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Recombinant Newcastle Disease Virus Expressing H9 HA Protects Chickens against Heterologous Avian Influenza H9N2 Virus Challenge

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Abstract

In order to produce an efficient poultry H9 avian influenza vaccine that provides cross-protection against multiple H9 lineages, two Newcastle Disease Virus (NDV) LaSota vaccine strain recombinant viruses were generated using reverse genetics. The recombinant NDV-H9Con virus expresses a consensus-H9 hemagglutinin (HA) that is designed based on available H9N2 sequences from Chinese and Middle Eastern isolates. The recombinant NDV-H9Chi virus expresses a chimeric-H9 HA in which the H9 ectodomain of A/Guinea Fowl/Hong Kong/WF10/99 was fused with the cytoplasmic and transmembrane domain of the fusion protein (F) of NDV. Both recombinant viruses expressed the inserted HA stably and grew to high titers. An efficacy study in chickens showed that both recombinant viruses were able to provide protection against challenge with a heterologous H9N2 virus. In contrast to the NDV-H9Chi virus, the NDV-H9Con virus induced a higher hemagglutination inhibition titer against both NDV and H9 viruses in immunized

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birds, and efficiently inhibited virus shedding through the respiratory route. Moreover, sera collected from birds immunized with either NDV-H9Con or NDV-H9Chi were able to cross-neutralize two different lineages of H9N2 viruses, indicating that NDV-H9Con and NDV-H9Chi are promising vaccine candidates that could provide cross-protection among different H9N2 lineage viruses.

Keywords

Influenza; recombinant NDV LaSota viruses; H9N2; Cross-protection

1. Introduction

Avian influenza is a contagious viral disease of birds caused by influenza A viruses (IAV). IAVs are classified into different subtypes based on the antigenic differences of two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [1]. Since 2009, two novel HA and NA subtypes have been discovered in New World bat species, resulting in a total of 18 HA subtypes (H1-18) and 11 NA subtypes (N1-11) [2]. The first isolation of an avian influenza virus (AIV) H9N2 subtype was reported in the US in 1966 [3]. Based on epidemiological and phylogenetic studies, the HA gene of the H9N2 influenza viruses is divided into Eurasian and American avian lineages [4]. To date, three distinct lineages of H9N2 viruses have been identified that caused outbreaks in domestic poultry in Asia [5–11]. Infection with an H9N2 virus in laying hens results in decreased egg production while co-infection with other viruses and/or bacteria can cause severe morbidity and high mortality in chickens [12, 13]. H9N2 avian influenza is endemic in poultry in Asian and Middle Eastern countries, causing a significant economic impact to their poultry industries. Furthermore, human infections with an H9N2 avian influenza virus have been reported [14, 15]. H9N2 viruses have been shown to be donors of internal genes to generate zoonotic influenza viruses such as the highly pathogenic H5N1 [16], the H7N9 [17–19] and the H10N8 viruses [20–22]; zoonotic infections with the H7N9 and H10N8 viruses were reported recently [17, 23, 24]. It is, therefore, important to control H9N2 spread in poultry in order to protect both animal and public health. Although a large amount of inactivated H9N2 vaccines have been used in endemic areas, outbreaks caused by H9N2 viruses are still not efficiently controlled. The currently used inactivated H9N2 vaccines have several disadvantages such as the cost of production, laborious administration, lack of long-term immunity and lack of cross-protection among different H9N2 lineages, all of which limit their efficacy in the field. Thus, it is necessary to develop an efficacious vaccine that can overcome some of the disadvantages of conventional inactivated vaccines and confer cross-protection against different lineages of H9N2 viruses.

Newcastle disease virus (NDV) is a single-stranded negative sense non-segmented RNA virus that belongs to the *Avulavirus* genus in the *Paramyxoviridae* family; it infects avian species. The NDV genome consists of six transcriptional units: NP, P, M, F, HN and L, which encode eight proteins in the order NP-P/V/I-M-F-HN-L. The NDV LaSota strain, a naturally occurring low-virulent NDV strain, has been routinely used as a live NDV vaccine throughout the world [25]. This vaccine strain grows to a high titer in embryonated chicken

eggs, induces strong humoral and cellular immunity and can be readily administered through drinking water supplies or via aerosols [26]. Moreover, the LaSota vaccine strain has been proven to be safe and stable, and there is no evidence of recombination with field strains. The LaSota vaccine strain and other NDV strains have been developed as a vector to express foreign antigens for vaccine or gene therapy purposes [27–31]. In the present study, we used the NDV LaSota strain as a vector to generate two NDV recombinant viruses that express either a consensus full-length HA based on available H9N2 sequences from Chinese and Middle Eastern isolates or an ectodomain of HA of the H9N2 A/Guinea Fowl/Hong Kong/WF10/99 [32, 33] fused with the transmembrane and cytoplasmic tail of the NDV fusion protein (F). Both NDV recombinant viruses were evaluated as H9 vaccine candidates and their efficacies were tested in chickens against challenge with a heterologous H9N2 virus.

2. Material and Methods

2.1. Cells, eggs, chickens and viruses

Adenocarcinomic human alveolar basal epithelial cells (A549) were used for transfection of NDV anti-genome cDNA with NDV supporting plasmids expressing NDV N, P and L to generate recombinant NDV viruses. Specific Pathogen Free (SPF) embryonated chicken eggs (Charles River Laboratories) were used to amplify recombinant NDV-H9 viruses. African green monkey kidney (Vero) cells were infected with recovered-recombinant NDV viruses. The aforementioned cells were maintained in 1X Dulbecco modified Eagle medium (DMEM) (Life Technologies) with 10% FBS (Gibco, Life Technologies) and 1% penicillin-streptomycin (Corning Cellgro). Two-week-old SPF White Leghorn chickens (purchased from Charles River Laboratories) were used to test vaccine efficacy. The A/chicken/Bangladesh/10450/2011 (H9N2) was propagated in SPF eggs and titrated in Madin-Darby Canine kidney (MDCK) cells using a standard plaque assay and virus aliquots were used for the challenge study.

