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Research Article

Combined insertion of basic and non-basic amino acids at hemagglutinin cleavage site of highly pathogenic H7N9 virus promotes replication and pathogenicity in chickens and mice

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ABSTRACT

Since mid-2016, the low pathogenic H7N9 influenza virus has evolved into a highly pathogenic (HP) phenotype in China, raising many concerns about public health and poultry industry. The insertion of a "KRTA" motif at hemagglutinin cleavage site (HACS) occurred in the early stage of HP H7N9 variants. During the co-circulation, the HACS of HP-H7N9 variants were more polymorphic in birds and humans. Although HP-H7N9 variants, unlike the H5 subtype virus, exhibited the insertions of basic and non-basic amino acids, the underlying function of those insertions and substitutions remains unclear. The results of bioinformatics analysis indicated that the PEVPKRKRTAR/G motif of HACS had become the dominant motif in China. Then, we generated six H7N9 viruses bearing the PEIPKGR/G, PEVPKGR/G, PEVPKRKRTAR/G, PEVPKGKRTAR/G, PEVPKGKRIAR/G, and PEVPKRKRR/G motifs. Interestingly, after the deletion of threonine and alanine (TA) at HACS, the H7N9 viruses manifested decreased thermostability and virulence in mice, and the PEVPKRKRTAR/G-motif virus is prevalent in birds and humans probably due to its increased transmissibility and moderate virulence. By contrast, the insertion of non-basic amino acid isoleucine and alanine (IA) decreased the transmissibility in chickens and virulence in mice. Remarkably, the I335V substitution of H7N9 virus enhanced infectivity and transmission in chickens, suggesting that the combination of mutations and insertions of amino acids at the HACS promoted replication and pathogenicity in chickens and mice. The ongoing evolution of H7N9 increasingly threatens public health and poultry industry, so, its comprehensive surveillance and prevention of H7N9 viruses should be pursued.

1. Introduction

Since its emergence in 2013, the novel H7N9 influenza virus has continued to circulate in Chinese mainland and has caused six waves of human infection (Zhu et al., 2018), while the first four of which causes 773 cases of human infection with a fatality rate of 42% (Wang et al., 2017). As of May 2021, a total of 1568 human cases and 616 related deaths had been reported. Previous studies indicated that the 2013 H7N9 influenza viruses isolated from birds were low pathogenic (LP) for chickens (Liu et al., 2013); however, by mid-2016, a novel H7N9 variant possessing combined insertions of basic and non-basic amino acids at the hemagglutinin cleavage site (HACS) was first isolated from Guangdong Province of China and was shown to be highly pathogenic (HP) in chickens (Jiang et al., 2017; Zhang et al., 2017). In the fifth wave that

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followed, the co-circulation of highly pathogenic avian influenza (HPAI) H7N9 viruses caused high genetic diversity and host adaption, posing serious public health concerns (Quan et al., 2018). Other studies have also indicated that those H7N9 variants are not only HP to mice but also highly transmissible in ferrets, posing the threats of pandemic potential in humans (Imai et al., 2017; Shi et al., 2018; Yang et al., 2018). After the H5/H7 bivalent vaccine for poultry was used in September 2017, the prevalence of H7N9 viruses in birds and humans decreased dramatically (Shi et al., 2018). However, these HP-H7N9 viruses re-emerged during 2019 and several immune escape mutations were observed (Zhang et al., 2020), indicative of the antigenic drift of H7N9 variants.

Hemagglutinin (HA) is particularly important to the infectivity and pathogenicity of the viral particles of influenza viruses (Webster and Rott, 1987). An essential step in the infection of influenza virus is the cleavage of the HA precursor, HA0, into subunits HA1 and HA2 through intracellular proteases (Rott, 1992). The insertion of multiple basic amino acids at the HACS had been described in the H5 subtype avian influenza virus (AIV) via repeated passaging in chickens (Ito et al., 2001). Unlike HP-H5 subtype viruses, HP-H7 subtype viruses showed the diversity of HACS, including the insertion of basic and non-basic amino acids at HACS. Previous studies have demonstrated that the insertion of basic amino acids at HACS in H7 subtype viruses plays an important role in HA cleavage, pH fusion, and viral pathogenesis (Abdelwhab et al., 2016; Seekings et al., 2020; Sun et al., 2019). Additionally, increased number of basic amino acids in combination with mutations adjacent to the HACS is important for viral virulence (Lee et al., 2006). These findings suggested that the insertions of polybasic amino acids and other amino acid substitutions of HA protein affected the virulence of H7 subtype viruses.

The HACS of emerging HP-H7N9 viruses in China were more polymorphic than their predecessors. Earlier reports confirmed four motifs at the HACS of HP-H7N9 viruses, including the motifs PEVPKRKRTAR/G, PEVPKGKRTAR/G, PEVPKGKRIAR/G, and PEVPKRKRAAR/G. The emphasis sign represents the mutation of amino acid, and the underline represents the insertion of amino acid. The PEVPKRKRTAR/G and PEVPKGKRTAR/G motifs were observed to circulate widely in poultry, whereas the PEVPKGKRIAR/G and PEVPKRKRAAR/G motifs were observed only in humans (Quan et al., 2018). Later, the PEVPKRKRTAR/G motif of the H7N9 virus became the dominant cleavage site motif in China (Fig. 1A). In our previous research, we observed that the four novel HPAI H7N9 variants carrying unique HACS motifs showed differences in virulence in chickens, mice, and ferrets (Bao et al., 2019; Qi et al., 2018), and we also found that isoleucine at site 335 of the most HP-H7N9 strains was mutated to valine. However, the contribution of basic and non-basic amino acids to the motifs remains unclear. In order to elucidate the function of those combined insertions and the substitution of adjacent residues, reverse genetics were conducted to generate six H7N9 recombinants containing different HACS motifs (i.e., PEIPKGR/G, PEVPKGR/G, PEVPKRKRTAR/G, PEVPKGKRTAR/G, PEVPKGKRIAR/G, and PEVPKRKRR/G) with the backbone of the A/chicken/Guangdong/G1/2013 [H7N9] (G1) virus and the HA gene of the A/chicken/Guangdong/HZ-3/2016 [H7N9] (HZ-3) virus (PEIPKGR/G cleavage site motif). We characterized the reconstituted viruses in terms of viral replication in avian and mammalian cells, thermostability and acid stability, cleavage efficiency, the virulence in mice, and pathogenicity and transmissibility in chickens.

2. Materials and methods

2.1. Data collection

All data regarding cases of influenza A (H7N9) were collected from the World Health Organization (http://www.who.int/en/), the EMPRES-I datasets of the Food and Agriculture Organization of the United Nations (http://empres-i.fao.org/eipws3g/#h=1), and the National Health Commission of the People's Republic of China (http://en.nhfpc.gov.cn/) before May 2021. The distributions of influenza A (H7N9) viruses available from GenBank (http://ncbi.nlm.nih.gov/genbank/) and Global Initiative on Sharing All Influenza Data (https://www.gisaid.org) before May 2021 were calculated in GraphPad Prism 7 (https://www.graphpad.com/) and ArcGIS 10.4 for Desktop (http://www.esri.com/software/arcgis/arcgis-for-desktop/).

