07M and JS10, respectively) (Figure 1b). However, as compared to the CIVs-infected animals, the CA04-infected dog showed lower frequency of sneezing and nasal discharge, less severe fever (peak body temperature, 39.6°C) and lower viral loads in the nasal swab (peak titres, 4.32 log EID_{50}/ml).

The lungs and trachea of the inoculated animals showed pathological changes (Figure 2). Animals infected with CA04 had minimal peribronchiolar and/or perivascular cuffing and suppurative bronchiolitis (Figure 2a). Dogs in JS10 infected group showed a severe suppurative bronchopneumonia and moderate degeneration of bronchial epithelium with loss of cilia (Figure 2b), which compared to KR07 (Figure 2c), which presented a mild form of suppurative bronchopneumonia, degeneration of bronchial epithelium with loss of cilia, peribronchiolar and/or perivascular cuffing and pulmonary haemorrhage. However, KR07M presented severe suppurative bronchointerstitial pneumonia with formation of numerous multinucleated syncytial cells (Figure 2d).

3.2 | Cell uptake efficiency

Figure 3 depicts the intracellular virus distribution as investigated by CLSM and flow cytometric analysis. Cellular uptake of virus by host cells is the basis of virulence in infection. Furthermore, endocytosis of the virus is crucial for induction of viral replication. Under the confocal microscope, the DiD fluorescence was observed in the cytoplasm, indicating that DiD-labelled virus entry by endocytosis. Subsequently, the infectivity of DiD-labelled influenza viruses was quantified by flow cytometry. In the DiD incorporated virus, CA04 and KR07M were highly distributed in the cytoplasm, but only lower amounts of KR07 and JS10 were seen following an incubation period of 6 hr. These results indicate that the morphological changes of viruses provide evidence of viral infectivity.

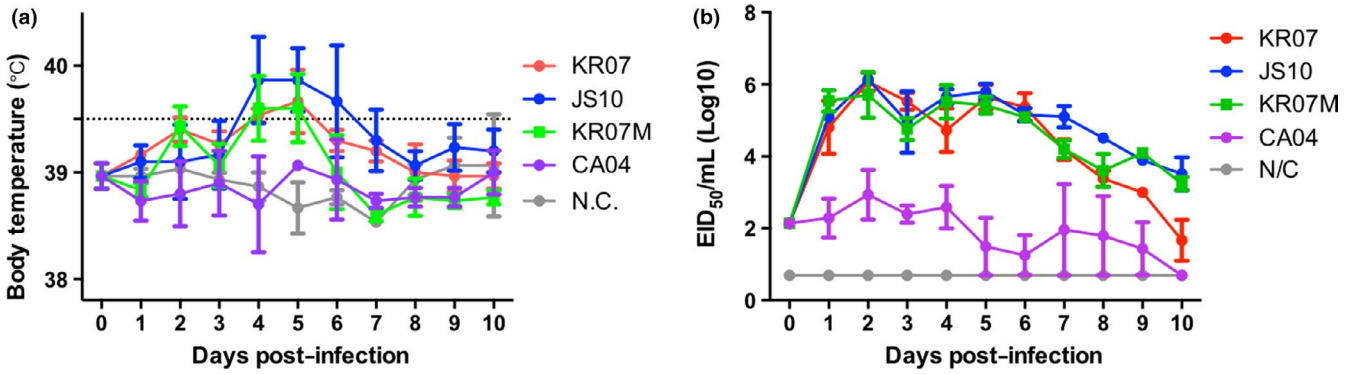

FIGURE 1 Daily estimated body temperature and nasal swab shedding of the viruses in different virus-inoculated dogs. (a) Change in body temperature of dogs infected with CA04, KR07M, KR07 and JS10, respectively. (b) Quantitation of viral shedding in virus-inoculated dogs. Each symbol represents the mean value of the temperatures from three dogs, and each error bar indicates standard deviation. The dotted line shows the upper limit of normal range of body temperature. Viral titres are expressed as $\log_{10}EID_{50}$ ml^{-1}

