Development of DIVA (differentiation of infected from vaccinated animals) vaccines utilizing heterologous NA and NS1 protein strategies for the control of triple reassortant H3N2 influenza in turkeys

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ABSTRACT

Since 2003, triple reassortant (TR) swine H3N2 influenza viruses containing gene segments from human, avian, and swine origins have been detected in the U.S. turkey populations. The initial outbreak that occurred involved birds that were vaccinated with the currently available H3 swine- and avian-origin influenza vaccines. Antigenically, all turkey swine-lineage TR H3N2 isolates are closely related to each other but show little or no antigenic cross-reactivity with the avian origin or swine origin influenza vaccine strains that are currently being used in turkey operations. These results call for re-evaluation of currently available influenza vaccines being used in turkey flocks and development of more effective DIVA (differentiation of infected from vaccinated animals) vaccines. In this study, we selected one TR H3N2 strain, A/turkey/OH/313053/04 (H3N2) that showed broad cross reactivity with other recent TR turkey H3N2 isolates, and created NA- and NS-based DIVA vaccines using traditional reassortment as well as reverse genetics methods. Protective efficacy of those vaccines was determined in 2-week-old and 80-week-old breeder turkeys. The reassortant DIVA vaccines significantly reduced the presence of challenge virus in the oviduct of breeder turkeys as well as trachea and cloaca shedding of both young and old breeder turkeys, suggesting that proper vaccination could effectively prevent egg production drop and potential viral contamination of eggs in infected turkeys. Our results demonstrate that the heterologous NA and NS1 DIVA vaccines together with their corresponding serological tests could be useful for the control of TR H3N2 influenza in turkeys.

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1. Introduction

Influenza A viruses are segmented, single stranded, negative sense RNA viruses which belong to the Orthomyxoviridae family. Due to the segmented nature of the viral genome, it is possible for genetic reassortment to occur in cells infected with two or more influenza viruses resulting in drastic change in virus genotype (genetic shift) [1]. In 1998, triple reassortant (TR) H3N2 viruses with gene segments from human (polymerase basic 1 (PB1), hemagglutinin (HA), and neuraminidase (NA)), swine (nucleoprotein (NP), matrix (M), and nonstructural (NS)), and avian (polymerase basic 2 (PB2) and polymerase acid (PA)) influenza viruses were first isolated from swine populations [2], supporting the hypothesis that pigs can serve as a mixing vessel for the generation of strains with interspecies transmission potential. In 2003, Choi et al. [3] reported the first isolation of TR H3N2 from turkeys in two geographically distant farms in the U.S. Further antigenic and genetic analysis led to speculation [3] and subsequent confirmation [4] that not only interspecies transmission from swine to turkeys but also intraspecies transmission among turkeys occur. Since 2003, TR H3N2 virus infection in turkeys has been reported in several farms in the U.S. and Canada [5,6], raising concerns about control of this new virus lineage.

It is well known that biosecurity is the first line of defense to protect birds from any pathogen. However, biosecurity alone

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sometimes is not enough to stop the spread of influenza virus [7]. To complement the prevention and control effort against influenza, vaccines have sporadically been used in the U.S. to control low pathogenic avian influenza outbreaks [8]. Vaccination can increase resistance of poultry to virus infection, protect poultry against morbidity and mortality, and reduce virus shedding and egg-production losses. The use of inactivated vaccines allowing differentiation of infected from vaccinated animals (DIVA), together with strict biosecurity and serological surveillance, have been successfully used to control H7N1 and H7N3 outbreaks in Italy [9]. For the control of turkey TR H3N2 avian influenza, a commercial inactivated mono-valent DIVA vaccine made with the A/duck/Minnesota/79 (H3N4) strain is available, however, this vaccine strain shows poor antigenic cross reactivity against currently circulating field strains and could not provide turkey breeder hens effective protection against recent TR H3N2 isolate challenge [10]. Thus, there is an urgent need to update vaccine seed strains and development of more efficacious vaccines.

Several strategies have been used to develop inactivated vaccines to comply with a DIVA program. The heterologous NA strategy is based on the rationale that the same HA in both vaccine strain and field viruses will provide protection to immunized birds, while a different NA in vaccine strain and field virus could be used to differentiate infected from vaccinated birds [9,11]. However, this strategy cannot be used in certain countries or regions where many subtypes of influenza virus are circulating in the field at the same time. To overcome this problem, vaccine strains with a rare NA have been generated by classical reassortment or reverse genetics methods [12–14]. On the other hand, a DIVA program based on antibody response against non-structural protein (NS1) has been tested in horses and poultry [15–17]. Since NS1 protein is a non-structural protein, theoretically anti-NS1 antibodies can be detected only in the serum samples of infected animals and not in vaccinated animals by serological tests [15,18,19]. Experimentally, this approach failed with commercial inactivated vaccines in chickens [16]. It was speculated that commercial inactivated vaccines are contaminated with small amounts of residual NS1 protein due to partial purification of vaccine virus after amplification in eggs, and thus vaccinated chickens will have antibodies against NS1 protein. By diluting serum before serological test, this strategy could differentiate infected from vaccinated birds experimentally [16]. However, field evidence on the efficacy of the NS1 DIVA strategy is still lacking. Recently, immunodominant 5B19 epitope of the S2 glycoprotein of murine hepatitis virus inserted into NA, or tetanus toxoid as exogenous markers were used in vaccines for serological differentiation between vaccinated and infected chickens [20,21]. The main drawback of both new marker vaccines is the inability to differentiate vaccinated and subsequently infected birds from vaccinated birds.