2.2. Cells and viruses

Human embryonic kidney (HEK293T) cells, Madin-Darby canine kidney (MDCK), chicken embryo fibroblast (CEF), and human lung adenocarcinoma (A549) cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, 11054020, Paisley, Scotland, UK) with 10% fetal bovine serum (FBS, Biological Industries, 04-011-1A, Kibbutz, Israel) and 1% penicillin/streptomycin (Gibco, 15140122) at 37 °C in an atmosphere of 5% CO_2. Both A/chicken/Guangdong/G1/2013 [H7N9] (G1) and A/chicken/Guangdong/HZ-3/2016 [H7N9] (HZ-3) of H7N9 were obtained from the National and Local Joint Engineering Laboratory for the Medicine of Zoonosis Prevention and Control at South China Agricultural University's College of Veterinary Medicine. Viruses were propagated in 9- to 11-day-old specific-pathogen-free (SPF) embryonated chicken eggs and stored at −80 °C. All experiments with H7N9 influenza viruses were conducted in an animal biosecurity level 3 (ABSL-3) laboratory.

2.3. Generation of recombinant viruses by reverse genetics

The viral RNA of the *HA* gene of HZ-3 was amplified, and the mutations were introduced into the cleavage sites by inducing site-directed mutagenesis using PCR. The six H7N9 recombinants were rescued by reverse genetics after cloning the different *HA* genes and the seven remaining genes of the amplified G1 into plasmid pHW2000 (Hoffmann et al., 2000). Human embryonic kidney cell monolayers in 6-well plates were transfected at 80%–90% confluency with 4 μg of the eight plasmids (i.e., 500 ng of each plasmid) by using Lipofectamine 3000 (Invitrogen, L3000015, Carlsbad, CA, USA) according to the instructions of manufacturer. DNA and transfection reagent were mixed, incubated at room temperature for 5 min, and added to the cells. After 4 h, the mixture was replaced with Opti-MEM (Gibco, 11058021) containing 0.2% bovine serum albumin (BSA) and 1 μg/mL tolylsulfonyl-phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Sigma-Aldrich, T4049, Saint Louis, USA). The supernatant collected after transfection was inoculated into SPF embryonated chicken eggs, and the full-length genomes of all of the H7N9 viruses were confirmed by sequencing.

2.4. Replication kinetics

Replication kinetics was determined by inoculating CEF or A549 cells in the presence or absence of 1 μg/mL TPCK-treated trypsin. Next, 90%-confluent CEF and A549 cells were infected at a multiplicity of infection (MOI) of 0.01 and 0.1, respectively, washed twice with phosphate-buffered saline (PBS) after 1 h of incubation, and incubated with 1 mL of DMEM containing 0.2% BSA at 37 °C with 5% CO_2. Supernatants were sampled at 12, 24, 48, and 72 h after inoculation, and viral titers were determined by 50% tissue culture infective dose ($TCID_{50}$) assay in MDCK cells.

2.5. Western blot

CEF and A549 cells were inoculated with the wild-type and HA mutant constructs at an MOI of 1 in the presence of either 1 μg/mL TPCK-treated trypsin or no protease for 6 h in minimal essential medium containing 0.2% BSA. Proteins from the cell cultures were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and were electro-transferred onto nitrocellulose (NC) membranes. Polyclonal rabbit anti-HA antibody from A/Shanghai/1/2013H7N9 (Sino

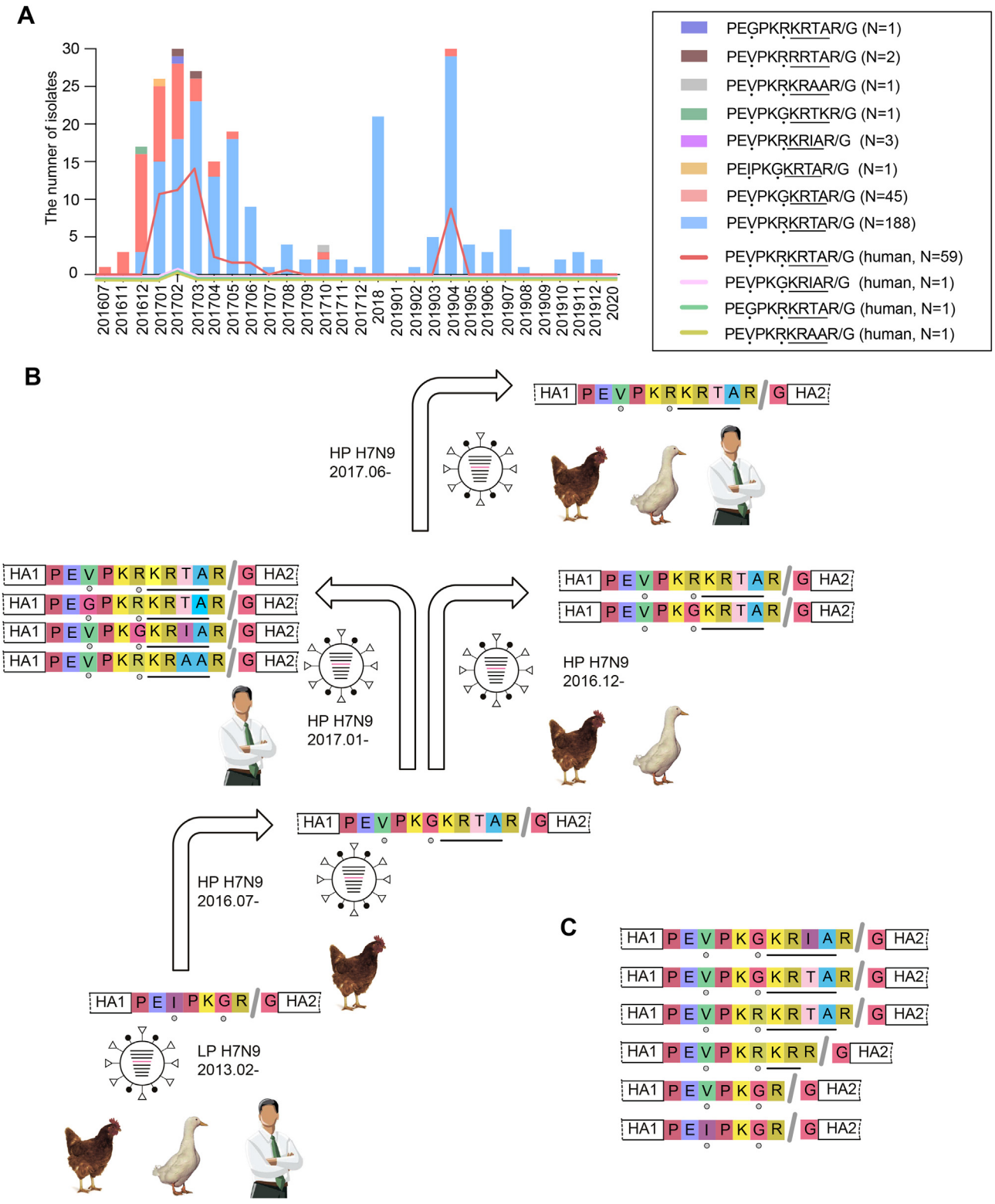

Fig. 1. The prevalence and evolution of HPAIV H7N9 in China. **A** Distribution of HPAIV H7N9 in China, 2016–2020. The bar chart represents HPAIV H7N9 in China, and the line graph represents human isolates. **B** Evolutionary history of HPAIV H7N9 in China. **C** The schematic diagram of cleavage site motifs of six recombinant viruses. HPAIV, highly pathogenic avian influenza virus; LP, Low pathogenic; HP, Highly pathogenic; HA, Hemagglutinin.

