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Brief Communication

A novel chimeric Newcastle disease virus vectored vaccine against highly pathogenic avian influenza virus

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ABSTRACT

Avian influenza (AI) is an economically-important disease of poultry worldwide. The use of vaccines to control AI has increased because of frequent outbreaks of the disease in endemic countries. Newcastle disease virus (NDV) vectored vaccine has shown to be effective in protecting chickens against a highly pathogenic avian influenza virus (HPAIV) infection. However, preexisting antibodies to NDV vector might affect protective efficacy of the vaccine in the field. As an alternative strategy, we evaluated vaccine efficacy of a chimeric NDV vectored vaccine in which the ectodomains of F and HN proteins were replaced by those of avian paramyxovirus serotype-2. The chimeric NDV vector stably expressed the HA protein *in vivo*, did not cross-react with NDV, was attenuated to be used as a safe vaccine, and provided a partial protection of 1-day-old immunized chickens against HPAIV subtype H5N1challenge, indicating its potential use for early protection of chickens.

1. Introduction

Highly pathogenic avian influenza virus (HPAIV) is an economically-important pathogen of poultry worldwide. The outbreaks involving H5 and H7 subtypes of influenza viruses resulted in lethal infections in poultry and the death of a limited number of people, thus affecting poultry production, public health and trade (OIE, 2015). Therefore, vaccination of poultry against HPAIV could play an important role in reducing virus shedding and raising the threshold for infection and transmission (Park et al., 2006). Inactivated, oil adjuvanted, whole virus vaccines are the most common vaccines available for AIV and account for 95.5% of AIV vaccine usage in poultry (Swayne et al., 2011). However, poor quality vaccines and inappropriate application have led to vaccine failures in the field. The use of live attenuated influenza viruses as vaccines in avian or mammalian species has a major biosafety concern, because the vaccine virus can become virulent through mutation or genetic reassortment with circulating strains (OIE, 2015).

Replicating viral vector vaccines offer a live vaccine approach without requiring involvement of the complete pathogen or cultivation of the pathogen (Bukreyev and Collins, 2008). Newcastle disease virus (NDV) is a fast replicating avian virus that is prevalent in all species of birds (Samal, 2011). NDV is a member of the family *Paramyxoviridae* and has a nonsegmented, negative-sense RNA genome that contains six genes (3′-N-P-M-F-HN-L-5′) (Samal, 2011). NDV isolates are categorized into three pathotypes based on their virulence in chickens: lentogenic (avirulent), mesogenic (moderately virulent), and velogenic (virulent) (Alexander, 1998). The amino acid sequence at the F protein cleavage site has been identified as the primary determinant of virulence (Panda et al., 2004; Peeters et al., 1999). Virulent NDV strains have multibasic cleavage sequences that contain the preferred cleavage site of the intracellular protease furin (Arg-X-Arg/Lys-Arg↓) available in most cell types (Samal, 2011). In contrast, avirulent NDV strains typically contain one or two basic residues at the F protein cleavage site and are delivered to the plasma membrane in an uncleaved form for cleavage by extracellular proteases. Naturally-occurring low virulent NDV strains, such as LaSota and B1 are widely used as live attenuated vaccines to control Newcastle disease in poultry.

Of the vectored vaccines, NDV can be an ideal vaccine vector for development of an avian influenza vaccine. NDV has similar tissue tropism to AIV and infects via the intranasal route, thus inducing both local and systemic immune responses at the respiratory tract (DiNapoli et al., 2007). NDV grows to high titers in cell cultures (10^8 pfu/ml) and in embryonated eggs (10^9 pfu/ml), thus making it cost-effective (Kim and Samal, 2016). NDV vectored vaccine can be easily manufactured in a biosafety level 2 facility and can be administered using automated methods as a spray or in drinking water. Therefore, it provides a convenient platform for rapid, efficient, and economical immunization. In fact, NDV has been evaluated as a vaccine vector against HPAIV in the laboratories (DiNapoli et al., 2007, 2010; Kim et al., 2014; Nayak et al., 2009, 2010; Park et al., 2006; Veits et al., 2006). However, pre-existing immunity to the vector due to vaccination against NDV has

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limited the protective efficacy of NDV vectored vaccines in the field (Spackman and Pantin-Jackwood, 2014).