In the present study, we selected a turkey TR H3N2 isolate which shows broad cross reactivity with other recent turkey TR H3N2 subtype viruses, and utilized heterologous NA and NS1 protein strategies to generate DIVA vaccines. Vaccine efficacy was evaluated in both 2-week-old and 80-week-old breeder turkeys and ND2- and NS1-enzyme-linked immunosorbent assay (ELISA), modified Neuraminidase inhibition (NI) assay, and NS1-fluorescence microsphere immunoassay (FMIA) were evaluated as DIVA serological tests.

2. Materials and methods

2.1. Viruses, cells and birds

The virus strains used in this study, A/turkey/OH/313053/04 (H3N2), A/duck/LA/B174/86 (H8N4), and A/turkey/OR/71-Dpc4-H7N3 [22] were obtained from the repository of the Food Animal Health Research Program (OSU, Wooster, OH) and were passaged once in 10-day-old specific pathogen free (SPF) embryonated chicken eggs (ECEs) (SPAFAS, Inc., Norwich, CT) to propagate viruses for the study from the stock strains. MDCK (Madin Darby canine kidney) and 293-T (human kidney cells, kindly provided by Dr. Yoshihiro Kawaoka at the University of Wisconsin) cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum and 1% gentamicin. Cells were incubated in a 37 °C incubator with 5% CO₂. Experimental turkeys were obtained from turkey flocks maintained at the Ohio Agricultural Research and Development Center, Wooster, OH.

2.2. Generation of reassortant viruses by traditional reassortment method

The parental viruses, A/turkey/OH/313053/04 (H3N2) and either A/duck/LA/B174/86 (H8N4) or A/turkey/OR/71-Ddel-pc4 (H7N3), were mixed in a proportion of 1:1 and inoculated into the allantoic cavity of 10-day-old SPF ECE [26]. Three days post inoculation, allantoic fluid was harvested and used for plaque assay to purify reassortant viruses as previously described [23]. In brief, confluent monolayers of MDCK cells in 6 well plates were infected with 10-fold serial dilutions of allantoic fluid. After adsorbing the virus at 37 °C for 1h, infected cells were overlaid with 1% agar in serum-free media supplemented with 0.75 μg/ml of trypsin (Sigma–Aldrich, St. Louis, MO). Three days after infection, a second agar overlay was done with an addition of neutral red (Sigma). Individual plaques were picked and resuspended into 0.5 ml sterile phosphate buffered saline (PBS) followed by inoculation into ECEs for amplification. Viral RNA was extracted with the RNeasy mini kit (Qiagen, Valencia, CA) from allantoic fluids as previously described [24]. Standard RT-PCR was first carried out with the Qiagen one-step RT-PCR kit (Qiagen) using H3 and N4 or NS specific primers followed by direct sequencing of the PCR product. Additional sequencing was performed on the remainder genes to confirm the genetic makeup of the reassortant viruses. Sequencing was done in an ABI PRISM 377 DNA sequencer at the Molecular and Cellular Imaging Center (Ohio Agricultural Research and Development Center, Wooster, OH).

2.3. Generation of reassortant viruses by reverse genetics

RNA from A/turkey/Ohio/313053/04 (H3N2) was extracted with the RNeasy mini kit (Qiagen, Valencia, CA) from infected allantoic fluid. All eight gene segments of A/turkey/OH/313053/04 (H3N2) were amplified by RT-PCR and cloned into pH21 transcripational vector between the promoter and terminator sequences of RNA polymerase I. The transcription plasmids that contain the NS genes of A/turkey/OR/71-Ddel-pc3 (H7N3) was previously described [22]. Four expression plasmids (pcAGGS:WSN-NP, pcDNA774–PB1, pcDNA762–PB2, and pcDNA787–PA) were kindly provided by Dr. Yoshihiro Kawaoka [25]. Recombinant viruses (rTK/0H/04-Ddel-pc3 (H3N2) and rTK/0H/04-Ddel-pc4 (H3N2)) containing the NS gene segment from either A/turkey/OR/71-Ddel-pc3 (H7N3) or A/turkey/OR/71-Ddel-pc4 (H7N3) and remaining gene segments from A/turkey/OH/313053/04 (H3N2) were generated in 293-T cells by reverse genetics as described previously [25]. The rescued viruses were propagated in 10-day-old ECE to make working stocks. The authenticity of the recovered reassortant viruses was verified by sequencing.
2.4. Immunization with inactivated vaccines and challenge studies in young and breeder turkeys

Vaccination studies were undertaken in four groups of 2-week-old turkeys (six birds per group), including three vaccine groups and one non-vaccination control group. Infectious anallantoic fluids containing wild type A/turkey/OH/313053/04 (H3N2) (TK/OH/04 wt), and reассortant DIVA vaccine strains, rH3N4 and rH3N3-NS-Ddel-pc4 (rH3N3-Nsdel), were inactivated with 0.1% β-propiolactone. MontanideTM ISA-70 VG adjuvant (Seppic, France) was mixed with inactivated viruses in a proportion of 2:3:1 and administered into turkeys subcutaneously in the nape of the neck (0.5 ml/turkey). Two weeks post vaccination, sera were collected to test for HA and NA specific antibody responses by the hemagglutination inhibition (HI) [26] and neuraminidase inhibition (NI) tests, respectively, followed by challenge with 10⁶ EID₅₀/0.2 ml of wild type A/turkey/OH/313053/04 (H3N2) by intranasal inoculation. At 2 and 5 days post challenge (DPC), tracheal and cloacal swabs were collected from all groups and were incubated at 35°C for 24 h. The fluids were then tested for viral RNA using the viral RNA kit (Qiagen). Virus load was quantified by real-time RT-PCR as described previously [12,24]. Birds were observed for 14 days for clinical signs of disease and serum samples were collected on the last day to check antibody response to HA and NA by HI and NI assays, respectively.