Biological, 40104-RP02, Beijing, China) (1:1000 dilution for 1 h at room temperature) was used to detect HA protein, whereas goat anti-rabbit IgG conjugated with horseradish peroxidase (LICOR, 926-32211, Lincoln, USA) (1:10,000 dilution for 1 h at room temperature) was used as a secondary antibody, followed by chemiluminescence detection (SuperSignal West Pico Chemiluminescent Substrate Kit; Pierce, Rockford, IL, USA). Last, the NC film was scanned with an Odyssey bicolor infrared laser imaging system (LI-COR Odyssey) to obtain a sample strip image. Grayscale analysis of individual bands was determined by ImageJ v1.8.0 (National Institution of Health, USA), and relative cleavage

efficiency of H7N9 viruses was calculated according to the equation HA1/(HA0 + HA1). Three independent replicates of HA cleavability were conducted in the experiment.

2.6. Viral thermostability and pH stability

Viral thermostability was measured by determining the loss of the virus titer after incubating the viruses at 50 °C for 0–5 h. Afterward, 1 mL (10^6 $TCID_{50}$/mL) virus-containing allantoic fluids were incubated at 50 °C for 0, 1, 2, 3, 4, and 5 h, respectively, and the infectivity of H7N9 viruses was assessed by $TCID_{50}$ assay in MDCK cells. Acid stability was determined by treating each virus at low pH. Different pH levels (4.0, 4.5, 5.0, 5.5, 6.0, 7.0, and 7.4) of PBS were mixed with 10^5 $TCID_{50}$/mL of H7N9 viruses, after which H7N9 viruses were incubated at 37 °C for 1 h. The viral titers of acid-treated H7N9 viruses were determined by $TCID_{50}$ assay in MDCK cells.

2.7. Animal experiments

In the first chicken experiment, the intravenous pathogenicity index (IVPI) test was performed according to the standard procedure of the World Organisation for Animal Health (https://www.oie.int/en/home/). Ten 6-week-old SPF chickens (Guangdong Dahuanong Animal Health Products Co., Ltd., Guangdong Province, China) were injected intravenously in the ulnar vein with 0.1 mL of a 1/10 dilution of H7N9 viruses. All chickens were observed for clinical symptoms for 10 days; at each observation, each chicken was classified as healthy (score of 0), sick (score of 1), severely sick (score of 2), or dead (score of 3). The IVPI index is the average score per chicken per day over 10 days.

In the second chicken experiment, nine chickens from each group were intranasally inoculated with 10^4 $TCID_{50}$/200 μL of six recombinant viruses, whereas nine chickens inoculated with 200 μL of PBS served as negative controls. After 24 h, nine naive chickens were introduced into a clean sealed containers with 60 × 26 × 27 cm^3 with infected chickens to assess transmission. Cloacal and throat swabs were collected at 3, 5, and 7 days post-infection (dpi) from the six infected and naive chickens. Three infected chickens in each group were euthanized at 3 dpi, and the lung and brain samples were collected for virus titration. In the same way, the contacted groups were euthanized at 4 dpi to test for the virus in the lungs and brains. Tissue and swab samples were collected for virus titration by $TCID_{50}$ assay. After 14 days, antibodies were detected in the serum samples collected from all surviving chickens.

In a separate experiment, nine 5-week-old SPF female BALB/c mice (Vital River Laboratory, Beijing, China) in six groups were anesthetized with CO_2 and inoculated intranasally with 10^6 $TCID_{50}$/50 μL H7N9 viruses. Nine mice were inoculated PBS as mock group. For 14 dpi, the mice in each group were monitored daily for clinical symptoms, weight loss, and mortality, and the mice were euthanized if they lost more than 25% of the initial body weight. Three mice in each group were euthanized at 4 dpi, after which the lung and brain samples were also collected. Viral titers were determined by $TCID_{50}$ assay on MDCK cells.

2.8. Statistical analyses

Data were presented as mean ± standard deviation and were analyzed with GraphPad Prism 5.0. The independent samples *t*-test was used for analysis, and *P* values less than 0.05 were considered to indicate statistical significance.

3. Results

3.1. Bioinformatic analysis of the H7N9 cleavage site

As of August 2021, 242 HA sequences of HP-H7N9 viruses were collected by the Global Initiative on Sharing All Influenza Data (https://www.gisaid.org), including 62 human-infecting H7N9 strains (Supplementary Table S1). Molecular characterization showed that the low pathogenic H7N9 viruses bearing the PEIPKGR/G cleavage site motif firstly emerged in China in February 2013. After mid-2016, H7N9 viruses containing PEVPKGKRTAR/G and PEVPKRKRTAR/G motifs, first isolated in Guangdong Province of China, had spread to more than 10 provinces (Supplementary Table S2). However, a steep increase in the PEVPKRKRTAR/G motif of HACS arose after January 2017 and had been stably maintained ever since (Fig. 1A and B). Additionally, approximately 95% of HACS in human-origin HP-H7N9 variants exhibited the PEVPKRKRTAR/G motif, while the HACS of two HP-H7N9 strains showed IA and AA alleles of non-basic amino acids (Fig. 1B, Supplementary Table S1). The amino acid residue at 335 site in the HA protein of the LP-H7N9 virus was more polymorphic, including changes with isoleucine (I) (99.6%), leucine (L) (0.16%), threonine (T) (0.12%), and valine (V) (0.16%). Interestingly, V residue at 335 site in HA protein appeared in 99.2% HP-H7N9 viruses (Supplementary Table S3). Taken together, our finding indicated that PEVPKRKRTAR/G-motif of bird- and human-origin H7N9 viruses had become the dominant motif in China.

3.2. Generation of H7N9 viruses

Eight-plasmid reverse genetic systems with the HA plasmid of HZ-3 and the seven plasmids of G1 were established. Six mutants differing in the HACS were generated by site-directed mutagenesis. The HACS of HZ-3 was mutated to generate the PEVPKGKRTAR/G and PEVPKGKRIAR/G motifs present in human cases. In order to assess the effects of non-basic amino acids TA of the HACS, the HACS of the PEVPKRKRR/G motif was also generated. In order to determine the function of I335V substitution, we replaced V with I in the 335 site of HA protein. The six recombinant H7N9 viruses were designated PEIPKGR/G, PEVPKGR/G, PEVPKRKRTAR/G, PEVPKGKRTAR/G, PEVPKGKRIAR/G, and PEVPKRKRR/G, respectively (Fig. 1C).