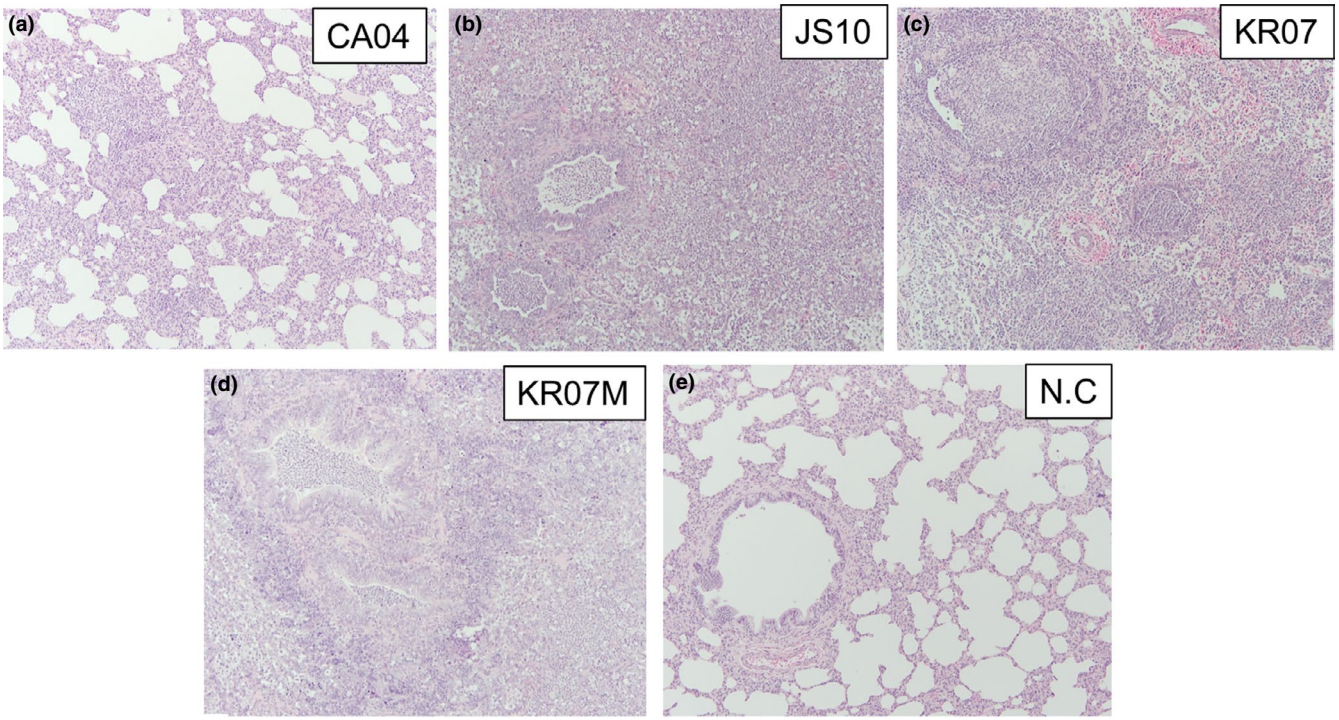

FIGURE 2 Lung histopathology of dogs infected with the viruses. Histopathology of lungs from dogs infected with each of CA04, KR07M, KR07 and JS10. Dogs were inoculated intranasally with each virus at a dose of $10^{6.75}$ of 50% egg infectious dose (EID50 (in a 500 μl volume), and lung tissues were collected on day 10 following virus inoculation. Images are representative of three dogs per group (a, CA04; b, JS10; c, KR07; d, KR07M; and e, negative control). The bar represents 100 μm. Images were obtained at 100× magnification

3.3 | Morphology of viruses

Viral particles have varying surface area of the viral envelop based on their morphology (Figure 4). As the difference in virion shape may affect the efficiency of virion-associated cell uptake, we examined the morphology of virus particles. Consistent with the previous reports (Seladi-Schulman, Steel, & Lowen, 2013), the pandemic strain of 2009 presented filamentous shape. However, the other CIVs produced spherical or small ovoid particles. KR07M negative-stained transmission electron microscope, concentrated through centrifugation with sucrose cushion, showed a wide range of virion shapes, including filaments and spheres. The larger spheres and irregular shapes depict damaged cell debris and filaments produced during preparation of the concentrated sample. It may reasonably be speculated that the M segment of CA04 conferred various degrees of filamentous morphology to CIV. This has correlation to the prevalence of filamentous particles and efficiency of cellular uptake (Figure 3), suggesting that pandemic M leads to increase virion-associated cellular uptake.

4 | DISCUSSION

Viral shedding is a critical parameter of viral pathogenicity, post-challenge in a host animal. In this study, three H3N2 CIV strains showed similar viral shedding albeit the ratio of cellular uptake appeared to be associated with their morphology in various ways. This trend is also observed between KR07 and KR07M, which carry identical gene segments and differ only in their M segment. Thus, our results show that the cellular uptake of influenza virus particles is dependent on the M segment and suggest that the efficiency of cell uptake is mediated by virion morphology. The gene product of the M segment is reported to affect the morphology of influenza virions (Bourmakina & Garcia-Sastre, 2003; Burleigh, Calder, Skehel, & Steinhauer, 2005; Elleman & Barclay, 2004; Roberts, Lamb, & Compans, 1998). Various residues of M protein have been identified as morphology determinants (Bourmakina & Garcia-Sastre, 2003; Roberts et al., 1998).

