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Short communication

Protective efficacy of an inactivated chimeric H7/H5 avian influenza vaccine against highly pathogenic avian influenza H7N9 and clade 2.3.4.4 H5 viruses

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ABSTRACT

The highly pathogenic avian influenza (HPAI) H5 and H7N9 viruses pose a serious challenge to public health and the poultry industry in China. In this study, we generated a chimeric H7/H5 recombinant virus that expressed the entire HA1 region of the HPAI A/chicken/Guangdong/RZ/2017(H7N9) virus and the HA2 region of the HPAI A/chicken/Fujian/5/2016(H5N6) viruses. The resulting chimeric PR8-H7/H5 virus exhibited similar growth kinetics as the parental PR8-H5 and PR8-H7 viruses *in vitro*. The inactivated chimeric PR8-H7/H5 vaccine induced specific, cross-reactive hemagglutination inhibition antibodies against the H7 virus only but induced serum-neutralizing antibodies against both H7 and H5 viruses. Furthermore, the inactivated chimeric PR8-H7/H5 vaccine significantly reduced virus shedding and protected chickens from challenge with the HPAI H5N6 and H7N9 viruses. Our results suggested that the inactivated chimeric PR8-H7/H5 vaccine was effective against HPAI H5 and H7N9 viruses in chickens.

1. Introduction

H5N1 avian influenza viruses (AIVs) have been detected in more than 60 countries and cause great economic losses to the worldwide poultry industry (Swayne, 2012). Of note, H5N1 AIVs have become enzootic in domestic poultry and wild birds in Asia, Europe, Africa, and North America (Claas et al., 1998; Eagles et al., 2009; Ip et al., 2015; Jiang et al., 2017; Monne et al., 2008; Pasick et al., 2015; Peiris et al., 2007; Zhao et al., 2013). Sequence analyses of hemagglutinin (HA) genes have shown that H5 viruses have evolved into diverse clades and subclades (Li et al., 2010). Viruses in clade 7.2 have been detected in chickens in several provinces in northern China and clades 2.3.2.1 and 2.3.4.4 continue to co-circulate in wild birds and poultry in several countries (Li et al., 2010). However, recent molecular epidemiological surveys of AIVs detected no clade 7.2 viruses and a significant reduction in the circulation of clade 2.3.2.1 viruses since 2015 (unpublished data). Currently, clade 2.3.4.4 viruses are the dominant epidemic strains and N6 is the main NA subtype.

Novel H7N9 viruses isolated from samples collected at live poultry markets in China in 2013 had low pathogenicity in poultry. However, since December 2016, H7N9 viruses with novel polybasic amino acid sequences at HA cleavage sites, which are associated with increased pathogenicity in poultry, have been detected in both human patients and chickens. These viruses were found to be highly pathogenic in chickens in particular (Wang et al., 2017). Since March 2017, H7N9 highly pathogenic AIVs (HPAIVs) have caused in excess of 26 outbreaks in 10 provinces in China, resulting in great economic losses to the poultry industry.

Vaccination is an important strategy used to control AIV infection in poultry (Swayne, 2012; Swayne et al., 2014). To address the problem of AIV infection, the government of China implemented a mass poultry vaccination program. Since August 2017, the H5 Re-8 and H7 Re1 combination vaccine has been used to control H5 and H7N9 HPAIVs throughout China. Despite the availability of this efficacious combination vaccine, vaccine production requires more embryonated chicken eggs (ECEs), an alternative vaccine (*i.e.* a bivalent recombinant vaccine expressing both corresponding antigens) would be more advantageous. Furthermore, one important problem during mass vaccination should

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be considered that avian influenza is characterized by non-sterile immunity, and immunized poultry with high antibody titers are sometimes viraemic (shedding and spreading viruses), but asymptomatic.

Killed influenza virus vaccines induce anti-HA antibodies that specifically target antigenic sites in the globular head domain of the HA1 region and block receptor binding (Chiu et al., 2009; Hashem, 2015). These responses are typically strain specific, as the induced antibodies have potent neutralizing activity against homologous strains but do not cross-react with HAs of other influenza virus subtypes (Sui et al., 2009). In contrast, the HA2 stalk domain, although less immunogenic (Tan et al., 2012; Toroghi and Momayez, 2006), is relatively well conserved among different subtypes (Pica and Palese, 2013). Furthermore, broadly neutralizing antibodies against the HA2 stalk domain have been isolated (Sui et al., 2009; Tan et al., 2012). Here, we generated a novel chimeric H7/H5 virus that expressed the entire HA1 portion of H7N9 and the HA2 region of the heterosubtypic virus H5N6. We then evaluated the immunogenicity and protective efficacy of the chimeric vaccine against clade 2.3.4.4 H5N6 and H7N9 HPAIV infections in chickens.

2. Materials and methods

2.1. Ethics statement

All procedures in animal experiments met the requirements and were approved by the Animal Welfare Ethics Committee of the China Animal Health and Epidemiology Center (Approval Number: # CAHEC-2017-017). All applicable international, national, and institutional guidelines for the care and use of animals were followed.