3.3. Cleavage efficiency of H7N9 viruses in CEF and A549 cells

The cleavage efficiency of the HA protein is a major determinant of the virulence of the AIV. In order to determine how the H7N9 viruses containing different cleavage sites influenced the activation of the cleavage of HA0, a Western blot analysis of CEF and A549 cells inoculated with the wild-type and HA mutant constructs at an MOI of 1 in the presence of either 1 μg/mL TPCK-treated trypsin or no protease for 6 h was performed. As expected, in the absence of TPCK trypsin, the HA protein of all mutants with monobasic HACS was not cleaved into HA1 and HA2; however, the HA0 carrying polybasic HACS was cleaved to HA1 and HA2 to varying degrees, whether in CEF or A549 cells (Fig. 2A and B). The HA protein of monobasic H7N9 viruses (PEVPKGR/G and PEIPKGR/G) can be cleaved into HA1 and HA2 in the presence of TPCK trypsin. Of the four H7N9 viruses with polybasic cleavage sites in the absence of TPCK-trypsin, PEVPKRKRTAR/G showed the most efficient cleavage efficiency and PEVPKGKRTAR/G the least, whereas the cleavage efficiency of PEVPKRKRR/G and PEVPKGKRIAR/G was moderate in CEF and A549 cells. Importantly, the cleavage efficiency of PEVPKRKRR/G ($P = 0.0085$) and PEVPKRKRTAR/G ($P = 0.0065$) were both higher than that of the PEVPKGKRTAR/G in CEF cells, whereas PEVPKGKRTAR/G showed more limited cleavage efficiency in A549 cells (Fig. 2B).

3.4. Impact of H7N9 viruses on viral thermostability and acid stability

The pH stability of HA protein has repeatedly been shown to be an important factor in the adaption of influenza viruses to efficiently transmit among chickens and ferrets (Russell et al., 2018). In order to investigate the effects of the acidic conditions of the viruses, six H7N9 recombinant viruses were incubated at pH levels ranging from 5.0 to 7.2 for 30 min. Viral titer assay showed that all H7N9 viruses displayed a decreased viral activity at pH 5.5, and PEIPKGR/G exhibited significantly reduced infectivity ($P < 0.001$) compared to the PEVPKGR/G (Fig. 2C).

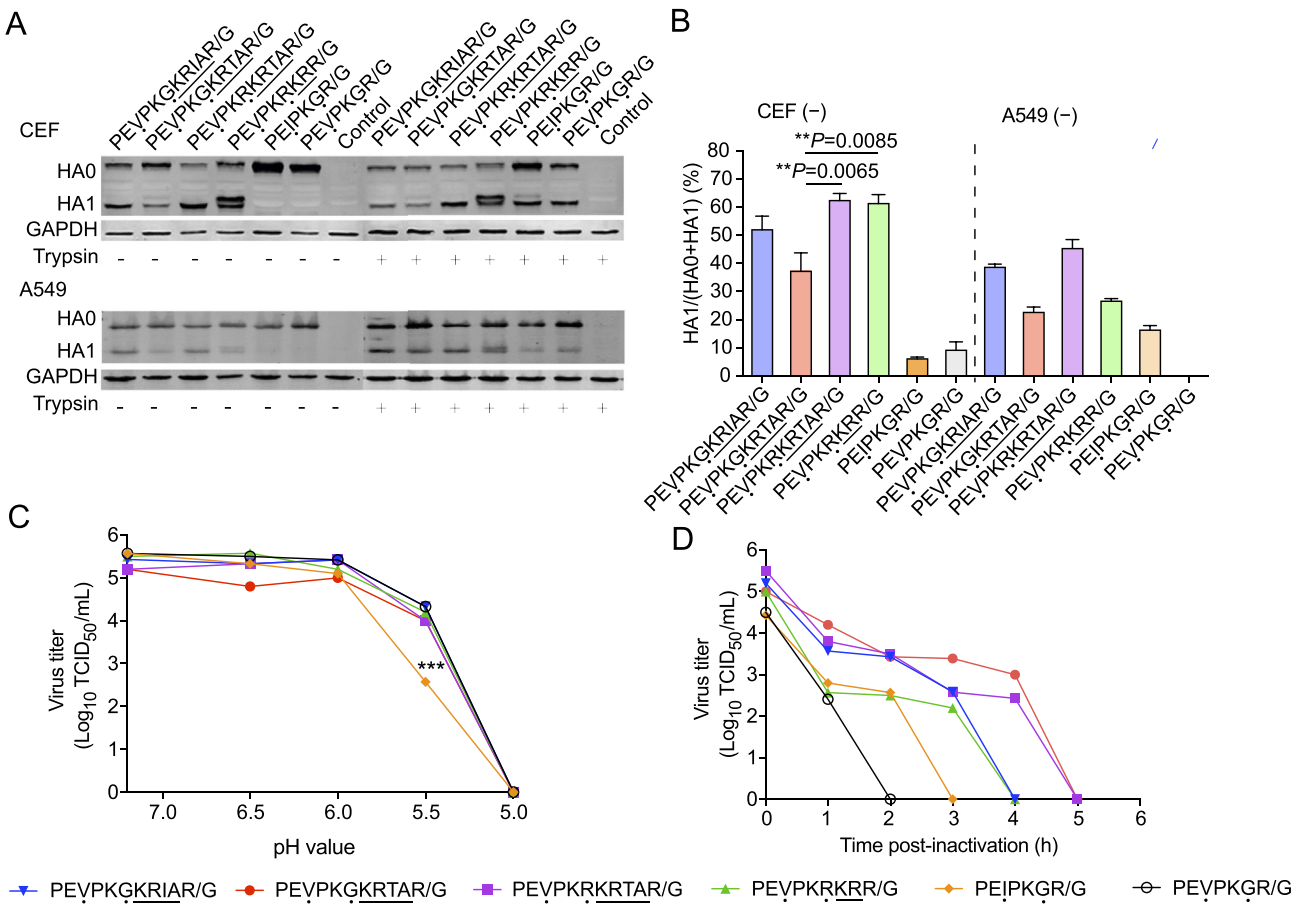

Fig. 2. HA cleavage efficiency and stability of H7N9 viruses. **A** Western blots of cell cultures from CEFs and A549 cell cultures inoculated with H7N9 viruses at a multiplicity of infection (MOI) of 1 for 6 h, treated with (+) or without (−) trypsin. **B** Grayscale analysis of H7N9 recombinant viruses. Cleavage efficiencies were calculated according to the equation HA1/(HA0 + HA1). Each point on the curve is the mean ± standard deviation from three independent experiments. The independent samples *t*-test was used for analysis. **C** Stability after incubation of viruses at pH 5, 5.5, 6, 6.5, or 7.2. **D** Heat stability at 50 °C for 1, 2, 3, 4, and 5 h. Statistical significance was analyzed using an unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

These results suggested that the I335V substitution increased the resistance against the acid of the H7N9 viruses. In addition, we investigated the thermostability of the H7N9 viruses containing different HACS. Our result showed that the monobasic PEIPKGR/G and PEVPKGR/G viruses lost all infectivity after incubation for 3 h. Conversely, PEVPKRKRR/G and PEVPKGKRIAR/G were inactivated after 4 h of incubation, suggestive of considerable tolerance to high temperatures. PEVPKRKRTAR/G and PEVPKGKRTAR/G, inactivated after 5 h of incubation, were the most resistant (Fig. 2D). Overall, these results indicated that the insertion of basic and non-basic amino acids enhanced the thermostability of the H7N9 viruses.