The genetic features of influenza A viruses allow continued emergence of new strains of the virus (Kasowski, Garten, & Bridges, 2011). Since the outbreak of the pH1N1 virus in 2009, new influenza A virus strains have developed by rearrangement events between the pH1N1 virus and other influenza A virus types. For example, H3N2 swine influenza virus (SIV) undergoes a rearrangement with the pH1N1 virus, resulting in a new H3N2 SIV strain carrying the M gene of the pH1N1 virus (Lindstrom et al., 2012). In previous studies, A/Puerto Rico/8/1934 (H1N1) containing the M gene of the pH1N1 and the remaining gene segments of A/Puerto Rico/8/1934 (H1N1) presented increased transmissibility in guinea pigs and mice (Chou et al., 2011; Lakdawala et al., 2011). Thus, it has been frequently reported that H3N2 SIV contains M gene from the pH1N1 virus.

To assess the replication and pathogenicity of CIVs in infection–naive hosts, we experimentally infected dogs with the viruses. Dogs showed efficient replication of KR07, KR07M and JS10 followed by morbidity (typical symptoms of respiratory disease); however, lower

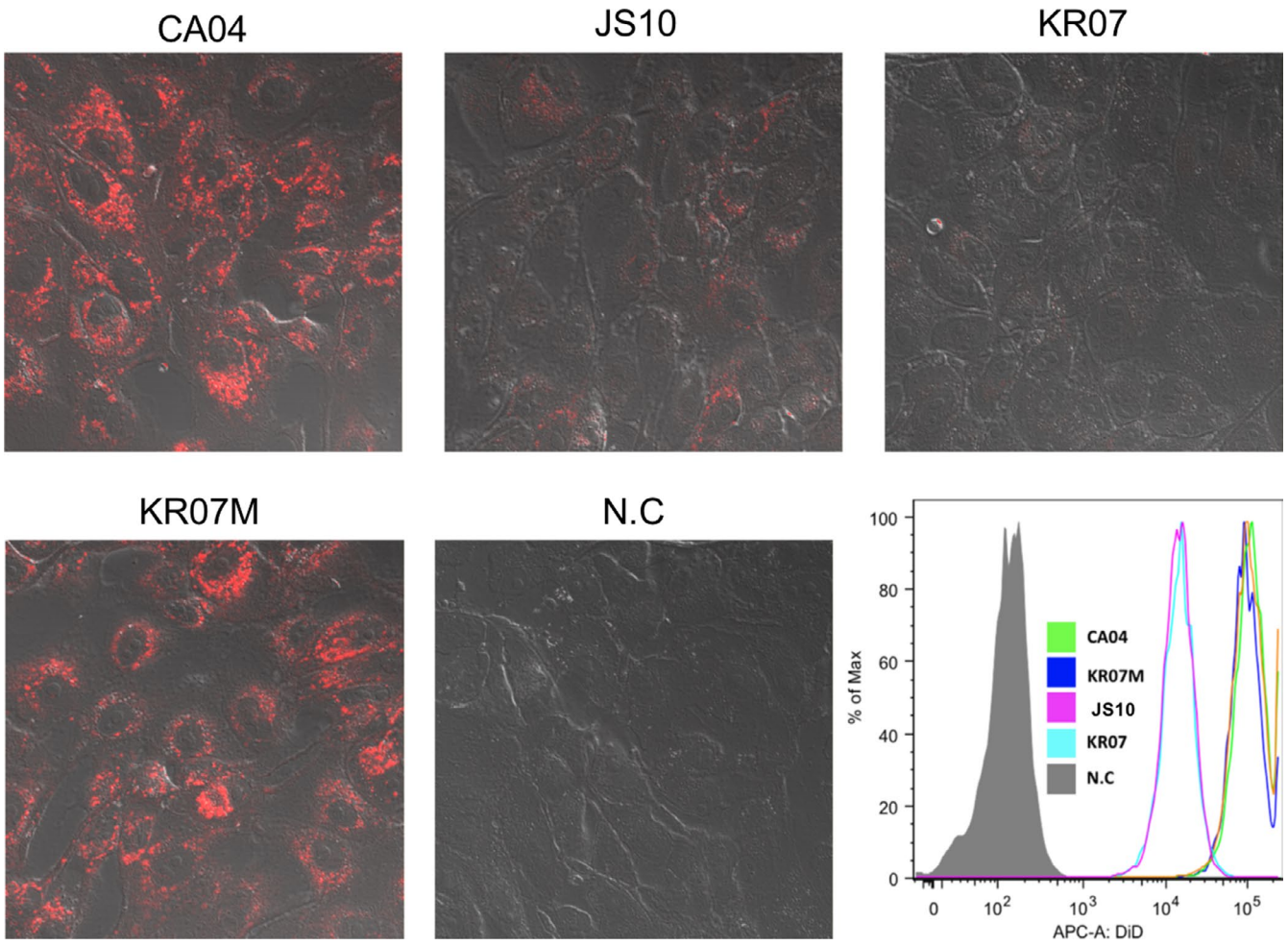

FIGURE 3 Intracellular virus distribution. 0.1 M.O.I. of purified influenza virus was labelled with DiD (Sigma-Aldrich, Korea) and incubated in MDCK cells for 1 hr at 37°C to allow virus binding. MDCK cells were imaged by CLSM and analysed by flow cytometry