Similar vaccination and challenge study was also done in four groups of 80-week-old breeder turkeys (n=8 per group). Vaccination and challenge protocols were the same as described above. At 2 and 4 DPC, tracheal and cloacal swabs were collected for virus isolation. At 7 DPC, 2 birds from each group were bled to collect sera and euthanized. Tissues (trachea, lung, kidney, spleen, portions of small and large intestine, and four different parts of the oviduct) were collected and preserved in 10% neutral buffered formalin for histopathology. Infundibulum, magnum, isthmus, and uterus (1 g each) of the oviduct were also collected separately to determine viral load. At 14 DPC, sera were collected from remaining birds to check HA and NA specific antibody responses by HI and NI tests, respectively.

Tissues fixed in 10% neutral buffered formalin were embedded in paraffin and 5 μm sections were prepared and stained with hematoxylin and eosin (HE) as previously described [27]. Based on the extent of histopathological lesions, tissues with no lesion were scored as “−”, mild lesions as “+”, moderate lesions as “++”, and severe lesions as “+++”.

2.5. Immunization with live vaccines and challenge studies in young turkeys

Two vaccine groups were inoculated with 10⁶ EID₅₀/0.2 ml of live vaccine candidates (rTK/OH/04-Ddel-pc3 (H3N2) and rTK/OH/04-Ddel-pc4 (H3N2)) by the intranasal route. Immunizations with live and inactivated vaccines in young turkeys were done at the same time and the same non-vaccinated challenge control group described above was used in both experiments. Two weeks post vaccination sera were collected to test specific influenza virus antibody responses by HI test. Virus challenge was done as described above for inactivated vaccines. At 2 and 5 DPC, tracheal and cloacal swabs from vaccinated and non-vaccinated challenged groups were collected for virus isolation. Birds were observed for 14 days for clinical signs and serum samples were collected at the end of the experiment to check HA antibody responses by HI test.

2.6. N2 and N51 protein-based serological tests

ELISA and FMIA were used to detect anti-N51 antibody as described [28]. For FMIA assay, purified recombinant NP and NS1 proteins were bound to carboxyl-functionalized fluorescent polystyrene microspheres (Luminex Inc., Austin, TX) using the Bio-Plex Amine Coupling Kit (BioRad Laboratories, Hercules, CA) as per the manufacturer’s instructions. Unlabeled sites in the coated microsphere were blocked with 1% BSA in PBS. Coupled microspheres were incubated with 100 μl serum diluted 1:100 in PBS (1% BSA) for 30 min at room temperature in the dark. Following two washes with 200 μl of assay buffer, plates were incubated with 50 μl of 0.5 μg/ml biotinylated donkey anti-chicken IgY (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) for 30 min at room temperature (RT). Plates were further washed twice with assay buffer and incubated with 50 μl of 10 μg/ml streptavidin–phycocerythrin conjugates for 30 min at RT. Following two additional washes, beads were resuspended in 125 μl assay buffer per well, analyzed with a Luminex100 instrument, data collected with the IS2.3 software (Luminex Inc., Austin, TX) and results were expressed as mean fluorescence intensity (MFI). The cut-off of N51- and NP-FMIA was set as 1500 and 5000, respectively, as previously described [28].

N2-ELISA was performed as previously described [29] using a baculovirus expressed N2 protein from A/turkey/OH/313053/04 (H3N2) virus. The expression of N2 protein was optimized, and purified protein was utilized for ELISA. The antigen and sera concentrations used in the ELISA were optimized using a homologous N2 turkey polyclonal antiserum.

N2 antibody titers were also determined using an NI assay. The substrate 2-(4-methylumbelliferyl)–a-D-N-acetylmuramiduronic acid sodium salt hydrate (MUN; Sigma Chemical Co., St. Louis, MO) reacts with N-acetylmuramiduronic acid yielding fluorescent 4-methylumbelliflurone [30]. Neuraminidase activity was determined by adding 7.6 μM MUN to a 2-fold dilution of BPL-inactivated A/turkey/OH/313053/04 (H3N2) in calcium saline (CaS; 20 mM sodium borate, 7 mM calcium chloride, 154 mM sodium chloride, and 12.5 mM sodium acetate; at pH 7.2). Relative 4-methylumbelliflurone fluorescence versus the inverse virus dilution (1/x, where x = log₂ dilution factor) was determined to find the fluorescence linear limit of the assay for this N2 protein. A 96-well plate was blocked with BSA (50 mM Tris–HCl, 0.14 M NaCl, 1% BSA at pH 8.0) for 30 min, and washed with wash buffer (50 mM Tris–HCl, 0.14 M NaCl, 0.05% Tween 20 at pH 8.0). Turkey sera were pretreated in 0.5% milk in PBS for 30 min. A black flat-bottom 96-well plate was blocked with BSA for 30 min and washed. Serum samples were diluted 1:5 in BPL-inactivated A/turkey/OH/313053/04 (H3N2) that was diluted 1:8 in PBS. BPL-inactivated A/turkey/OH/313053/04 (H3N2) was diluted 2-fold in PBS to determine the fluorescence signal. Serum samples were incubated with the virus at room temperature for 15 min. After incubation, 7.6 μM MUN CaS (1:1 with the virus dilution) was added to each well. Normal turkey serum was added to BPL-inactivated A/turkey/OH/313053/04 (H3N2) that was diluted 1:8 in CaS to account for non-specific antibody binding to the NA that would block the fluorescent signal. The kinetic production of 4-methylumbelliflurone was measured every 3 min for 15 min at 360 nm excitation wavelength and at 460 nm emission wavelength using a Synergy HT Multi-Detection microplate reader that was warmed to 37 °C (Bio-Tek, Winooski, VT). Fluorescence readings lower than the normal sera were considered as neuraminidase inhibitory. Sera that were neuraminidase inhibitory above 3 log₂ were considered N2-positive. Selected samples were also tested with A/turkey/MO/24093/99 (H1N2), which has a related neuraminidase gene but a heterologous hemagglutinin gene using the same procedure as described above.