3.5. Replication of H7N9 viruses in CEF and A549 cells

To compare the replicative abilities of H7N9 recombinant viruses containing different multibasic cleavage sites (MBCSs), viral replication kinetics in A549 and CEF cells was determined. Six H7N9 viruses showed different replicative phenotypes in CEFs (Fig. 3A and B). Statistical analysis of PEVPKGR/G was conducted compared to the PEIPKGR/G. Statistical analysis of PEVPKGKRTAR/G, PEVPKRKRTAR/G, and PEVPKRKRR/G were conducted compared to the PEVPKGKRIAR/G, respectively. Among them, PEVPKGKRTAR/G replicated more efficiently after 12 h and attained the highest titer, and PEVPKRKRR/G had the same replicative tendency as PEVPKRKRTAR/G. By contrast, PEVPKGKRIAR/G decreased the viral replication after reaching the peak at 24 h. Interestingly, PEVPKGR/G and PEIPKGR/G with monobasic mutants exhibited differences in viral titer at different time points, although the viral titers of PEVPKGR/G were higher than those of PEIPKGR/G, especially in the prophase of replication (Fig. 3A).

In A549 cells, the growth rate of PEVPKRKRR/G was significant higher ($P < 0.05$) than that of PEVPKGR/G at 24 h in the presence of TPCK-treated trypsin (Fig. 3D). In the absence of TPCK-treated trypsin, however, the replicative ability of H7N9 viruses containing different MBCSs was not significantly different, nor was the monobasic H7N9 viruses in A549 cells (Fig. 3C). These findings indicated that the insertion of amino acids KRTA promoted the replicative ability in CEFs and A549 cells, and the I335V substitution promoted the early replication of the H7N9 virus in CEF cells.

3.6. Pathogenicity and transmission of H7N9 viruses in chickens

The virulence of six H7N9 viruses was assessed by intravenously inoculating 0.1 mL of a 10-fold diluted allantoic fluid into 6-week-old SPF chickens according to the standard procedure of the World Organisation for Animal Health. The IVPI index of the PEVPKGKRTAR/G, PEVPKRKRTAR/G, PEVPKRKRR/G, and PEVPKGKRIAR/G strains was similar (2.79, 2.7, 2.73, and 2.78, respectively), suggestive of HP phenotype in chickens. All chickens infected with HP-H7N9 viruses died within three days (Fig. 4A). However, chickens infected with PEVPKGR/G and PEIPKGR/G, with an IVPI of 0, did not show any symptoms of the disease during the 10-day period, indicative of LP phenotype in chickens.

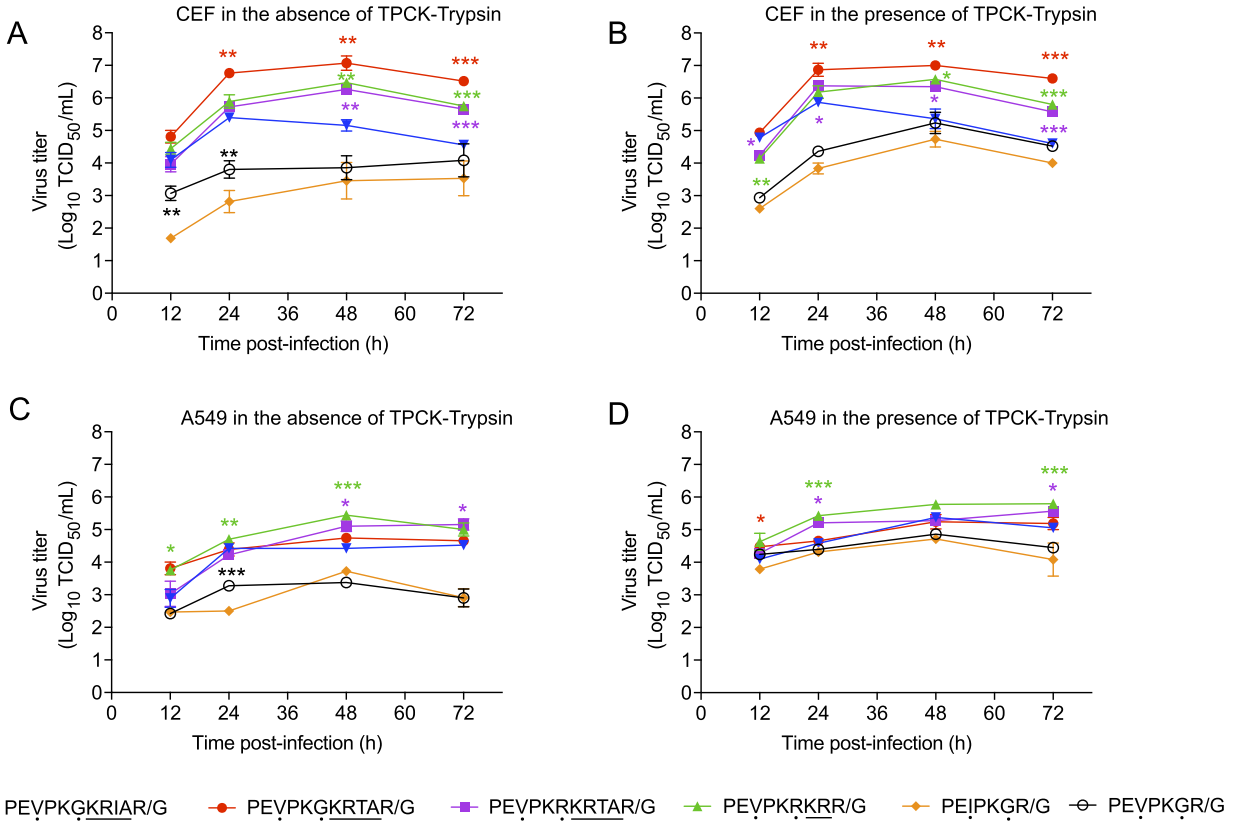

Fig. 3. The growth kinetics of H7N9 viruses. **A** CEFs were inoculated with viruses at a multiplicity of infection (MOI) of 0.01 in the absence of trypsin. **B** CEFs were inoculated with viruses at an MOI of 0.01 in the presence of trypsin. **C** A549 cells were infected with the indicated viruses at an MOI of 0.1 in the absence of trypsin. **D** A549 cells were infected with the indicated viruses at an MOI of 0.1 in the presence of trypsin. Samples were collected at 12, 24, 48, and 72 h post-inoculation (hpi). Viral titers were determined via 50% tissue culture infectious dose ($TCID_{50}$) assay. Each point on the curve is the mean ± standard deviation from three independent experiments. Statistical analysis of PEVPKGR/G was conducted compared to the PEIPKGR/G. Statistical analysis of PEVPKGKRTAR/G, PEVPKRKRTAR/G, and PEVPKRKRR/G were conducted compared to the PEVPKGKRIAR/G, respectively. Statistical significance was analyzed using an unpaired *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

In order to determine which mutations at cleavage sites abolished virulence and transmissibility in chickens, six-week-old SPF chickens were intranasally inoculated with 10^4 $TCID_{50}$/200 μL of H7N9 viruses. After 24 h, nine uninfected chickens were placed in a clean sealed container and housed with the inoculated chickens. Four groups of chickens with recombinant viruses containing MBCSs showed clinical symptoms and tissue lesions and died within 5–7 dpi (Fig. 4B). However, chickens infected with PEVPKGR/G and PEIPKGR/G did not exhibit any clinical symptoms. Three inoculated and exposed chickens in each group were dissected in order to evaluate viral titers in lung and brain at 3 dpi. PEVPKGKRTAR/G, PEVPKRKRR/G, PEVPKGKRIAR/G, and PEVPKRKRTAR/G achieved relatively high but not significantly different viral titers in lungs compared to the monobasic mutants. By contrast, in brain, PEVPKRKRTAR/G was detected only in one of the three chickens. Importantly, PEVPKGR/G replicated more efficiently than PEIPKGR/G in lungs (Fig. 4D).