2.2. Construction and rescue of recombinant viruses

Two recombinant viruses were generated and designated as NDV-H9Con and NDV-H9Chi. For the NDV-H9Con construction, the full-length consensus H9 sequence was computationally designed using Geneious (Biomatters, New Zealand) based on available H9 sequences from Middle East and Chinese isolates. The consensus sequence was codon-optimized based on chickens (*Gallus gallus*) (<https://www.idtdna.com/CodonOpt>). Moreover, a Kozak sequence was inserted upstream of the start codon for optimal initiation of translation [34]. The consensus sequence was synthesized (Genewiz, USA) and received as pUC57-H9Con plasmid that was digested using SacII and HindIII to generate the H9Con sequence which was subsequently cloned into P-M junction of pNDV LaSota anti-genomic cDNA that is between the T7 promoter (P_{T7}) and the T7 terminator sequence (T_{T7}) containing the Hepatitis Delta Ribozyme (HDR) cleavage (Fig. 1). The second construct was designated as NDV-H9Chi in which the codon-optimized ectodomain part of HA of A/Guinea fowl/Hong Kong/WF10/99 (H9N2) was fused with the sequence of the transmembrane and cytoplasmic tail of the NDV fusion protein and subsequently cloned into the pNDV antigenome cDNA between the T7 promoter (P_{T7}) and the T7 terminator sequence (T_{T7}) containing the Hepatitis Delta Ribozyme (HDR) cleavage (Fig. 1) (Primers

used in generating both constructs available upon request). Infectious recombinant viruses NDV-H9Con or NDV-H9Chi were rescued by reverse genetics as described previously [35]. Briefly, A549 cells were infected with T7 RNA polymerase-expressing recombinant vaccinia virus MVA/T7 at a multiplicity of infection of approximately 3 PFU/cell in 500 µl PBS/Bovine albumin (BA) and incubated for 1h at 37°C in the presence of 5% CO₂ with shaking every 10 min. The cells were washed with 1ml 1X DMEM (10% FBS, Hyclone), then covered by 1ml 1X DMEM (10% FBS) and 200 µl Opti-MEM (Life Technologies) containing the lipofectamine (Life Technologies) and DNA mix (1µg rNDV cDNA, and the supporting plasmids; 0.5 µg NP, 0.25 µg P and 0.25 µg L), and incubated for 24 h at 37°C in the presence of 5% CO₂. After 24 hours incubation, cells were detached and inoculated into the allantoic cavity of nine-day-old SPF chicken eggs. Seventy two hours post inoculation, allantoic fluids were harvested and tested by hemagglutination assay using 0.5% turkey red blood cells. Presence of inserted genes was confirmed by RT-PCR and sequencing. Vaccine stocks were prepared and amplified into SPF eggs, then were titrated in Vero cells and stored at -80°C until use. To titrate the recombinant NDV stocks, confluent monolayers of Vero cells in 96-well tissue culture plates were infected with serial dilutions of NDV-H9Con or NDV-H9Chi vaccine stocks (starting at dilution 1:10⁴). After 24 h cells were fixed and the number of cells infected was determined by immunofluorescence, using a rabbit polyclonal serum against NDV (diluted 1:1000) and Alexa Fluor 488 labelled anti-rabbit secondary antibody (Invitrogen, 1:1000) under a fluorescence microscope. The virus titer was expressed as focus forming units per milliliter (FFU/mL).

2.3. Characterization of recombinant viruses

2.3.1. Indirect immunofluorescence assay—Confluent monolayer Vero cells in 96-well tissue culture plates were infected with 10-fold serial dilutions of NDV-H9Con or NDV-H9Chi vaccine stocks. After 1 h of adsorption at 37°C in the presence of 5% CO₂, the virus inoculum was removed and cells were washed once with phosphate-buffered saline (PBS), and then incubated with 1X DMEM with 10% FBS. After 24 h, cells were fixed in methanol for 15 min at room temperature, permeabilized and washed with PBS-Tween 0.05% and incubated overnight with mouse-H9 monoclonal (G9-25) antibody (1:1000) or rabbit polyclonal NDV (1:1000) overnight at 4°C. Infected cells were then washed five times with PBS-Tween 0.05% followed by adding secondary Alexa fluor anti-mouse FITC-conjugated antibody (Invitrogen) with dilution 1:1000 or Alexa fluor anti-rabbit-FITC antibody (Invitrogen) with dilution (1:1000) and visualized under a fluorescence microscope (Evo-digital inverted microscope) at 20X magnification.

2.3.2. Western Blot—Confluent monolayers of Vero cells in 24 well tissue culture plates were infected with 10-fold serial dilutions of NDV-H9Con or NDV-H9Chi, and incubated 1 h for virus adsorption at 37°C in the presence of 5% CO₂. Virus inoculum was then removed and cells were washed once with PBS, then incubated with 1X DMEM with 10% FBS for 24 h. After 24 h, medium was removed and cells were lysed and boiled for five min at 100°C in loading buffer containing SDS and 0.6 M DTT (Bio-Rad). The samples were loaded in 4–15% Tris-HCL SDS gel, and samples were blotted onto a nitrocellulose membrane (Whatman). Blotted membranes were blocked in 3% diluted-skimmed milk in Tris-buffered saline with Tween (TBST) for 1h at room temperature. H9 and NDV proteins were detected

using rabbit monoclonal anti-H9 antibody (Gentex) with dilution 1:800 in TBST-1% BA or rabbit NDV-polyclonal antibody with a dilution 1:1000 in TBST-1% BA respectively and incubated at 4°C overnight with gentle shaking. After washing three times with TBST, the second antibody goat anti-rabbit IgG-HRP (Southern Biotech) was added with a dilution of 1:1000 in TBST-1% BA and incubated for 1h at room temperature with gentle shaking followed by washing three times with TBST, then the substrate (Supersignal WestFemto, Thermo Scientific) was added to the blot for 2 min in the dark at room temperature.

