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Evaluation of avian influenza virus isolated from ducks as a potential live vaccine candidate against novel H7N9 viruses

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ABSTRACT

Recent outbreaks of a novel H7N9 avian influenza virus in humans in China raise pandemic concerns and underscore an urgent need to develop effective vaccines. Theoretically, live influenza vaccines are of multiple advantages over traditional inactivated influenza vaccines to be used in a pandemic, because they can be produced rapidly, safely, and inexpensively. However, studies on live vaccines against the novel H7N9 virus are limited. In this study, we evaluated a potential live influenza vaccine candidate using an H7N3 avian influenza virus isolated from ducks with controls of two recombinant viruses generated through reverse genetics. The potential candidate could be produced efficiently using chicken embryonated eggs, and is homogenous to the novel H7N9 virus in their viral hemagglutinin genes. The potential candidate is likely low pathogenic to birds and mammals, and likely sensitive to oseltamivir and amantadine, as suggested by its genomic sequences. Its low pathogenicity was further supported through inoculation in mice, chicken embryonated eggs and chickens. Specific antibodies elicited in mice were detectable at least during the period between day 14 and day 56 after intranasal administration of the candidate for one time. Titers of the specific antibodies increased significantly with a boost intranasal administration or a higher inoculation dose. The induced specific antibodies were of substantial cross-reactivity with the novel H7N9 virus. These primary but promising evaluation data suggest that the duck influenza virus could be used as a potential live vaccine candidate, favorably through a prime-boost route, to mitigate the severity of the possible pandemic caused by the newly emerging H7N9 virus, and is valuable to be further evaluated.

1. Introduction

A previously unrecognized H7N9 subtype avian influenza virus (AIV) first identified in March 2013 has since caused more than 350 human cases in China. The virus carries some mutations associated with enhanced ability to bind to human-like receptors [1,2]. Recent studies further indicated that the novel zoonotic H7N9 virus can replicate efficiently in mice, ferrets, nonhuman primates and in human airway cells [3,4], and the virus has spread to the live poultry markets (LPMs) in more than 10 provinces of China, suggesting that the virus presents a severe pandemic threat to the world [5].

To be properly prepared for the pandemic threat caused by the novel H7N9 virus, studies on effective vaccines are highly needed. Traditionally, inactivated influenza vaccines (IIVs) were used to mitigate the severity of influenza pandemics. However, IIVs cannot be produced rapidly to meet the huge demand in the event of a pandemic [6,7]. Stockpiling of adequate IIVs in advance is not feasible, because we do not know whether and when a pandemic threat comes true, and the stored IIVs would have to be thrown away if they are not needed in a couple of years after production. In contrast, live influenza vaccines (LIVs) can be produced much more rapidly and inexpensively than IIVs [8]. Moreover, production of LIVs is of less biosafety concerns than production of IIVs using highly pathogenic viruses, and needle-free (e.g. nasal spray) and adjuvant-free delivery of LIVs is of less pain and side-effect than the injection delivery of IIVs [8]. Therefore, theoretically, LIVs are of multiple advantages over IIVs for combating the potential H7N9 pandemic influenza, and a few H7 and LIVs have been exam-

Abbreviations: AIV, avian influenza virus; LPM, live poultry market; IIV, inactivated influenza vaccine; LIV, live influenza vaccine; SPF, specific-pathogen-free; EID_{50}, 50% egg infection dose; HI, hemagglutination inhibition; GMT, geometric mean titer; SN, serum neutralization.

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ined in animal models and/or in human clinical trials [9–12], but relevant experimental data about the safety and efficacy of LIVs remain limited. Here we report a series of primary but promising evaluation data about a potential LIV candidate using a H7N3 AIV isolated from ducks and two controls of recombinant viruses generated through reverse genetics.

2. Materials and methods

2.1. Ethics statement

This study was conducted according to the animal welfare guidelines of the World Organization for Animal Health (Terrestrial Animal Health Code), and approved by the Animal Welfare Committee of China Animal Health and Epidemiology Center.

2.2. Embryonated eggs and mice

Specific-pathogen-free (SPF) chicken embryonated eggs were purchased from Merial-Vital Laboratory Animal Technology Co., Ltd. (Beijing, China). SPF female BALB/c mice were purchased from Vital River Laboratories (Beijing, China).

2.3. Isolation of field AIVs

The H7N3 subtype AIV, A/duck/Zhejiang/1028/2009(H7N3) abbreviated as H7N3-zj2009 below, was isolated from a duck in a LPM in Zhejiang Province in 2009. The H5N1 subtype highly pathogenic AIV, A/chicken/Hubei/RA18/2010(H5N1), was isolated from a chicken in a LPM in Hubei Province in 2010. The H7N9 subtype AIV, A/chicken/Jiangsu/K89/2013(H7N9) abbreviated as H7N9-js2013 below, was isolated from a chicken in a LPM in Jiangsu Province in 2013. All these field viruses were isolated by us using the method described previously [13]. They were propagated using 10-day-old SPF chicken embryonated eggs at 35 °C for three days.