Contacted chickens of the PEVPKGKRTAR/G, PEVPKRKRR/G, and PEVPKRKRTAR/G viruses died within 7 days; however, 50% of the chickens directly exposed to PEVPKGKRIAR/G survived, which underscored the difference of transmission in chickens (Fig. 4C). Beyond the contacted group, the viral titer of lung samples in the PEVPKGKRTAR/G group was significantly higher than that of the PEVPKRKRTAR/G group ($P = 0.015$). Only PEVPKRKRR/G, with an average titer of 2.73 lg$TCID_{50}$/100 μL (Fig. 4E), was detected in the brains of the contacted chickens. Furthermore, virus shedding was detected at 3 and 5 dpi both in throat and cloacal swabs. The chickens that survived from PEVPKGKRIAR/G contact, showed virus shedding at 5 dpi and seroconversion on 14 dpi. Hemagglutinin inhibition assay showed that four contacted chickens in the PEVPKGR/G group were infected, whereas only one was infected in the PEIPKGR/G group (Table 1), which indicated that I335V substitution enhanced the susceptibility, virulence, and transmissibility of H7N9 virus in chickens.

3.7. Pathogenicity of H7N9 viruses in mice

In order to investigate the effects of H7N9 viruses with different HACS in mammals, nine five-week-old female BALB/c mice weighing 14g–16 g were intranasally inoculated with 10^6 $TCID_{50}$/50 μL of each H7N9 viruses. The body weight of the mice infected with PEVPKGR/G virus increased during the observation period. Mice infected with PEVPKRKRR/G and PEVPKGKRIAR/G showed slight weight loss (< 5%) at about 6 dpi, and one mouse infected with PEVPKGKRIAR/G died at 8 dpi (Fig. 5B). However, mice infected with PEVPKRKRTAR/G showed significant weight loss at 6 dpi compared with the PEVPKGR/G group ($P = 0.0003$), and mice infected with PEVPKGKRTAR/G showed significant weight loss at 7 dpi compared with PEVPKGR/G ($P = 0.0001$) (Fig. 5A). Surprisingly, the monobasic mutants (i.e., PEIPKGR/G) showed clear symptoms of infection, including shaggy hair and abdominal breathing, after the second day of infection, followed by 15% of weight loss compared to the initial weight and death, with a mortality rate of 35%. In addition, each H7N9 viruses can replicate efficiency in lungs of the infected mice (Fig. 5C). The PEVPKGKRTAR/G ($P = 0.0081$) and

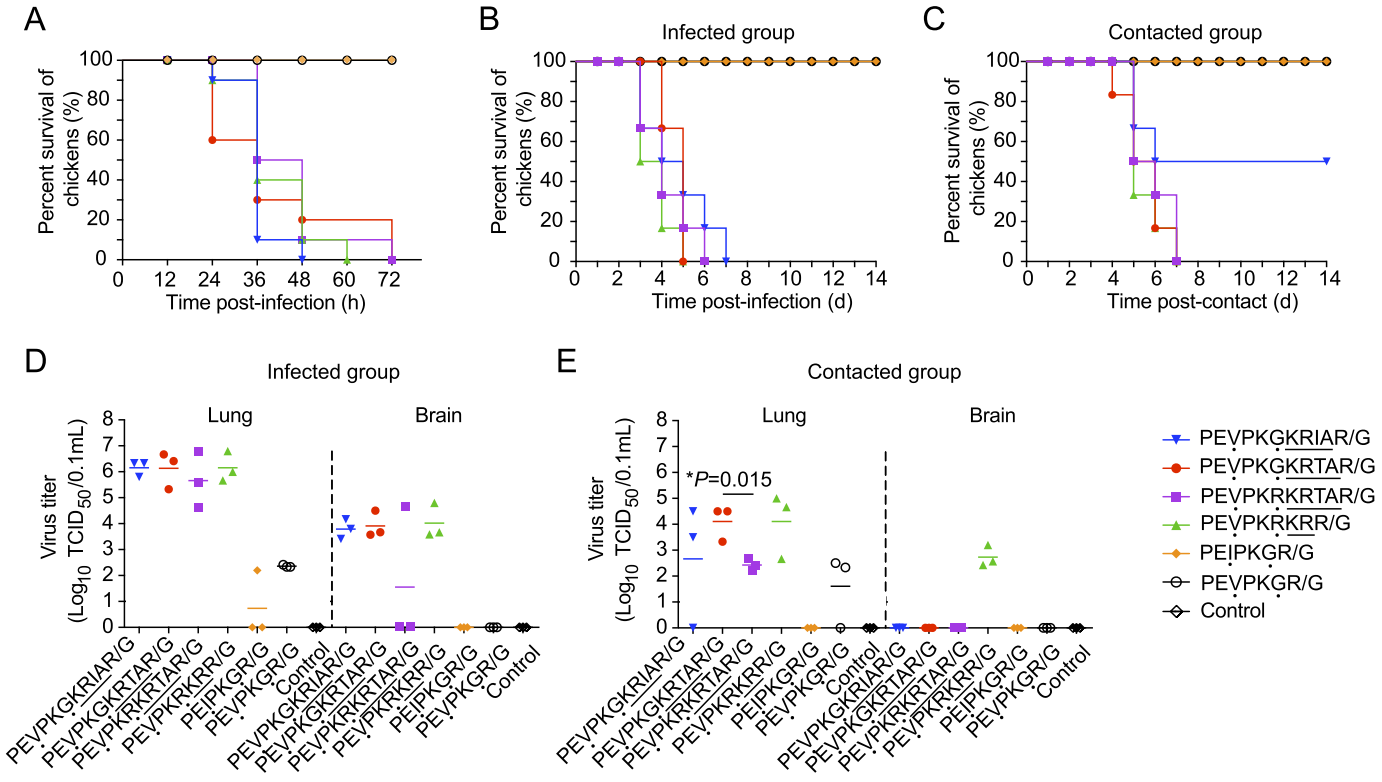

Fig. 4. Pathogenicity of the H7N9 viruses in chickens. **A** Survival rate of IVPI chickens. **B** Survival rate of inoculated chickens. Six groups of nine 6-week-old SPF chickens were infected intranasally with 10^4 $TCID_{50}$/200 μL of each virus. **C** Survival rate of SPF chickens in direct contact with infected chickens. **D** Viral titers in lungs and brains of three infected chickens. **E** Viral titers in lungs and brains of three contacted chickens. Each point on the curve is the mean ± standard deviation from three independent experiments. Statistical significance was analyzed using an unpaired *t*-test: $^*P < 0.05$. Viral titers were determined via $TCID_{50}$ assay. IVPI, intravenous pathogenicity index; $TCID_{50}$, 50% tissue culture infective dose.