2.4. Vaccine efficacy testing in chickens

Sixty, two-week-old SPF white leghorn chickens (Charles River Laboratories) were purchased and divided into three groups (20 chickens/group). The chickens from groups one and two were immunized with 10^7 focus forming unit (FFU/0.1ml) of NDV-H9Con or NDV-H9Chi respectively, using either the oculonasal (ON) or the intramuscular (IM) route (10 birds/route). Birds of group three were inoculated with sterile PBS as a control through the same routes. Two weeks post-vaccination, vaccinated birds received a booster with the same dose of vaccines via the aforementioned routes. Birds from each group were challenged oculonasally with 10^7 plaque forming unit (PFU/0.1ml) of a heterologous (A/chicken/Bangladesh/10450/2011) H9N2 virus at two weeks post-booster. Chickens were monitored daily for clinical signs. Oropharyngeal and cloacal swabs were collected on 0, 1, 3, 5, 7 and 9 days post-challenge (dpc). Five birds from each group were necropsied on 3 and 5 dpc. Trachea, lung, spleen, intestine, liver and pancreas were collected for histopathological analysis. The remaining chickens were kept and euthanized at the end of the study (14 dpc). Virus titers in oropharyngeal, cloacal swabs and lung tissues were analyzed by virus isolation on MDCK cells as described previously [36]. Virus titers were calculated by TCID₅₀/mL using the Reed and Munch method.

2.5. Hemagglutination inhibition and micro-neutralization assays

Hemagglutination inhibition (HI) assay was performed to determine the immunogenicity of developed vaccines as described previously [37]. Briefly, 25µl of each heat-inactivated chicken serum samples were tested in duplicate and diluted two-fold in PBS against four hemagglutination units (4 HAU) of the H9N2 challenge virus in V-bottom plates and then incubated at room temperature for 30 min. After incubation, 50 µl of 0.5% turkey red blood cells were added to virus-serum mixture and incubated for another 30 min at room temperature. Cross-reactive antibodies elicited by either NDV-H9Con or NDV-H9Chi were tested using standard HI and micro-neutralization (MN) assays as described previously [38]. Briefly, 10 µl of heat-inactivated serum was added to 90 µl of 1X MEM media with TPCK-treated trypsin (1µg/ml) then, two-fold serial dilutions were done. Approximately, 100 TCID₅₀/50 µl of each virus was added, mixed with the sera and incubated for 1h at 37°C in the presence of 5% CO₂. Then, 100 µl of the virus and serum mixture was transferred to confluent MDCK monolayers in a 96-well culture plates, and incubated at 37°C for three days in the presence of 5% CO₂. The presence of cytopathic effect (CPE) was examined daily and was confirmed by a standard immunocytochemistry assay using pH1N1 mouse anti-influenza A NP monoclonal antibody.

2.6. Histopathology and immunohistochemistry

Tissue samples (lung, trachea, spleen, pancreas, intestine and liver) were collected from euthanized chickens on 3 and 5 dpc and were preserved in 10% neutral buffered formalin. All tissue samples were routinely processed by the histopathology section of the Kansas State Veterinary Diagnostic Laboratory (KSVDL). Histopathological examination was performed with H&E staining and immunohistochemistry (IHC) staining was performed by a standard procedure using rabbit anti-influenza A NP antibody (Genscript) at 1:4000. Heat retrieval with EDTA at pH 9 was performed for 10 minutes at 100°C. Bond polymer refine detection kit (Leica) was used and DAB was applied. Hematoxylin was used for the counterstain. Lesions in the respiratory tract were graded on epithelial changes and degree of inflammation in trachea, bronchi, atria and air capillaries. The grading scale for epithelial changes in the respiratory system was a 5 point scale: 0= no changes, 1=focal to multifocal loss of cilia and early epithelial degeneration, 2=mild epithelial flattening, 3=moderate flattening with decreased thickness of the respiratory epithelium and loss of cilia, and 4=severe necrosis to where the submucosa is covered by a single cell layer thick of flattened epithelium. Inflammation in the respiratory system was graded using a 4 point scale of 0=no inflammation, 1= mild categorized as 3 cell layers thick, 2=moderate inflammation characterized by inflammation greater than 3 cells thick that extends around the mucous glands and 3=severe inflammation that extends to and surrounds the cartilage of overlapping tracheal rings. The other organs were graded on necrosis and inflammation on a 0–4 point scale, 0=no necrosis or inflammation, 1=focal necrosis or inflammation, 3=multifocal necrosis or inflammation and 4=diffuse necrosis and inflammation.

2.7. Statistical analysis

Virus titers and antibody titers among groups were analyzed using One-way ANOVA in GraphPad Prism version 6.0 (GraphPad Software Inc., CA). Those response variables were subjected to comparisons for all pairs by using the Tukey-Kramer test. Pairwise mean comparisons between vaccinated and mock groups were made using the Student t-test. A *p* value of 0.05 was considered as a significant difference.

3. Results

3.1. Generation and characterization of recombinant NDV viruses

The full-length cDNA clones carrying the complete antisense genome of the NDV LaSota vaccine strain with a consensus H9 open reading frame (ORF) or the ectodomain part of HA of the H9N2 A/Guinea Fowl/Hong Kong/WF10/99 virus fused with the transmembrane and cytoplasmic tail of the NDV F protein (Fig. 1A and 1B) were co-transfected with the supporting plasmids (NP, P and L) into A549 cells. The rescued viruses were amplified in SPF embryonated chicken eggs. The recombinant LaSota viruses expressing H9Con or H9Chi were successfully recovered and the presence of inserted genes was confirmed by RT-PCR and sequencing. Expression of the inserted gene in the NDV-H9Con or NDV-H9Chi viruses was confirmed by IFA assay (Fig. 2A) and western blotting (Fig. 2B & C). Moreover, the recovered viruses were serially passaged four times in SPF eggs to determine whether the recombinant NDV viruses were stable and efficiently express the respective H9 protein. The H9 antigen from each passage was detected by both IFA and western blotting in

recombinant NDV-infected Vero cells (data not shown), indicating that the recombinant NDV viruses expressing the respective H9 antigen are stable. Furthermore, both recombinant NDV-H9Con and NDV-H9Chi viruses replicated very efficiently in SPF eggs and their titers reached up to 3.8×10^8 FFU/mL and 6.6×10^8 FFU/mL, respectively.