To overcome this limitation, we have evaluated potential use of antigenic chimeric NDV strain (Beaudette C) in which the F and HN ectodomains of avian paramyxovirus serotype-2 (APMV-2) were used to replace the counterpart of NDV (Kim et al., 2011). NDV and APMV-2 represent two distinct serotypes, and their F and HN glycoproteins have 41% and 35% amino acid sequence identity, respectively (Subbiah et al., 2008). In fact, AMPV-2 has shown the lowest cross-reactivity with antiserum against NDV among the tested avian paramyxovirus serotypes 2–10 (Tsunekuni et al., 2014). In commercial poultry, seroconversion rates to NDV and APMV-2 were 71% and 15%, respectively (Warke et al., 2008). Previously, our study suggested that the chimeric virus was attenuated to be safe for administration to chickens, but replicate efficiently *in vivo* (Kim et al., 2011). Therefore, in this study, the chimeric NDV was evaluated as an alternative vaccine vector against HPAIV by determining its antigenic cross-reactivity with NDV and protective efficacy of the chimeric vectored vaccine against H5N1 HPAIV.

2. Results

2.1. Generation of chimeric NDV expressing the HA protein of H5N1 HPAIV

Due to a polar gradient transcription, foreign genes are expressed more efficiently when placed closer to 3´ end of the genome (Bukreyev and Collins, 2008). Therefore, we engineered chimeric NDVs expressing the HA protein of H5N1 HPAIV strain A/Vietnam/1203/2004 by placing the HA gene between the N and P or the P and M genes of NDV (Fig. 1A). To evaluate the genetic stability, the recovered viruses were passaged seven times in 9-day-old embryonated chicken eggs and of 1-day old chicks. All of the viruses replicated efficiently in the eggs ($> 2^8$ HAU/ml), and the sequence of the HA genes were found to be correct after the final passage. We further determined the cytopathic effect (CPE) of chimeric NDVs *in vitro*. NDV is a typical syncytia-forming paramyxovirus; whereas, APMV-2 produces single cell infection leading to cell rounding and detachment of infected cells (Fig. 1B). Chimeric NDV/2 F-HN produced CPE similar to that of the APMV-2 parent. In addition, the insertion of the HA genes into the genomes of chimeric virus did not affect the CPE of the parental virus. The multicycle growth kinetics of the chimeric NDVs bearing the H5N1 HPAIV HA gene showed that insertion location site can affect the virus replication (Fig. 1C). Growth kinetics between the parental virus and NDV containing the HA gene at the junction of the P and M genes was similar. However, replication of chimeric NDV containing the HA gene at the junction of the N and P genes was inefficient, suggesting that this insertion affected the ratio of N and P protein (Zhao and Peeters, 2003).

Expression of HA by the NDVs was evaluated in DF1 cells by Western blotting (Fig. 1D). Chimeric NDV containing the HA between the N and P genes (NDV/2 F-HN N-P) expressed only uncleaved precursor (HA_0, molecular weight 70 kDa). In contrast, chimeric NDV containing the HA between the P and M genes (NDV/2 F-HN HA P-M) expressed the HA protein in the form of HA_0 and the larger subunit of the cleaved form, HA_1 (molecular weight 50 kDa). Further, this virus expressed higher level of HA_0 than NDV/2 F-HN N-P. This indicates that the insertion location of HA gene greatly affects the expression