Table 1
Virus titers in chicken cloacal and oropharyngeal swabs[a].

Virus		Oropharyngeal swabs, n			Cloacal swabs, n			Seroconversion HI antibody titers (14 dpi), n (range)	IVPI	Cleavage site sequence
		3 dpi	5 dpi	7 dpi	3 dpi	5 dpi	7 dpi			
PEVPKRKRTAR/G	Infected	4/6	2/2	–	2/6	1/2	–	–	2.7	PEVPKRKRTAR/GLF
	Contact	1/6	3/3	–	1/6	1/3	–	–		
PEVPKGKRTAR/G	Infected	6/6	–[b]	–	6/6	–	–	–	2.79	PEVPKGKRTAR/GLF
	Contact	4/6	3/3	–	2/6	3/3	–	–		
PEVPKRKRR/G	Infected	1/4	–	–	0/4	–	–	–	2.73	PEVPKRKRR/GLF
	Contact	0/6	1/2	–	0/6	2/2	–	–		
PEVPKGKRIAR/G	Infected	3/4	2/2	–	2/4	1/2	–	–	2.78	PEVPKGKRIAR/GLF
	Contact	0/6	2/4	1/3	0/6	1/4	1/3	1/3 (1024)		
PEVPKGR/G	Infected	3/6	1/6	0/6	0/6	1/6	0/6	6/6 (256–1024)	0	PEVPKGR/GLF
	Contact	2/6	2/6	1/6	1/6	1/6	1/6	4/6 (64–256)		
PEIPKGR/G	Infected	2/6	3/6	0/6	2/6	2/6	0/6	6/6 (256–1024)	0	PEIPKGR/GLF
	Contact	0/6	0/6	0/6	1/6	0/6	0/6	1/6 (64)		
MOCK infection		0/10	0/10	0/10	0/10	0/10	0/10	0	0	

IVPI, intravenous pathogenicity index; *HI*, hemagglutinin inhibition assay.

[a] Nine 5-week-old SPF chickens were inoculated with 10^4 $TCID_{50}$ of six recombinant viruses in a volume of 200 μL. Cloacal and oropharyngeal swabs were collected on 3, 5, and 7 days post-infection (dpi) from infected and naive chickens.

[b] The chickens have died out at sample collecting time point.

PEVPKRKRTAR/G ($P = 0.0109$) significantly increased viral titers in the lungs of mice compared to that of the PEVPKGR/G group, indicating that the insertion of KRTA increased the pathogenicity of mice. Moreover, none of each H7N9 viruses were detected in brain of mice (Fig. 5C). All surviving mice recovered at 14 dpi.

4. Discussion

The HP-H7N9 viruses emerged in Guangdong Province since mid-2016 and widely spread in poultry populations. In our study, we investigated the biological function of the unique HACS motifs of H7N9 viruses by focusing on the effects of the combination of basic and non-basic amino acids in H7N9 viruses. Related to the findings proposing that the cleavability closely related to virulence (Horimoto and Kawaoka, 1997), our results revealed that the insertion of MBCSs significantly increased the cleavage efficiency of HA protein when compared to that of the monobasic H7N9 viruses. PEVPKRKRTAR/G showed significantly higher cleavage efficiency than that of PEVPKGKRTAR/G, likely because the presence of arginine at the 338 site increased the cleavage efficiency of HA protein. At the same time, PEVPKRKRR/G manifested high

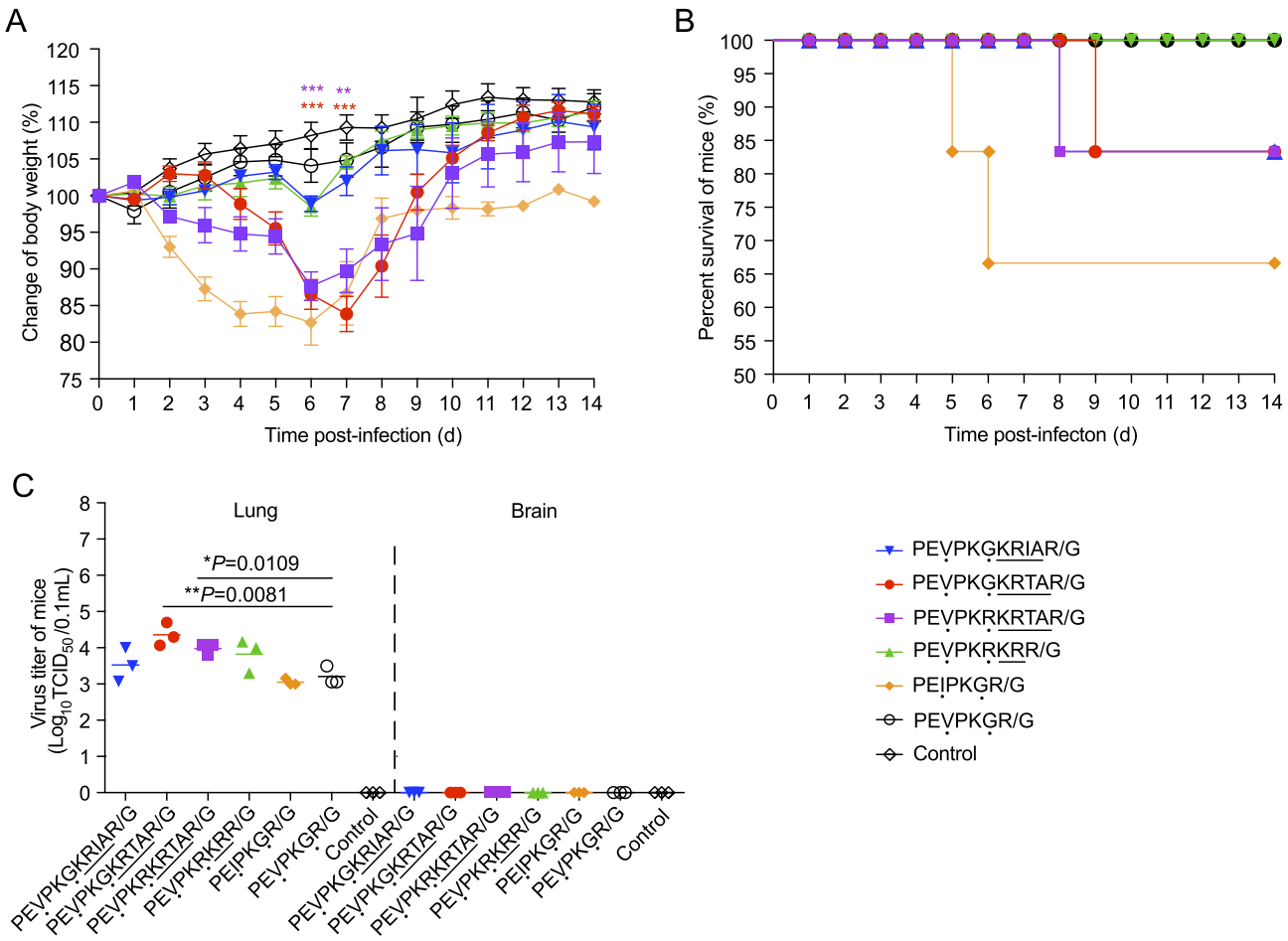

Fig. 5. Pathogenicity of the H7N9 viruses in mice. Six groups of nine female BALB/c mice were intranasally inoculated with 10^6 $TCID_{50}$/50 μL of the viruses or with PBS as negative controls. Mice that lost more than 25% of their initial weight were found dead or euthanized. **A** Relative weight changes in the mice compared to the initial body weight. **B** Survival rate of mouse. **C** Viral titers in lungs and brains of mice at necropsy 4 days post-infection. Each point on the curve is the mean ± standard deviation from three independent experiments. Statistical significance was analyzed using an unpaired *t*-test: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

cleavage efficiency in avian cells, which suggested the broader tissue tropism of different MBCSs in H7N9 viruses.