3.2. Recombinant NDV viruses are immunogenic and efficacious

The vaccinated and mock-vaccinated birds did not show any clinical signs during vaccination and gained weight equally. No HI titer against H9 and NDV viruses was detected in mock-vaccinated chickens two weeks after the first and second vaccination. All birds vaccinated with either the NDV-H9Con or the NDV-H9Chi via both routes (ON or IM) seroconverted after the first vaccination, and an HI titer against both NDV and H9N2 viruses, was detected in each vaccinated group. Interestingly, the HI titer in chickens vaccinated via IM route was higher than that from birds immunized via the ON route at 14 days post first vaccination; however, this was not statistically significant (Fig. 3A & B). After booster vaccination, the HI titers against both NDV and H9N2 viruses, in chickens immunized with either NDV-H9Con or NDV-H9Chi increased. Interestingly, the HI titers against the H9N2 virus in chickens immunized with NDV-H9Con were significantly higher than those of birds immunized with NDV-H9Chi with both inoculation routes (Fig. 3A). However, no significant difference was observed in regards to HI titers against the NDV virus in NDV-H9Con and NDV-H9Chi vaccinated groups (Fig. 3B).

After challenge with a heterologous low pathogenic H9N2 A/chicken/Bangladesh/10450/2011 virus (Table 1), 10 out of 20 chickens in the mock-vaccinated group showed clinical signs e.g., depression and conjunctivitis at 1 dpc, 18 out of 20 birds showed conjunctivitis at 2 dpc. In contrast, all chickens vaccinated with either the NDV-H9Con or NDV-H9Chi virus through either IM or ON routes displayed no clinical signs post challenge. Virus was detected in lung tissues of 3 out of 5 mock-vaccinated chickens that were necropsied at 3 dpc, whereas no virus was detected in the lung tissues of either NDV-H9Con or NDV-H9Chi vaccinated chickens on 3 dpc. No virus was detected in chicken lung tissues of each group at 5 dpc. Virus was detected in both oropharyngeal and cloacal swabs collected from variable numbers of mock-vaccinated chickens at 1, 3 and 5 dpc (Table 2). In contrast to the mock-vaccinated group, fewer chickens in either NDV-H9Con or NDV-H9Chi vaccinated groups shed virus via the oropharyngeal and cloacal routes. Interestingly, no vaccinated chicken except one from the NDV-H9Chi group shed virus through the cloacal route at early time points (1 and 3 dpc). However, at 5 dpc virus was detected in cloacal samples of vaccinated chickens to a similar amount as in mock-vaccinated birds (Table 2). No chickens in the NDV-H9Con vaccinated group shed virus through the oropharyngeal route at 5 dpc, whereas 2 out of 15 chickens from the NDV-H9Chi vaccinated group and 4 out of 15 in the mock-vaccinated group shed virus (Table 2). Virus titers in either oropharyngeal or cloacal swabs were much lower in NDV-H9Con vaccinated chickens when compared to the NDV-H9Chi or mock-vaccinated birds at both 3 and 5 dpc (Table 2). No virus was detected in either oropharyngeal or cloacal swabs in birds of each group at 7 and 9 dpc.

Histopathological changes and immunohistochemistry detection of viral antigen in chicken tissues

On routine histopathological examination, in the mock-vaccinated group 2 out of 5 chickens on 3 dpc and 4 out of 5 chickens on 5 dpc had multifocal mild to moderate tracheitis which consisted of heterophils, lymphocytes and plasma cells present in the mucosa and submucosa (Table 3). Similar lesions were seen in the trachea of 1 out of 5 birds on 3 dpc and 1 out of 5 birds on 5 dpc in the NDV-H9Chi group. No tracheal lesions were observed in the NDV-H9Con group (Table 3). One out of 5 chickens in the mock-vaccinated group and 1 out of 5 chickens in the NDV-H9Chi had a focal area of mild epithelial flattening. In the lung, mild to moderate lymphocytic inflammation was present in 4 out of 5 birds on 3 dpc and 3 out of 5 birds on 5 dpc in the mock-vaccinated group. Similar lesions were observed in 4 out of 5 chickens in the NDV-H9Con on 3 dpc and in 2 out of 5 chickens in the NDV-H9Chi group on 3 dpc (Table 3). No lesions were present in any of the chickens on 5 dpc in either the NDV-H9Con or the NDV-H9Chi group. In all 3 groups the pancreas and liver had mild multifocal lymphocytic infiltrates that were insignificant and represented background lesions (Table 3). No lesions were seen in the spleen or intestine of any of the groups.

No influenza NP antigen was detected in tissues including spleen, pancreas, liver and intestine collected from all chickens of each group by the IHC assay at 3 dpc. NP antigen was detected in the cytoplasm and nucleus of the respiratory epithelium of the trachea of 4 out of 5 mock-vaccinated chickens on 3 dpc and 2 out of 5 birds in NDV-H9Chi vaccinated chickens, whereas no positive staining was detected in NDV-H9Con vaccinated chickens (Fig. 4). Lung was positive in airway epithelium in one mock-vaccinated chicken that was also positive in the trachea for the influenza NP staining. The remaining chickens from each group of the study were negative in the lung at 3 dpc.