2.7. Statistical analyses

To determine the significant differences of virus shedding in the trachea and cloaca of turkeys between vaccinated and
Table 1
Protection of 2-week-old turkeys vaccinated with inactivated DIVA vaccines after challenge with 10^6 EID_{50} of A/turkey/OH/313053/04 (H3N2) virus.

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Viral load*</th>
<th>2 DPC^b</th>
<th>5 DPC</th>
<th>14 DPC^d</th>
<th>14 DPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TK/OH/04 wt</td>
<td>Tracheal</td>
<td>3/6*</td>
<td>0.9±0.7±</td>
<td>2/6</td>
<td>0.6±0.3</td>
</tr>
<tr>
<td></td>
<td>Cloacal</td>
<td>2/6</td>
<td>1.5±0.1</td>
<td>5/6</td>
<td>1.3±0.3</td>
</tr>
<tr>
<td>rH3N4</td>
<td>Tracheal</td>
<td>5/6</td>
<td>1.3±0.4</td>
<td>6/6</td>
<td>1.6±0.6</td>
</tr>
<tr>
<td></td>
<td>Cloacal</td>
<td>6/6</td>
<td>1.5±0.5</td>
<td>3/6</td>
<td>1.3±0.7</td>
</tr>
<tr>
<td>rH3N3-NSdel</td>
<td>Tracheal</td>
<td>5/6</td>
<td>1.2±0.7</td>
<td>5/6</td>
<td>0.8±0.5</td>
</tr>
<tr>
<td></td>
<td>Cloacal</td>
<td>4/6</td>
<td>1.6±0.3</td>
<td>3/6</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>Non-vaccinated control</td>
<td>Tracheal</td>
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<td>2.2±0.8</td>
<td>6/6</td>
<td>2.2±1.0</td>
</tr>
<tr>
<td></td>
<td>Cloacal</td>
<td>5/6</td>
<td>1.1±0.4</td>
<td>5/6</td>
<td>2.3±1.0</td>
</tr>
</tbody>
</table>

* Virus titers were obtained by extrapolation from real-time RT-PCR assay.
^b Days post-challenge.
^c The HI titer is expressed as the log_{10} reciprocal of the endpoint in a twofold dilution of sera.
^d Days post-vaccination.
^e Number of birds positive/number tested.
^f Average virus titer is expressed as log_{10} EID_{50}/ml + standard deviation.

Indicates P<0.05 compared to virus titer from unvaccinated-challenge control group.

non-vaccinated control, non-parametric Mann–Whitney test was performed. P-values were set at 0.05 (P<0.05).

3. Results

3.1. Screening and selection of reassortant viruses for seed vaccine

Infectious allantoic fluid from eggs co-inoculated with A/turkey/OH/313053/04 (H3N2) and either A/duck/LA/B174/86 (H8N4) or A/turkey/OR/71-Ddel-pc4 (H7N3) were extensively screened by plaque purification followed by gene segment-specific RT-PCR. All eight gene segments of several plaque purified viruses that had HA gene from A/turkey/OH/313053/04 (H3N2) and NA or NS gene from other two viruses were sequenced and two DIVA vaccine strains, rH3N4 and rH3N3-NSdel, were selected. After desired reassortant viruses, NA-based DIVA strain rH3N4 and NS1-based DIVA strain rH3N3-NSdel, were identified, the composition of internal genes were also determined by sequencing. Reassortant rH3N4 virus has HA, NP, and M genes from A/turkey/OH/313053/04 (H3N2), and remaining genes from A/duck/LA/B174/86 (H8N4). Reassortant rH3N3-NSdel virus has HA and PB2 genes from A/turkey/OH/313053/04 (H3N2), and remaining genes from A/turkey/OR/71-Ddel-pc4 (H7N3). Both rH3N4 and rH3N3-NSdel viruses replicating well in ECE and the titers of stock viruses (10^{1.7} and 10^{4.2} EID_{50}/ml, respectively) were comparable to that of wild type A/turkey/OH/313053/04 (H3N2) (10^{2.9} EID_{50}/ml) virus. Two recombinant viruses (rTK/OH/04-Ddel-pc3 (H3N2) and rTK/OH/04-Ddel-pc4 (H3N2)) containing the NS gene segment from either A/turkey/OR/71-Ddel-pc3 (H7N3) or A/turkey/OR/71-Ddel-pc4 (H7N3) and remaining gene segments from A/turkey/OH/313053/04 (H3N2), generated by reverse genetics showed titers of 10^{6.8} and 10^{6.0} EID_{50}/ml, respectively.