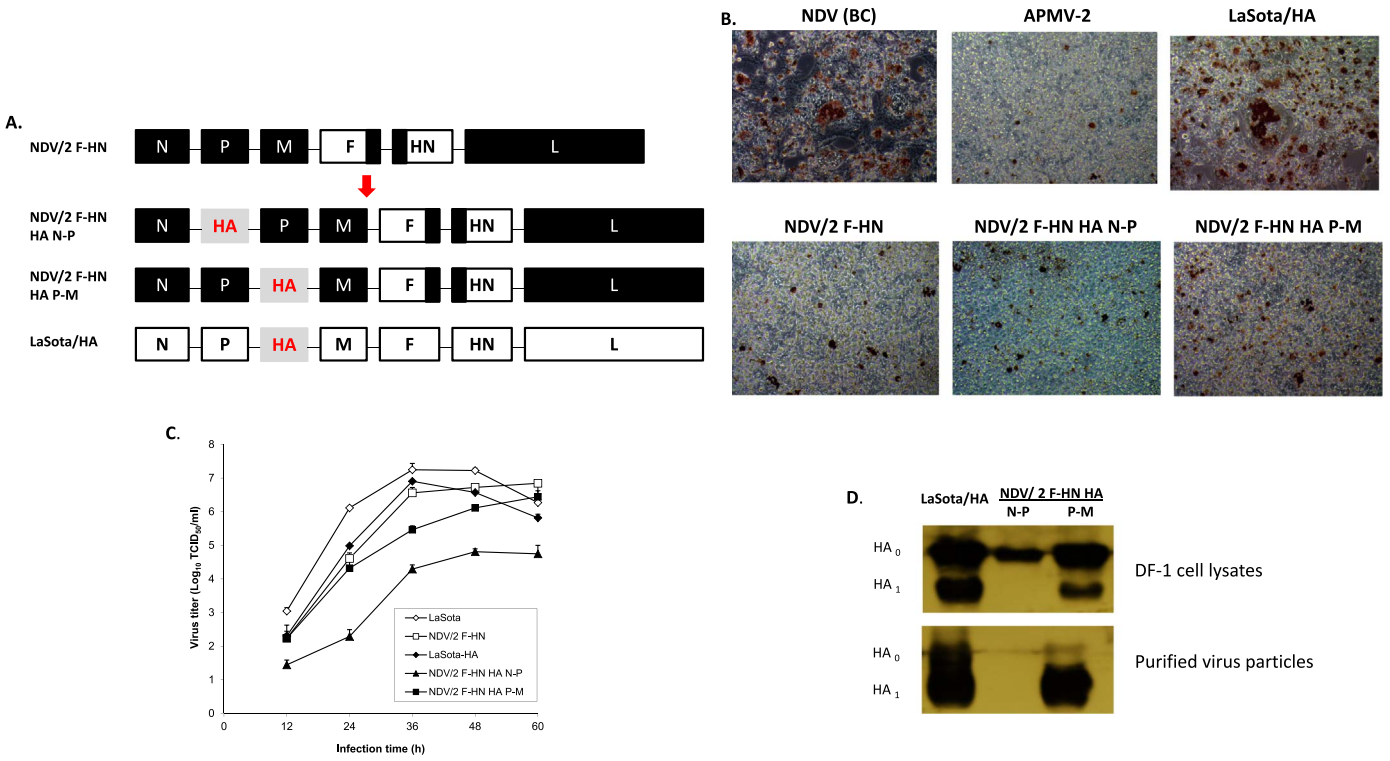

Fig. 1. Generation of antigenic chimeric NDVs expressing the H5N1 HPAIV HA gene. (A) The HA ORF of HPAIV H5N1 strain A/Vietnam/1203/04 was engineered to be flanked by the NDV gene-start and gene-end signals and was inserted into the intergenic region between the N and P or between the P and M genes in the chimeric NDV vector. Ectodomains of the F and HN genes derived from APMV-2 are shown as white rectangle. LaSota containing the HA ORF (LaSota/HA) was generated in our previous study (Kim et al., 2014). (B) To evaluate the syncytium formation of chimeric vaccine viruses, DF1 cells in six-well plates were infected with the indicated viruses at a multiplicity of infection (MOI) of 0.01 pfu/cell, incubated for 72 h, and conducted immunoperoxidase staining using antiserum against the N protein of NDV, with viral antigen stained red. (C) The growth kinetics was determined by infecting DF1 cells with each virus at an MOI of 0.01. (D) Expression of H5N1 HPAIV HA protein by NDV vectors in DF1 cells and incorporation of HA into NDV virions were analyzed by Western blot. DF1 cells were infected with each virus at MOI 1, and cell lysates were collected at 24 h post-infection for Western blot analysis using convalescent serum from chickens that had been infected with H5N1 HPAIV to visualize the HA protein. Incorporation of the HA protein into the NDV vector particle was detected by harvesting viruses from allantoic fluids of infected eggs at 72 hpi, purifying through a 30% sucrose cushion, and analyzing by Western blot.