3.3 Recombinant NDV viruses induced cross-neutralizing antibody

We tested pooled sera collected from birds vaccinated with either the NDV-H9Con or the NDV-H9Chi 14 days post booster against different lineages of H9N2 viruses including A/chicken/Bangladesh/10450/2011 (G1-Asia lineage), A/quail/Hong Kong/G1/1997 (G1-Asia lineage) and A/swine/Guangxi/9/2007 (Y280B lineage) (Table 1) by both HI and MN assays. Pooled sera from NDV-H9Con vaccinated birds showed a mean H9 HI antibody titer of 512, 64 and 256, and a mean H9 MN antibody titer of 640, 40 and 80 against the H9N2 A/chicken/Bangladesh/10450/2011, A/quail/Hong Kong/G1/1997 and A/swine/Guangxi/9/2007 viruses, respectively. The NDV-H9Chi pooled sera showed a mean H9 HI antibody titer of 64, 512 and 256, and a mean H9 MN antibody titer of 80, 640 and 40 against the H9N2 A/chicken/Bangladesh/10450/2011, A/quail/Hong Kong/G1/1997 and A/swine/Guangxi/9/2007 viruses, respectively. These results indicate that the recombinant NDV-H9Con and NDV-H9Chi induced cross-reactive antibodies against different heterologous H9N2 viruses.

4. Discussion

The H9N2 avian influenza cannot be efficiently controlled despite the large amount of inactivated vaccines used in endemic areas [6, 39–45]. One of the major challenges is that

vaccine production cannot keep up with rapid changes of the influenza virus, resulting in mismatch between virus strain used for the vaccine and the locally circulating virus. This results in failure of vaccination to control H9N2 avian influenza, and underscores the importance to produce an efficacious vaccine that is able to provide cross-protection among different lineages of H9 viruses. Another disadvantage of current widely used inactivated vaccines is that these kind of vaccines are not able to differentiate infected from vaccinated animals (DIVA), resulting in the difficulty of tracking animals infected with a circulating virus when conventional inactivated whole-virus vaccines are used [10, 46]. In this study we generated two NDV-based H9 vaccine candidates (NDV-H9Con and NDV-H9Chi) that have DIVA capability since the recombinant NDV viruses do not express the influenza NP that is normally used for the diagnostic purpose as described previously [30, 47–49]. Furthermore, both vaccines are highly immunogenic in chickens and provide protection against challenge with a heterologous H9N2 virus. This is evidenced by blocking virus replication in lungs, reducing virus shedding via both the respiratory and cloacal routes and less lesions in lung and trachea of vaccinated chickens when compared to mock-vaccinated birds. The results indicate that both vaccine candidates are able to provide cross-protection among different lineages of H9N2 viruses. When compared to generating a traditional inactivated H9 avian influenza vaccine, a vaccine candidate based on recombinant NDV expressing an HA derived from a currently circulating strain could be generated in approximately 4 to 5 weeks [35, 47]. This approach would enable us to produce a matching vaccine rapidly to meet the needs of the poultry industry.

The antigenic match between vaccine and challenge virus is one of the most important factors in determining influenza vaccine efficacy in blocking virus replication and virus transmission [48]. This point has also been confirmed in the present study, i.e., both vaccine candidates provide incomplete protection against challenge with a heterologous H9N2 virus. This is most likely due to the fact that the H9 expressed by the recombinant NDV-H9Con or NDV-H9Chi virus shows only 96% or 91% homology to the HA of the challenge H9N2 virus at the amino acid level, respectively. This is probably one of the reasons why the NDV-H9Con vaccine was more efficient than the NDV-H9Chi, evidenced by significantly less chickens shedding virus with a lower titer through the respiratory route, and by less or no lesions observed in the trachea and lung of vaccinated birds. The more efficient protection is most likely also related to the significant higher HI titer induced by the NDV-H9Con vaccine. The antibodies induced by either vaccine showed different abilities to cross-neutralize 2 different lineages of H9N2 AIVs. Both HI and MN titers of pooled sera from birds immunized with the recombinant NDV-H9Chi are either higher or equal to these of pooled sera from birds immunized with the recombinant NDV-H9Con when tested against the H9N2 A/quail/Hong Kong/G1/1997 or the A/swine/Guangxi/9/2007 virus. It should be noted that the H9 expressed by the NDV-H9Chi or NDV-H9Con virus shows 99% or 92% homology to the HA of the H9N2 A/quail/Hong Kong/G1/1997 virus, and 91% homology to the HA of the H9N2 A/swine/Guangxi/9/2007 at the amino acid level (Table 1). These data indicate that both vaccine candidates could be able to provide cross-protection against different lineages of H9N2 viruses, and also underscore the importance of updating the vaccine seed virus timely when the antigenic drift is present in the field circulating influenza viruses.

NDV inactivated and live vaccines are widely used in poultry worldwide, resulting in the high seropositive prevalence of NDV in chickens in the field. Based on our results on the specific HI titers against NDV, an anamnestic booster response was found in chickens which had HI titers against NDV after the first vaccination via either the IM or ON administration route; this is consistent with our previous finding [49] and suggests that both the NDV-H9Con and NDV-H9Chi vaccines could work in chickens containing passively or actively acquired HI titers against NDV. However, the possible use of these vaccines in ovo or in hatchlings with or without maternal antibodies needs to be investigated.

In summary, we show that two recombinant vaccine candidates are able to provide protection against a heterologous H9N2 challenge when applied via different immunization routes (such as IM and ON) in chickens. Moreover, the vaccine candidates also induced broad cross-reactive antibody responses against different lineages of H9N2 viruses, indicating that they are promising vaccine candidates for the control of H9N2 avian influenza in the field. However, further studies are needed to test the efficacy of these vaccine candidates in a single dose of regiment, in animals with maternal antibodies and in other poultry species such as ducks, turkeys and quails.