3.2. Efficacy of inactivated vaccines in 2-week-old turkeys

Two weeks after a single vaccination, both rH3N4 and rH3N3-NSdel inactivated vaccines elicited similar antibody immune responses against HA protein as the vaccine made with wild type strain (Table 1). Compared to the non-vaccinated control group, vaccination with rH3N4 significantly reduced virus shedding in the trachea at 2DPC, and vaccination with rH3N3-NSdel significantly prevented virus shedding in trachea at 2 and 5 DPC and in cloaca at 5 DPC. In birds vaccinated with wild type vaccine, the level of virus shedding in trachea and cloaca at 2 and 5 DPC was similar to birds vaccinated with recombinant vaccines; however, less number of birds was positive for virus shedding in trachea at 2 and 5 DPC and cloaca at 2 DPC. On the contrary, less number of birds vaccinated with recombinant vaccines shed viruses in cloaca at 5 DPC (Table 1).

3.3. Efficacy of inactivated vaccines in 80-week-old breeder turkeys

Both reassortant DIVA vaccines induced similar HA antibody immune responses to the wild type vaccine after a single immunization in breeder turkeys (Table 2). Vaccination with rH3N4 and rH3N3-NSdel significantly reduced virus shedding in cloaca at 4 DPC compared to the non-vaccinated control group. In addition, compared to non-vaccinated challenge control group, immunization with TK/OH/04 wt (H3N2) and DIVA vaccines significantly reduced challenge virus shedding in all four sections of the oviduct, as no virus or very low levels of virus (10^{1.3} EID_{50}/ml) were detected in vaccinated birds compared to higher virus levels (10^{2.5}–10^{3.9} EID_{50}/ml) in non-vaccinated control birds (Table 2). In the non-vaccinated control group, the higher levels of virus present in the oviduct explain the effect of virus infection in breeder turkeys observed in the field in which decreased in egg production is characteristic.

Histopathologic examination of trachea, lung, spleen, and kidney collected at 7 DPC showed no difference in these organs among vaccinated and non-vaccinated groups (data not shown). Lesions consisted of mild to moderate hyperplasia of tracheal epithelium with mild infiltration of lymphocytes and heterophils; mild congestion and peribronchial lymphohistiocytic infiltration in lungs; mild lymphoid depletion in spleen; and mild to severe tubule necrosis and associated lymphocytic interstitial nephritis. However, lesions in the large intestine and especially in the oviduct were more severe in the non-vaccinated group compared to the vaccinated groups, and included moderate lymphocytic infiltration in large intestines, and mild to moderate degeneration of the oviduct epithelium and generalized glandular atrophy as well as cysts present in the glands especially in the uterus.

3.4. Efficacy of live vaccine candidate in 2-week-old turkeys

At 2 weeks post vaccination, immune response induced by the live vaccines (Table 3) were about 3 log_{2} lower than that induced by inactivated wild type vaccine (Table 1). Compared to non-vaccinated challenge control group, rTK/OH/04-Ddel-pc3 (H3N2)
Table 2: Protection of 80-week-old turkeys vaccinated with inactivated DIVA vaccines after challenge with 10<sup>6</sup> EID<sub>50</sub>/0.2 ml of A/turkey/OH/313053/04 (H3N2) virus.

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Viral load&lt;sup&gt;a&lt;/sup&gt;</th>
<th>2 DPC&lt;sup&gt;b&lt;/sup&gt;</th>
<th>4 DPC</th>
<th>7 DPC</th>
<th>14 DPC&lt;sup&gt;c&lt;/sup&gt;</th>
<th>14 DPC</th>
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<tbody>
<tr>
<td></td>
<td>Tracheal</td>
<td>Cloacal Infundibulum</td>
<td>Magnum</td>
<td>Isthmus</td>
<td>Uterus</td>
<td></td>
</tr>
<tr>
<td>TK/OH/04 wt</td>
<td>6/8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6/8&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0/2</td>
<td>1/2</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1.6 ± 0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>2.0 ± 0.5&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.7 ± 0.3&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.6 ± 0.5&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.5 ± 0.3&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
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<td>Cloacal</td>
<td>Infundibulum</td>
<td>7/8</td>
<td>7/8</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>1.7 ± 0.4&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.8 ± 0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.5 ± 0.2&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.6 ± 0.4&lt;sup&gt;f&lt;/sup&gt;</td>
<td>0.4 ± 0.2&lt;sup&gt;f&lt;/sup&gt;</td>
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</table>

<sup>a</sup> Virus titers were obtained by extrapolation from real-time RT-PCR assay.
<sup>b</sup> Days post-challenge.
<sup>c</sup> The HI titer is expressed as the log<sub>2</sub> reciprocal of the endpoint in a twofold dilution of sera.
<sup>d</sup> Days post-vaccination.
<sup>e</sup> Number of birds positive/number tested.
<sup>f</sup> Average virus titer is expressed as log<sub>10</sub> EID<sub>50</sub>/ml ± standard deviation.
<sup>g</sup> Indicates P<0.05 compared to virus titer from unvaccinated-challenge control group.

and rTK/OH/04-Ddel-pc4 (H3N2) vaccination significantly reduced both the number of birds shedding and amount of virus shedding in trachea (Table 3). Although the antibody immune response induced by live vaccination was lower than by inactivated vaccines, live vaccines provided comparable protection to inactivated vaccines (Tables 1 and 3).