2.2. Viruses and cells

The HPAIVs A/chicken/Fujian/5/2016(H5N6) (FJ/5; clade 2.3.4.4) and A/chicken/Guangdong/RZ/2017(H7N9) (GD/RZ) were isolated from dead chickens and propagated in 10-day-old specific pathogen-free (SPF) ECEs. Human embryonic kidney (HEK) 293 T cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum at 37 °C in an atmosphere of 5% CO_2.

2.3. Construction of the pHW-H7/H5 HA plasmid

To construct the chimeric H7/H5 HA gene, the HA1 sequence from GD/RZ and the HA2 sequence of FJ/5 were amplified. The chimeric H7/H5 HA gene was generated by overlapping PCR and cloned into pHW2000 as previously described (Jiang et al., 2014).

2.4. Generation of recombinant viruses

The chimeric H7/H5 influenza virus was generated by standard reverse genetics methods using eight bidirectional pHW2000 plasmids (Hoffmann et al., 2000). Briefly, HEK 293 T cells were co-transfected with 0.8 μg of each of the six pHW-plasmids (pHW191-PB2, pHW192-PB1, pHW193-PA, pHW195-NP, pHW197-M, and pHW198-NS), pHW-H7/H5 HA and pHW-NA encoding for the neuraminidase (NA) gene of FJ/5, using Lipofectamine 3000 (Life Technologies, Carlsbad, CA, USA). The chimeric HA gene contained the amino acid motif RETRG at the cleavage site. After 24 h, TPCK-treated trypsin (Sigma-Aldrich) was added to a final concentration of 2 μg/ml. After 72 h, the supernatants of transfected cells were collected and inoculated into 10-day-old SPF ECEs, which were then incubated at 37 °C for 72 h. The genetic composition of the viruses was confirmed by full-length sequencing. Vaccine batches were produced over five passages of viral constructs in SPF ECEs.

Recombinant PR8-H5 and PR8-H7 viruses containing the HA and NA protein sequences of the FJ/5 or GD/RZ viruses were generated and attenuated by removing the multi-basic amino acid motif located in the HA cleavage site.

2.5. In vitro growth kinetics

Ten-day-old SPF ECEs were infected with 10^4 50% egg infective dose (EID_{50}) of PR8-H5, PR8-H7, or PR8-H7/H5 viruses. Samples of allantoic fluid from five eggs infected with each virus were taken at 12, 24, 36, and 48 h. The viral titer in the samples was determined by EID_{50} analysis.

2.6. Virus titration

Virus titers in virus stocks were determined by standard methods using 10-day-old SPF ECEs. Viral suspensions were prepared in phosphate-buffered saline (PBS; pH 7.2) and the allantoic cavities of five ECEs were infected with 0.1 ml of each dilution. The ECEs were incubated at 37 °C with a relative humidity of 60% for 72 h. The presence of viruses was determined by the hemagglutination assay. Viral titers were expressed as log10 EID_{50}/ml.

2.7. Antigenic analyses

Antigenic analyses were performed using cross haemagglutination inhibition (HI) tests with polyclonal antisera against the indicated viruses. To generate the antisera, 21-day-old SPF chickens were injected with 1 ml of oil emulsion-inactivated vaccines derived from the rescued viruses, and sera were collected 3 weeks after the injection. Antibodies to HI were tested with 0.5% (vol/vol) chicken erythrocytes.

2.8. Preparation of vaccines

Vaccines were prepared from the PR8-H5, PR8-H7, and PR8-H7/H5 viruses grown in 10-day-old ECEs at 37 °C for 72 h. Formalin (final concentration, 0.1%) was added to inactivate the virus in the allantoic fluid at 4 °C for 72 h. Inactivated viruses were concentrated, purified by a 10%–50% sucrose density gradient, and resuspended in PBS. The presence of viruses in the pellet was confirmed by a hemagglutination assay. The inactivated virus was then mixed with mineral oil adjuvant at a ratio of 1:2 (v/v) and emulsified to prepare the oil-adjuvant whole-virus inactivated vaccine. The HA protein content in the final vaccine preparation is about 9.24 μg/ml, which was quantified as previous described (Tian et al., 2005).

2.9. Vaccination and challenge tests

Groups of 20 3-week-old White Leghorn SPF chickens were injected intramuscularly (i.m.) with 0.3 ml of each formalin-inactivated vaccine. Group of 20 chickens were injected i.m. with 0.3 ml of PBS as controls. Vaccines were administered to all subjects 14 days apart. Two weeks after each vaccination, serum samples were taken from all chickens for antibody detection. Two weeks after the second vaccination, the birds were challenged intranasally with 10^5 EID_{50} of lethal H5 or H7 virus. Oropharyngeal and cloacal swabs were collected on day 3 and day 5 post-challenge for virus isolation and titration in ECEs. All birds were observed for signs of disease or death during a 14-days period after challenge and then humanely euthanized.

2.10. HI assays

HI assays were performed as described previously (Hirst, 1942). Post-immunization sera were two-fold serially diluted in PBS in a 25 μl volume in a 96-well plate and an equal volume of virus (four hemagglutination units in 25 μl) was added to each well. The plate was incubated at room temperature for 30–60 min. Then, 25 μl of 0.5% (vol/vol) red blood cell suspension were added to each well on the plate. The titer was calculated as the highest dilution of serum that