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References

1. Fouchier RA, Munster V, Wallensten A, Bestebroer TM, Herfst S, Smith D, et al. Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from blackheaded gulls. *J Virol*. 2005 Mar; 79(5):2814–22. [PubMed: 15709000]
2. Tong S, Zhu X, Li Y, Shi M, Zhang J, Bourgeois M, et al. New world bats harbor diverse influenza A viruses. *PLoS Pathog*. 2013; 9(10):e1003657. [PubMed: 24130481]
3. Homme PJ, Easterday BC. Avian influenza virus infections. I. Characteristics of influenza A-turkey-Wisconsin-1966 virus. *Avian Dis*. 1970 Feb; 14(1):66–74. [PubMed: 4314007]
4. Guo YJ, Krauss S, Senne DA, Mo IP, Lo KS, Xiong XP, et al. Characterization of the pathogenicity of members of the newly established H9N2 influenza virus lineages in Asia. *Virology*. 2000 Feb 15; 267(2):279–88. [PubMed: 10662623]
5. Xu KM, Smith GJ, Bahl J, Duan L, Tai H, Vijaykrishna D, et al. The genesis and evolution of H9N2 influenza viruses in poultry from southern China, 2000 to 2005. *J Virol*. 2007 Oct; 81(19):10389–401. [PubMed: 17652402]
6. Sun Y, Pu J, Jiang Z, Guan T, Xia Y, Xu Q, et al. Genotypic evolution and antigenic drift of H9N2 influenza viruses in China from 1994 to 2008. *Vet Microbiol*. 2010 Dec 15; 146(3–4):215–25. [PubMed: 20685047]
7. Bashashati M, Vasfi Marandi M, Sabouri F. Genetic diversity of early (1998) and recent (2010) avian influenza H9N2 virus strains isolated from poultry in Iran. *Arch Virol*. 2013 Oct; 158(10):2089–100. [PubMed: 23640582]

8. Golender N, Panshin A, Banet-Noach C, Nagar S, Pokamunski S, Pirak M, et al. Genetic characterization of avian influenza viruses isolated in Israel during 2000–2006. *Virus Genes*. 2008 Dec; 37(3):289–97. [PubMed: 18712589]
9. Kayali G, Kandeil A, El-Shesheny R, Kayed AS, Gomaa MM, Maatouq AM, et al. Active surveillance for avian influenza virus, Egypt, 2010–2012. *Emerg Infect Dis*. 2014 Apr; 20(4):542–51. [PubMed: 24655395]
10. Lee DH, Song CS. H9N2 avian influenza virus in Korea: evolution and vaccination. *Clin Exp Vaccine Res*. 2013 Jan; 2(1):26–33. [PubMed: 23596587]
11. Shanmuganatham K, Feeroz MM, Jones-Engel L, Walker D, Alam S, Hasan M, et al. Genesis of avian influenza H9N2 in Bangladesh. *Emerg Microbes Infect*. 2015 Dec; 3(12):e88.
12. Kim JA, Cho SH, Kim HS, Seo SH. H9N2 influenza viruses isolated from poultry in Korean live bird markets continuously evolve and cause the severe clinical signs in layers. *Vet Microbiol*. 2006 Dec 20; 118(3–4):169–76. [PubMed: 16930870]
13. Kishida N, Sakoda Y, Eto M, Sunaga Y, Kida H. Co-infection of *Staphylococcus aureus* or *Haemophilus paragallinarum* exacerbates H9N2 influenza A virus infection in chickens. *Arch Virol*. 2004 Nov; 149(11):2095–104. [PubMed: 15503199]
14. Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, et al. Human infection with influenza H9N2. *Lancet*. 1999 Sep 11; 354(9182):916–7. [PubMed: 10489954]
15. Shanmuganatham K, Feeroz MM, Jones-Engel L, Smith GJ, Fourment M, Walker D, et al. Antigenic and molecular characterization of avian influenza A(H9N2) viruses, Bangladesh. *Emerg Infect Dis*. 2013; 19(9)
16. Guan Y, Shortridge KF, Krauss S, Webster RG. Molecular characterization of H9N2 influenza viruses: were they the donors of the “internal” genes of H5N1 viruses in Hong Kong? *Proc Natl Acad Sci U S A*. 1999 Aug 3; 96(16):9363–7. [PubMed: 10430948]
17. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, et al. Human infection with a novel avian-origin influenza A (H7N9) virus. *N Engl J Med*. 2013 May 16; 368(20):1888–97. [PubMed: 23577628]
18. Lam TT, Zhou B, Wang J, Chai Y, Shen Y, Chen X, et al. Dissemination, divergence and establishment of H7N9 influenza viruses in China. *Nature*. 2015 Jun 4; 522(7554):102–5. [PubMed: 25762140]
19. Lam TT, Wang J, Shen Y, Zhou B, Duan L, Cheung CL, et al. The genesis and source of the H7N9 influenza viruses causing human infections in China. *Nature*. 2013 Oct 10; 502(7470):241–4. [PubMed: 23965623]
20. Chen H, Yuan H, Gao R, Zhang J, Wang D, Xiong Y, et al. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. *Lancet*. 2014 Feb 22; 383(9918):714–21. [PubMed: 24507376]
21. Deng G, Shi J, Wang J, Kong H, Cui P, Zhang F, et al. Genetics, Receptor Binding, and Virulence in Mice of H10N8 Influenza Viruses Isolated from Ducks and Chickens in Live Poultry Markets in China. *J Virol*. 2015 Jun; 89(12):6506–10. [PubMed: 25855738]
22. Liu M, Li X, Yuan H, Zhou J, Wu J, Bo H, et al. Genetic diversity of avian influenza A (H10N8) virus in live poultry markets and its association with human infections in China. *Sci Rep*. 2015; 5:7632. [PubMed: 25591167]
23. Vachieri SG, Xiong X, Collins PJ, Walker PA, Martin SR, Haire LF, et al. Receptor binding by H10 influenza viruses. *Nature*. 2014 Jul 24; 511(7510):475–7. [PubMed: 24870229]
24. Xiao C, Ma W, Sun N, Huang L, Li Y, Zeng Z, et al. PB2–588 V promotes the mammalian adaptation of H10N8, H7N9 and H9N2 avian influenza viruses. *Sci Rep*. 2016; 6:19474. [PubMed: 26782141]
25. Goldhaft TM. Historical note on the origin of the LaSota strain of Newcastle disease virus. *Avian Dis*. 1980 Apr-Jun; 24(2):297–301. [PubMed: 7002145]
26. Meulemans, G. Control by vaccination. In: Alexander, DJ., editor. *Newcastle disease*. Boston, MA: Kluwer Academic Publishers; 1988. p. p318–32.
27. Schirmacher V, Fournier P. Newcastle disease virus: a promising vector for viral therapy, immune therapy, and gene therapy of cancer. *Methods Mol Biol*. 2009; 542:565–605. [PubMed: 19565923]
28. Vigil A, Martinez O, Chua MA, Garcia-Sastre A. Recombinant Newcastle disease virus as a vaccine vector for cancer therapy. *Mol Ther*. 2008 Nov; 16(11):1883–90. [PubMed: 18714310]