3.5. N2 and NS1 serological tests to differentiate infected from vaccinated birds

To evaluate the usefulness of N2-ELISA and NI assays in differentiation of infected and vaccinated birds, serum samples collected from young turkeys and breeder turkeys at 2 weeks post-vaccination (wpv) by inactivated vaccines and at 2 weeks post challenge (wpc) were tested (Table 4). In the young turkey experiment, serum samples from all non-vaccinated and vaccinated groups were anti-N2 antibody negative at 2 wpv according to the N2-ELISA; however, 3 out of 6 TK/OH/04 wt (H3N2), rH3N4, or rH3N3-NS1del vaccinated young turkeys were N2-positive 2 wpv by NI assay. At 2 wpv, all 6 birds in the non-vaccinated and wild type vaccinated groups, 3 out of 6 birds in the rH3N4 group and none in the rH3N3-NSdel group were positive for N2 antibodies by ELISA. Based on NI assay result, the rH3N3-NS1del vaccinated young turkeys had a four-fold increase in N2 antibody titers and the non-vaccinated young turkeys increased by 5.0 log<sub>2</sub> ± 0.6. Selected samples from the young turkeys were also tested with the

Table 3: Protection of 2-week-old turkeys vaccinated with live DIVA vaccines after challenge with 10<sup>6</sup> EID<sub>50</sub>/0.2 ml of A/turkey/OH/313053/04 (H3N2) virus.

| Vaccine group | Viral load<sup>a</sup> | 2 DPC<sup>b</sup> | 5 DPC | Average HI titer<sup>c</sup> |
|---------------|------------------|------------------|-------|------------------|-------|
|               | Tracheal         | Cloacal Infundibulum | Magnum | Isthmus | 1/2 |
| rTK/OH/04-Ddel-pc3 (H3N2) | 3/6<sup>e</sup>  | 5/6<sup>e</sup>   | 4/6   | 3.0 ± 0.6<sup>f</sup> | 1/2 |
|               | 1.2 ± 0.4<sup>e</sup> | 1.4 ± 0.3<sup>e</sup> | 1.1 ± 0.1<sup>e</sup> | 2.2 ± 0.8<sup>e</sup> | 1.1 ± 0.4<sup>e</sup> |
| Non-vaccinated control | Cloacal         | Infundibulum      | 2/6   | 5/6   | 2/2 |
|               | 2.2 ± 0.8<sup>e</sup> | 2.2 ± 1.1<sup>e</sup> | 2.1 ± 1.0<sup>e</sup> | 1.1 ± 0.4<sup>e</sup> | 1.0 ± 0.3<sup>e</sup> |

<sup>a</sup> Virus titers were obtained by extrapolation from real-time RT-PCR assay.
<sup>b</sup> Days post-challenge.
<sup>c</sup> The HI titer is expressed as the log<sub>2</sub> reciprocal of the endpoint in a twofold dilution of sera.
<sup>d</sup> Days post-vaccination.
<sup>e</sup> Number of birds positive/number tested.
<sup>f</sup> Average virus titer is expressed as log<sub>10</sub> EID<sub>50</sub>/ml ± standard deviation.
<sup>g</sup> Indicates P<0.05 compared to virus titer from unvaccinated-challenge control group.
Table 4
Antibody response to N2 protein in turkeys vaccinated with inactivated wild type and DIVA vaccines and challenged with A/turkey/OH/313053/04 (H3N2) virus as determined by an indirect N2-ELISA and neuraminidase inhibition (NI) assay.

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>N2-ELISA</th>
<th>NI Assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Young Turkeys</td>
<td>Breeder Turkeys</td>
</tr>
<tr>
<td></td>
<td>2 wpc&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 wpc&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TK/OH/04 H3N2 wt</td>
<td>0/6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>6/6</td>
</tr>
<tr>
<td>rH3N4</td>
<td>0/6</td>
<td>3/6</td>
</tr>
<tr>
<td>rH3N3-NS1del</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Non-vaccinated control</td>
<td>0/6</td>
<td>6/6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Week post vaccination.
<sup>b</sup> Week post challenge.
<sup>c</sup> Number of birds positive/number tested.
<sup>d</sup> Mean ± SEM N2-positive antibody titre.

<sup>1</sup> Indicates P < 0.05 difference between 2 wpc and 2 wpc within turkey age and vaccine group.

A/turkey/MO/24093/99 (H1N2) antigen because of concerns about steric hindrance from the HA protein giving false positive results. All the samples tested after vaccination, but before challenge were seronegative with this antigen. Several of the birds after challenge seroconverted, but at lower titers than when using the H3N2 antigen (data not shown). In the case of 80-week-old breeder turkeys, in contrast to the results in young turkey experiment, some of the birds in the vaccinated group were N2 positive at 2 wpc. As in young turkey experiment, all birds in wild type vaccinated group were anti-N2 sero-positive after challenge but not all serum samples from H3N4 and rH3N3-NSdel vaccinated groups and unvaccinated control group turned to anti-N2 sero-positive after challenge. Previous avian influenza virus infection history of the breeder turkeys was unknown.