HA stability was regarded as an important contributor to the infectivity, transmissibility, and pathogenicity of influenza viruses in different hosts. After the virus internalized by endocytosis, the acidic pH in the endosomes triggers an irreversible conformational change in HA protein, that creates a viral envelope with an endosomal membrane (Bullough et al., 1994). High temperatures at a neutral pH can also induce conformational changes in HA that prompt protein inactivation (Carr et al., 1997; Scholtissek, 1985). In this study, the environmental stability of the H7N9 viruses at different pH levels was consistent; however, PEVPKRKRTAR/G and PEVPKGKRTAR/G became inactive after 5 h of incubation, which suggested that the insertion of non-basic amino acids (TA) increased the resistance to higher temperature. As reported, influenza viruses with high stability were more infectious in ferrets (Cotter et al., 2014). Compared to TA, amino acid IA exhibited low virulence and transmissibility in contacted chickens, which might have been responsible for the lower viral thermostability. Although the insertion of amino acid KRTA in HACS did not influence the pH stability of the H7N9 virus, the I335V substitutions in HA protein enhanced the pH stability. Additionally, other studies have also demonstrated that a lysine to glutamic amino acid mutations at the HA2-64 position increases the threshold pH for HA activation in HP-H7N9 viruses (Sun et al., 2019), indicating that the mutations in the HA protein of H7N9 viruses affect the pH fusion.

In *in vivo* experiment, H7N9 viruses containing MBCS were high pathogenicity in chickens. However, all of the HP-H7N9 viruses exhibited significant differences in contacted groups. PEVPKGKRIAR/G showed limited transmission in chickens via direct contact. In addition, we found that the replicative ability of PEVPKGKRIAR/G virus was lower than that of the other H7N9 viruses in CEF cell and SPF chickens, which indicated that it had not adapted in poultry. Interestingly, the viral titers in lung samples of infected and contacted chickens infected with PEVPKGR/G were higher than that of PEIPKGR/G virus, indicating that I335V substitution increased the transmission in chickens. The viral titers in lung samples of chickens infected with PEVPKGKRTAR/G virus were higher than that of the other groups, whereas PEVPKRKRTAR/G showed the mild virulence in chickens than other H7N9 viruses containing MBCS. In our assessment, PEVPKRKRTAR/G, a moderately virulent strain, though exhibited the high pathogenicity and strong transmission in chickens, might leave individuals of the species with greater fitness to circulate in poultry.

In previous studies, HP-H7N9 was not lethal in mice (Shi et al., 2017) or else killed certain mice (Liu et al., 2018) in absence of mutations at the PB2 protein, which was corroborated in our results. The phenomenon that we observed, namely, increased virulence when infected mice and varying virulence between different MBCSs, suggested that the MBCSs represented an important factor in the virulence in mice. Another study reported that the single amino acid of the HACS affected the pathogenicity and neurotoxicity of the H5N1 influenza virus in mice (Zhang et al., 2012). Notably, we observed that the insertion of non-basic amino acids TA and IA in H7N9 viruses caused death in mice despite having the same viral titer, which suggested that HPAIV H7N9 inserted with a

non-basic amino acid posed a potential threat to humans. PEVPKRKRR/G does not contain non-basic amino acids, despite being highly pathogenic in chickens. However, the virulence in mice was not more significant than that of PEVPKRKRTAR/G, which was consistent with the cleavage efficiency in A549 cells. We also observed that mice infected with the PEIPKGR/G strain showed a sharp drop in body weight, followed by death. In our previous study, we also found polymorphic mutations in the PB2 protein of dead mice infected with HP-H7N9 viruses by next-generation sequencing (Qi et al., 2018). Consequently, the death of mice might be caused by dynamic mutations of other genes.

There remains an important question to answer regarding the mechanism of the insertion of amino acids at the HACS in H7N9 viruses. Researchers have reported that influenza viruses cause the insertion of exogenous nucleotide sequences into the HACS by nonhomologous recombination (Khatchikian et al., 1989; Orlich et al., 1994; Suarez et al., 2004), and that all intersegmental recombination involving HA described thus far has only involved the H7 subtype (Khatchikian et al., 1989). Therefore, we hypothesized that the amino acid KRTA at the initial HACS of the HP-H7N9 virus could be obtained by inserting exogenous nucleotides. The BLAST search for that nucleotide sequence in the influenza database revealed that the most closely related sequence was the PB1 genome of many viruses of the H6 subtype, with 12 nucleotide identities at positions 2226–2251. We also found entirely identical nucleotides in chromosome 1 of gallus, located in the range of dihydrolipoamide dehydrogenase, and found that the two ends contained two nucleotides that matched the viral sequence, that was, the matching of 16 nucleotides. The results above suggest that the occurrence of the insertion of amino acids at the HACS is probably due to recombination events.

5. Conclusions

In conclusion, we found that the combination of basic and non-basic amino acids improved the cleavage efficiency and stability of H7N9 viruses while reducing their virulence in chickens. Notably, H7N9 viruses with non-basic amino acid insertion (TA) caused the death of mice. The PEVPKRKRTAR/G motif has been widespread in poultry and human isolates. Therefore, the comprehensive surveillance and evaluation of the mutations identified should be urgently undertaken in order to assess the potential of the influenza virus epidemic to morph into a pandemic.

Data availability

All the data generated during the current study are included in the manuscript and/or the Supplementary data. Additional data related to this article may be requested from the authors.

Ethics statement

All animal experiments were carried out in ABSL-3 facilities in compliance with the biosafety committee of ABSL-3 Laboratory of South China Agriculture University (CNAS BL0011) protocols. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at SCAU and were carried out in accordance with the approved guidelines (2017A002).

Author contributions

Zhou Aobaixue: conceptualization, investigation, data curation, formal analysis, writing-original draft. Zhang Jiahao: conceptualization, investigation, data curation, formal analysis, writing-original draft, writing-review & editing. Li Huanan: investigation, formal analysis. Xu Qiang: investigation. Chen Yiqun: investigation. Li Bo: investigation. Liu Wanying: investigation. Su Guanming: investigation. Ren Xingxing: investigation. Lao Guangjie: investigation. Luo Baozheng: writing-review & editing. Liao Ming: conceptualization, data curation, formal analysis, funding acquisition, project administration. Qi Wenbao: conceptualization, data curation, formal analysis, funding acquisition, project administration, writing-review & editing. All the authors reviewed and revised the first and final drafts of this manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2022.01.001.

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