29. Zhao H, Peeters BP. Recombinant Newcastle disease virus as a viral vector: effect of genomic location of foreign gene on gene expression and virus replication. *J Gen Virol*. 2003 Apr; 84(Pt 4): 781–8. [PubMed: 12655078]
30. Park MS, Steel J, Garcia-Sastre A, Swayne D, Palese P. Engineered viral vaccine constructs with dual specificity: avian influenza and Newcastle disease. *Proc Natl Acad Sci U S A*. 2006 May 23; 103(21):8203–8. [PubMed: 16717196]
31. Bukreyev A, Collins PL. Newcastle disease virus as a vaccine vector for humans. *Curr Opin Mol Ther*. 2008 Feb; 10(1):46–55. [PubMed: 18228181]
32. Choi YK, Ozaki H, Webby RJ, Webster RG, Peiris JS, Poon L, et al. Continuing evolution of H9N2 influenza viruses in Southeastern China. *J Virol*. 2004 Aug; 78(16):8609–14. [PubMed: 15280470]
33. Perez DR, Lim W, Seiler JP, Yi G, Peiris M, Shortridge KF, et al. Role of quail in the interspecies transmission of H9 influenza A viruses: molecular changes on HA that correspond to adaptation from ducks to chickens. *J Virol*. 2003 Mar; 77(5):3148–56. [PubMed: 12584339]
34. Kozak M. An analysis of 5'-noncoding sequences from 699 vertebrate messenger RNAs. *Nucleic Acids Res*. 1987 Oct 26; 15(20):8125–48. [PubMed: 3313277]
35. Ayllon J, Garcia-Sastre A, Martinez-Sobrido L. Rescue of recombinant Newcastle disease virus from cDNA. *J Vis Exp*. 2013; (80)
36. Qiao C, Liu Q, Bawa B, Shen H, Qi W, Chen Y, et al. Pathogenicity and transmissibility of reassortant H9 influenza viruses with genes from pandemic H1N1 virus. *J Gen Virol*. 2012 Nov; 93(Pt 11):2337–45. [PubMed: 22875253]
37. World Organization for Animal Health. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. 2012. [cited; Available from: www.oie.int/en/international-standard-setting/terrestrial-manual/access-online/]
38. World Health Organization. Serological detection of avian influenza A (H7N9) infections by microneutralization assay. 2013. [cited; Available from: http://www.who.int/influenza/gisrs_laboratory/cnic_serological_diagnosis_microneutralization_a_h7n9.pdf?ua=1]
39. Pu J, Wang S, Yin Y, Zhang G, Carter RA, Wang J, et al. Evolution of the H9N2 influenza genotype that facilitated the genesis of the novel H7N9 virus. *Proc Natl Acad Sci U S A*. 2014 Jan 13; 112(2):548–53. [PubMed: 25548189]
40. Zhang P, Tang Y, Liu X, Peng D, Liu W, Liu H, et al. Characterization of H9N2 influenza viruses isolated from vaccinated flocks in an integrated broiler chicken operation in eastern China during a 5 year period (1998–2002). *J Gen Virol*. 2008 Dec; 89(Pt 12):3102–12. [PubMed: 19008399]
41. Li C, Yu K, Tian G, Yu D, Liu L, Jing B, et al. Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. *Virology*. 2005 Sep 15; 340(1):70–83. [PubMed: 16026813]
42. Bahari P, Pourbakhsh SA, Shoushtari H, Bahmaninejad MA. Molecular characterization of H9N2 avian influenza viruses isolated from vaccinated broiler chickens in northeast Iran. *Trop Anim Health Prod*. 2015 Aug; 47(6):1195–201. [PubMed: 26055889]
43. Banet-Noach C, Perk S, Simanov L, Grebenyuk N, Rozenblut E, Pokamunski S, et al. H9N2 influenza viruses from Israeli poultry: a five-year outbreak. *Avian Dis*. 2007 Mar; 51(1 Suppl): 290–6. [PubMed: 17494569]
44. Naeem K, Ullah A, Manvell RJ, Alexander DJ. Avian influenza A subtype H9N2 in poultry in Pakistan. *Vet Rec*. 1999 Nov 6; 145(19):560. [PubMed: 10609578]
45. Park KJ, Kwon HI, Song MS, Pascua PN, Baek YH, Lee JH, et al. Rapid evolution of low-pathogenic H9N2 avian influenza viruses following poultry vaccination programmes. *J Gen Virol*. 2010 Jan; 92(Pt 1):36–50. [PubMed: 20861321]
46. Wu R, Chen Q, Zheng L, Chen J, Sui Z, Guan Y, et al. Generation and evaluation of an H9N1 influenza vaccine derived by reverse genetics that allows utilization of a DIVA strategy for control of H9N2 avian influenza. *Arch Virol*. 2009; 154(8):1203–10. [PubMed: 19543688]
47. Veits J, Wiesner D, Fuchs W, Hoffmann B, Granzow H, Starick E, et al. Newcastle disease virus expressing H5 hemagglutinin gene protects chickens against Newcastle disease and avian influenza. *Proc Natl Acad Sci U S A*. 2006 May 23; 103(21):8197–202. [PubMed: 16717197]