2.4. Generation of recombinant AIVs

The recombinant H7N2 subtype AIV, designated as rH7N2-2002 below, was generated through reverse genetics according to the approach reported previously [14], as a parallel control of this study. Its HA and NA genes were synthesized according to the HA and NA sequences of A/chicken/Hebei/1/2002(H7N2) with the GenBank accession numbers of AY724257 and AY724264 [15]. Its six internal genes were synthesized according to the genomic sequences of the PR8/34 virus with the GenBank accession numbers of EF467817 to EF467820, EF467822, and EF467824, except that the mutation K627E was introduced into the viral PB2 gene, and this mutation could mitigate the virulence of an influenza virus in mammalians [16].

Another recombinant H5N1 AIV, designated as rH5N1-2010 below, was also generated through reverse genetics according to the approach reported previously [14], as the negative control of this study. It shares the same six internal genes with rH7N2-2002, and its HA and NA genes were derived from the aforementioned field virus A/chicken/Hubei/RA18/2010(H5N1). A mutation to delete multiple basic amino acid residues at the cleavage site in the HA gene was introduced into the genome of rH5N1-2010 according to the method reported previously [17], and this mutation can change the virus from a highly pathogenic AIV into a low pathogenic AIV [16,18,19].

2.5. RNA extraction and genome sequencing

Viral RNA was extracted using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany), and kept at −80 °C until use. The full-length genome was amplified using the PrimeScript One-step RT-PCR Kit (Takara, Dalian, China) according to the method reported previously [20]. The RT-PCR was performed in a 50-μl reaction system with incubation at 50 °C for 30 min and denaturation at 94 °C for 2 min, followed by 30 cycles at 94 °C for 30 s, 57 °C for 30 s and 72 °C for 2 min. RT-PCR products were purified with an agarose gel DNA extraction kit (Takara, Dalian, China) and ligated into the pMD19-T Easy vector (Takara, Dalian, China). Positive clones were sequenced using the ABI 3730xl DNA Analyzer.

2.6. Propagation and titration of the field and recombinant viruses

H7N3-zj2009, rH7N2-2002, and rH5N1-2010 were propagated using 10-day-old SPF chicken embryonated eggs at 35 °C. The allantoic fluids were harvested on the fourth day after inoculation. All the fluids were found to be free from contamination of Newcastle disease virus, infectious bronchitis virus, egg drop syndrome virus, chicken infectious anemia virus, and avian leukemia virus, using relevant RT-PCR or PCR assays. They were divided into aliquots and stored at −80 °C until use. The 50% egg infection doses (EID_{50}) of each of the stock viruses was determined using 10-day-old SPF chicken embryonated eggs according to the routine method developed by Reed and Muench [21].

2.7. Phylogenetic analysis

Phylogenetic relationship of the HA gene sequences of the H7N3-zj2009, rH7N2-2002 viruses, and some H7N9 viruses isolated from humans and birds in 2013 including the one H7N9-js2013 reported here, were analyzed using the software MEGA 6.0 with the neighboring-joining method [22,23]. Sequences were aligned using the software MUSCLE [24]. Nucleotide substitutions were set under the Kimura 2-parameter model, and substitution rates among sites were set in gamma distribution. The gaps were handled by pairwise deletion. Bootstrap values were calculated out of 1000 replicates [23,25].

2.8. Pathogenicity evaluation of the field and recombinant viruses

To investigate the pathogenicity of the viruses of H7N3-zj2009, rH7N2-2002, and rH5N1-2010 in birds, each of them was inoculated into 10-day-old SPF chicken embryonated eggs using the dose of 10^8 EID_{50} per egg, and then the eggs were examined at 24-hour intervals for four days.

To investigate the pathogenicity of H7N3-zj2009 in chickens, each of the viruses was intravenously inoculated into 10 6-week-old SPF chickens using 0.1 ml of a 1/10 dilution of the infectious allantoic fluid, to determine its intravenous pathogenicity index [26].

To investigate the pathogenicity of these three viruses in mice, each of the viruses was inoculated into 10 5-week-old female BALB/c mice (18.0 ± 1.0 g). The mice were randomly divided into four groups, with eight mice a group. These four groups were lightly anesthetized with CO_2 and administrated intranasally with H7N3-zj2009, rH7N2-2002, rH5N1-2010, and PBS, respectively. Each mouse was administrated with 10^8 EID_{50} of viruses or 50 μl PBS. Then the mice were monitored daily for the general appearance and development of adverse reactions and weighted at 1-week intervals.

To further investigate the pathogenicity of these three viruses in mice, each of the viruses was inoculated again into 5-week-old female BALB/c mice as the same as mentioned above. On the second, third and fourth days after inoculation, three mice of each group were euthanatized after anesthetization with isoflurane, and the nasal turbinates, brains, lungs, and spleens of the mice of each

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