NS1-ELISA and FMIA were used to evaluate NS1 as a marker to differentiate infected from vaccinated birds. In the young turkey experiment with killed vaccines, serum samples from the non-vaccinated group and vaccine groups of wild type virus and rH3N3-NSdel were anti-NS1 antibody negative tested by either NS1-ELISA or FMIA at 2 wpc (Table 5). Only one bird from the rH3N4 vaccinated group tested had anti-NS1 antibodies by both assays at 2 wpc. In contrast, some birds from all vaccinated groups, two from wild type vaccinated group, three from rH3N4 group, and five from rH3N3-NSdel group, had anti-NP antibodies by NP-FMIA at 2 wpc. At 2 wpc, 4 out of 6 birds in the non-vaccinated group, 2 out of 6 birds in the rH3N4 group, 1 out of 6 in the wild type virus vaccinated group, and none in the rH3N3-NSdel group were positive for NS1 antibodies tested by NS1-ELISA, while five from the non-vaccinated group, one from either wild type virus or rH3N4 vaccine groups, and none in the rH3N3-NSdel group were positive for NS1 antibodies tested by NS1-FMIA. All serum samples from non-vaccinated and vaccinated groups were positive for NP antibody by NP-FMIA. In the case of breeder turkeys, all inactivated vaccine groups and the non-vaccinated control group showed 100% positivity for anti-NS1 antibody at 2 wpc (data not shown), suggesting that these breeder turkeys may have previous influenza virus infection.

The two live vaccine strains with NS deletion genes, rTK/OH/04-Ddel-pc3 (H3N2) and rTK/OH/04-Ddel-pc4 (H3N2), were also used to vaccinate 2-week-old turkeys. The NS1-ELISA and FMIA serological results are shown in Table 6. Similar to results of birds with killed vaccines, all birds from the two live vaccinated groups were negative for anti-NS1 antibodies by both NS1-ELISA and FMIA at 2 wpc. There was only one bird in live rTK/OH/04-Ddel-pc3 (H3N2) vaccine group positive for NP antibodies at 2 wpc. At 2 wpc, only one bird from rTK/OH/04-Ddel-pc4 (H3N2) group was positive for anti-NS1 antibodies by both NS1-ELISA and FMIA. In contrast, with the NP-FMIA serological test, all birds from both live vaccinated groups and nonvaccinated control group were positive for NP antibody.

4. Discussion

Since 1998, TR H3N2 virus has been successfully established in swine population. Since influenza interspecies transmission between swine and turkeys had previously occurred, turkey producers started immunizing turkey breeders with autogenous killed swine TR H3N2 virus vaccines or the A/duck/Minnesota/79 (H3N4) vaccine commercially available that could potentially be used as a DIVA vaccine [10]. However, since 2003, several TR H3N2 outbreaks have been reported not only from unvaccinated but also from vaccinated turkey flocks. Subsequent genetic and antigenic studies demonstrated that despite a high percentage of genetic similarity between them, turkey H3N2 viruses were antigenically poorly related to the swine-origin TR vaccine virus. In addition, both poor antigenic reactivity and low genetic similarity were observed between the turkey isolates and the commercial H3N4 duck-origin vaccine strain, which was further confirmed by a recent protection-challenge study [10]. Taken together, field and

Table 5
Antibody response to NS1 protein in 2-week-old turkeys immunized with killed vaccines as detected by an indirect NS1-ELISA and fluorescence microsphere immunoassays (FMIA).

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>NS1-ELISA</th>
<th>NS1-FMIA</th>
<th>NP-FMIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 wpc&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2 wpc&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2 wpc</td>
</tr>
<tr>
<td>TK/OH/04 wt</td>
<td>0/6 (0)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1/6 (0.09)</td>
<td>0/6 (0)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>rH3N4</td>
<td>1/6 (0.42)</td>
<td>2/6 (0.19 ± 0.09)</td>
<td>1/6 (7018)</td>
</tr>
<tr>
<td>rH3N3-NSdel</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>Unvaccinated control</td>
<td>0/6 (0)</td>
<td>4/6 (0.132 ± 0.04)</td>
<td>0/6 (0)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Week post vaccination.
<sup>b</sup> Week post challenge.
<sup>c</sup> Number of birds positive/number tested.
<sup>d</sup> Mean OD value ± standard deviation.
<sup>e</sup> Mean fluorescent intensity ± standard deviation.
experimental evidence of currently available vaccines indicates an urgent need for update of vaccine strain and development of effective DIVA vaccines to control turkey TR H3N2. In the present study, we chose the A/turkey/OH/313053/04 (H3N2) strain characterized by its broad cross-reactivity with other recent TR H3N2 turkey isolates and developed NA-based and NS1-based DIVA vaccines.

To reduce the possibility of the same NA of field viruses with heterologous NA-based DIVA vaccine strain, we chose a rare N4 and created NA-based vaccine strain, rH3N4, which has not been previously identified in commercial poultry in the U.S. The NS1-based DIVA vaccine strains, rH3N3-Nsdel, rTK/OH/04-Ddel-pc3 (H3N2) and rTK/OH/04-Ddel-pc4 (H3N2) encode a truncated NS1 protein [22]. Our previous study demonstrated that the amount of NS1 proteins in cells infected with A/turkey/OR/71-Ddel-pc3 (H7N3) or A/turkey/OR/71-Ddel-pc4 (H7N3) or A/turkey/OR/71-Ddel-pc4 (H7N3) or A/turkey/OR/71-Ddel-pc3 (H7N3) was below detection limits or much less than that of wild type virus, respectively [22]. Therefore, incorporation of NS1 truncation into NS1-based DIVA vaccine strains would drastically decrease possibility of NS1 protein contamination in the vaccine strains, and thus reduce or avoid the anti-NS1 immune response after vaccination, even if small residual quantities of NS1 protein contamination occurs due to insufficient purification of the vaccine strain [16]. It should be noted that vaccine strains used in our study were all prepared from allantoic fluid of ECe. One turkey from killed rH3N4 vaccine group was positive for anti-NS1 antibodies by either NS1-ELISA or -FMIA, which is most likely due to the contamination of NS1 protein in the vaccine strain, as previously described [16]. In contrast, our NS1 serological data demonstrated that all turkeys immunized with either killed or live vaccine strains containing NS1 truncation were negative for anti-NS1 antibodies at 2 wpv (Tables 5 and 6). Thus, the vaccine candidates developed in our study are less likely to interfere with DIVA vaccine strategy.