48. Ge J, Deng G, Wen Z, Tian G, Wang Y, Shi J, et al. Newcastle disease virus-based live attenuated vaccine completely protects chickens and mice from lethal challenge of homologous and heterologous H5N1 avian influenza viruses. *J Virol.* 2007 Jan; 81(1):150–8. [PubMed: 17050610]
49. Liu Q, Mena I, Ma J, Bawa B, Krammer F, Lyoo YS, et al. Newcastle Disease Virus-Vectored H7 and H5 Live Vaccines Protect Chickens from Challenge with H7N9 or H5N1 Avian Influenza Viruses. *J Virol.* 2015 Jul; 89(14):7401–8. [PubMed: 25926639]

Fig. 1. Schematic design of recombinant NDV constructs used in this study

(A) The chicken codon-optimized ectodomain of hemagglutinin gene from the A/Guinea fowl/Hong kong/WF10/99 H9N2 was fused with the transmembrane and cytoplasmic tail of fusion (F) protein of NDV-LaSota strain, and cloned onto P and M junction of the NDV antigenomic cDNA that is under the T7 promoter (P_{T7}) and the T7 terminator sequence (T_{T7}) containing the Hepatitis Delta Ribozyme (HDR) cleavage by using SacII site. The H9 ectodomain was placed under the control of a set of NDV gene start (GS) and gene end (GE) transcription signals directing its expression as a separate mRNA. (B) The chicken codon-optimized consensus-H9 gene was designed based on available published H9N2 sequences of Middle Eastern and Chinese isolates and was cloned onto P and M junction of NDV-LaSota strain using the same method described aforementioned for the chimeric construct.

Fig. 2. Characterization of NDV-H9Con and NDV-H9Chi viruses in Vero cells

(A) H9 protein was expressed and detected in NDV-H9Con and NDV-H9Chi virus infected Vero cells using an H9 mouse-monoclonal antibody. NDV proteins were detected in Vero cells infected with the NDV-H9Con or NDV-H9Chi virus using rabbit polyclonal NDV antibody. (B) H9 protein was expressed in Vero cells infected with NDV-H9Con or NDV-H9Chi virus (62.6 kDa and 57.5 kDa protein). (C) NDV F protein (49 kDa) was detected in Vero cells infected with the NDV-H9Con or NDV-H9Chi virus.

Fig. 3. Serum mean hemagglutination inhibition (HI) titers of chicken post first and boost vaccination

(A) H9 HI antibody titers induced by NDV-H9Con (ON and IM) or NDV-H9Chi (ON and IM) in chickens post first and booster vaccination. (B) NDV HI antibody titers induced by NDV-H9Con (ON and IM) or NDV-H9Chi (ON and IM) in chickens post first and booster vaccination.

Fig. 4. Histological and immunohistochemical staining of trachea of vaccinated or mock-vaccinated chicken on 3 dpc

(A) Severe inflammation in the layers of the Oropharyngeal with loss of cilia in mock-challenged birds. (B) Moderate to severe inflammation in the layers of the Oropharyngeal with loss of cilia in NDV-H9Chi vaccinated birds. (C) Mild inflammation in the layers of Oropharyngeal in NDV-H9Con vaccinated birds. (D) Positive IHC staining in mock-vaccinated birds was detected in the Oropharyngeal of mock-vaccinated chickens. (E) Positive IHC staining was detected in the Oropharyngeal of NDV-H9Chi vaccinated birds. (F) No IHC positive staining was detected in NDV-H9Con vaccinated birds. Anti-influenza A polyclonal pH1N1 antibody (Genscript) was used for the immunohistochemical staining.

Table 1

Identities of the HA expressed by NDV vaccines with those of different H9N2 viruses used in the study at amino acid level.

H9N2 virus Strain	Consensus-H9 full	Chimeric-H9 ectodomain
A/chicken/Bangladesh/10450/2011	95.22%	90.84%
A/swine/Guangxi/9/2007	90.45%	90.45%
A/quail/Hong Kong/G1/1997	91.60%	99.04%

Table 2

Virus titers in Oropharyngeal and cloacal swabs of vaccinated and mock-vaccinated chickens infected with a heterologous

	NDV-H9Con group		NDV-H9Chi group		Mock-vaccinated group	
	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal	Oropharyngeal	Cloacal
1 dpc	(1.04±0.36) ^a 6/20 ^b	(0.0±0.0) 0/20	(2.24±0.38) 13/20	(0.0±0.0) 0/20	(4.33±0.30) 19/20	(1.81±0.35) 12/20
3 dpc	(0.77±0.31) 5/20	(0.0±0.0) 0/20	(2.65±0.38) 15/20	(0.0±0.0) 1/20	(4.98±0.37) 19/20	(1.33±0.34) 9/20
5 dpc	(0.0±0.0) 0/15	(0.72±0.31) 4/15	(0.35±0.24) 2/15	(1.39±0.40) 7/15	(1.37±0.60) 4/15	(1.65±0.55) 5/15

^a Virus titer is expressed as Log₁₀ TCID₅₀± SEM.

^b Number of birds shed the virus/total numbers of birds.

Table 3

Histopathology scores in different tissues of vaccinated and mock-vaccinated chickens infected with a heterologous H9N2

	NDV-H9Con			NDV-H9Chi			Mock-vaccinated		
	Liver	Lung	Trachea	Liver	Lung	Trachea	Liver	Lung	Trachea
3 dpc	(1.20±0.20) ^a 5/5	(0.80±0.20) 4/5	(0.00±0.00) 0/5	(1.00±0.44) 0/5	(0.40±0.24) 2/5	(0.20±0.16) 1/5	(2.00±0.00) 5/5	(1.40±0.40) 4/5	(0.60±0.32) 2/5
5 dpc	(2.00±0.00) 5/5 ^b	(0.00±0.00) 0/5	(0.00±0.00) 0/5	(1.00±0.44) 3/5	(0.00±0.00) 0/5	(0.20±0.20) 1/5	(2.00±0.00) 5/5	(1.00±0.44) 3/5	(1.00±0.44) 3/5

^a Histopathological score was calculated as the mean ± SEM

^b Number animal with lesion/total animals