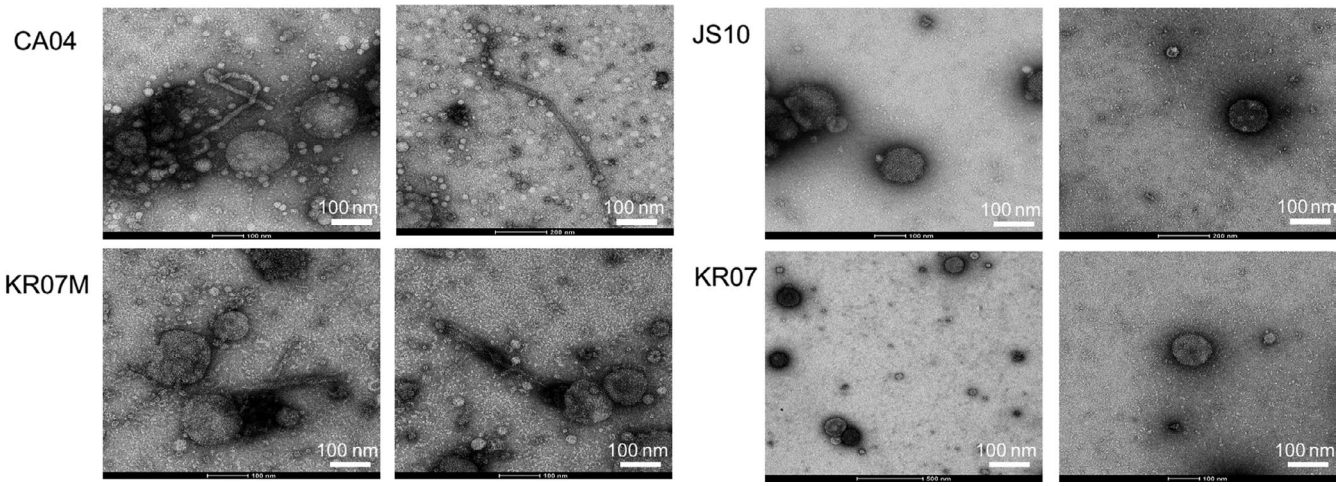

FIGURE 4 Virion morphology of CA04, KR07M, JS10 and KR07 virus. Virion morphologies shown as negative-stain images of influenza virions, as indicated, grown in embryonated chicken egg for 72 hr and concentrated through sucrose-cushioned centrifugation before fixing and staining

replication was observed in CA04. The infection of dogs with KR07M produced efficient virus shedding throughout the experiment, with high viral loads (5.9 log EID_{50}/mL). Consistent with the pathogenicity of KR07M, our study of cellular uptake indicated that the M segment of the pandemic strain is critical. These findings support the previous observation that the M gene segment of the pH1N1 virus

promotes replication of influenza A virus in the guinea pig model of influenza infection (Chou et al., 2011).

JS10 has naturally occurring two amino acid (2-aa) insertions at the distal end of the neuraminidase (NA) stalk. Consistent with previous studies, the long stalk strain showed more severe pathologic damage in mice and guinea pigs (Xie et al., 2018). However, no significant difference in cellular uptake between KR07 and JS10 was observed in this study. For the attachment and endocytosis of influenza virus, HA initiates the recognition and attachment of receptors on target cells, albeit NA plays the role of sialidase, facilitating release of the virus during budding from the host cell, which rarely affects cellular uptake. Therefore, it may reasonably be speculated that the 2-aa insertion of NA may not contribute to the cellular uptake of canine influenza viruses, even though the long stalk strain, JS10, has higher infectivity and wider organ tropism than KR07 (Xie et al., 2018).

To summarize, we characterized a strain of CIV that carries the M gene segment from the pH1N1 virus. KR07M showed efficient replication in natural host. Notably, it showed increased cellular uptake in vitro, compared to that of KR07 and JS10. The presence of the M gene from the pH1N1 virus is hypothesized to increase the replication of influenza viruses. Therefore, it may be assumed that the M gene contributes to the higher replication of canine influenza viruses.

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ETHICAL APPROVAL

The project was performed under the approval of Institutional Animal Care and Use Committee of Nanjing Agricultural University (approval no. SYXK 2017–0027).

DATA AVAILABILITY STATEMENT

All data will be available in the manuscript.

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