In vivo evaluation of DIVA vaccine efficacy was carried out in 2-week-old and 8-week-old breeder turkeys. At 2 weeks post vaccination, a single immunization of inactivated vaccine induced good HI antibody titers in turkeys (Tables 1 and 2). Overall, immunization with rH3N4 and rH3N3-Nsdel induced similar protection as A/turkey/OR/313053/04 (H3N2) wild type vaccine regardless of ages, indicating that the heterologous NA did not affect vaccine efficacy.

Although HI antibody titers induced by live vaccination were 3 log2 lower than those of killed vaccines, protection induced by rTK/OH/04-Ddel-pc3 (H3N2) and rTK/OH/04-Ddel-pc4 (H3N2) was comparable to that of killed vaccines, as they significantly reduced virus shedding in trachea (Table 3). In breeder turkeys, rH3N4 and rH3N3-Nsdel killed vaccines significantly reduced virus shedding in cloaca at 4 DPC (Table 2). Although both killed and live vaccines reduced virus shedding in trachea and cloaca, most of vaccinated birds regardless of ages still shed challenge virus in trachea and cloaca at both time points tested. One possible explanation could be the high challenge dose (106 EID50/0.2 ml) used in our study. Capua et al. demonstrated that challenge dose is important when determining vaccine protection rates [31]. In the aforementioned study, three challenge doses (104 EID50, 105 EID50, and 106 EID50) were tested, and only 106 EID50 challenge dose was able to evaluate the efficacy of inactivated vaccine. However, even with a high challenge dose (106 EID50), TR H3N2 viruses replicated to relatively low titers in trachea and cloaca of non-vaccinated control groups in both ages of turkeys (Tables 1 and 2), demonstrating that trachea and cloaca swabs may not adequately determine vaccine efficacy against TR H3N2 viruses in turkeys. In contrast, in our protection-challenge study with breeder turkeys, vaccination with reassortant inactivated vaccines significantly prevented virus replication in the oviduct, while lesions and virus replication was present in the oviduct of most birds in the non-vaccinated challenge control group (Table 2), which emphasizes the effect of virus infection on egg production.

Histopathology results also supported the capability of inactivated vaccine to reduce virus damage in the oviduct, as less severe lesions were observed in the oviduct of vaccinated birds compared to non-vaccinated birds. Thus, our study provides additional support to the idea that testing of vaccine efficacy in turkey against H3N2 influenza should be done using laying hens and an important sampling target should include the oviduct as well as the trachea and cloaca to obtain a clear evaluation of the effects of vaccination on challenge virus replication, as suggested in our previous study [27]. We were not able to assess egg production as a measure of vaccine protection since we used 80-week-old breeder turkeys for the study, and these older breeder turkeys were almost at the end of their egg production. However, difference in viral amount and histopathology observed in the oviduct between vaccinated and non-vaccinated groups suggests that immunization with both NA-based and NS1-based DIVA vaccines could reduce the drop in egg production.

A practical DIVA strategy should have an accompanying serological test that is capable of high-throughput serological monitoring. Indirect immunofluorescent antibody test (iFAT) [11], ELISA [14], and micro NA inhibition (micro-NI) assays [32] have been used for NA-based DIVA program, and recently ELISA [15–17] and FMIA [28] were developed for NS1-based DIVA approach. These assays are limited to be used in each laboratory and there is no commercial DIVA serological test available. In our study, N2-ELISA was used to monitor N2 antibodies for differentiation between vaccinated and infected birds. N2-ELISA has the ability to differentiate between non-infected and infected young turkeys and between infected and vaccinated young turkeys (Table 4). In the case of monitoring vaccinated groups, none of the A/turkey/OH/313053/04 (H3N2) wild type vaccinated birds were positive for N2 antibodies at 2 wpv, although 100% of birds in this group tested to be positive by N2-ELISA after challenge. A similar result was obtained by N2-ELISA in a previous study with wild type H9N2 vaccinated birds [14]. Possible explanations could be that the structure of NA protein is affected during the chemical inactivation process or that the immune response to NA is significantly suppressed due

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Table 6

Antibody response to NS1 protein in 2-week-old turkeys immunized with live vaccines as detected by an indirect NS1-ELISA and fluorescence microsphere immunoassays (FMIA).

<table>
<thead>
<tr>
<th>Group</th>
<th>NS1-ELISA</th>
<th>FMIA</th>
<th>NP-FMIA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 wpv</td>
<td>2 wpc</td>
<td></td>
</tr>
<tr>
<td>rTK/OH/04-Ddel-pc3 (H3N2)</td>
<td>0/6 (0)⁴</td>
<td>0/6 (0)</td>
<td>1/6 (6578)</td>
</tr>
<tr>
<td>rTK/OH/04-Ddel-pc4 (H3N2)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>Unvaccinated Control</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td></td>
<td>2 wpv</td>
<td>2 wpc</td>
<td></td>
</tr>
<tr>
<td>rTK/OH/04-Ddel-pc3 (H3N2)</td>
<td>0/6 (0)⁴</td>
<td>0/6 (0)</td>
<td>1/6 (6578)</td>
</tr>
<tr>
<td>rTK/OH/04-Ddel-pc4 (H3N2)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
</tr>
<tr>
<td>Unvaccinated Control</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
<td>0/6 (0)</td>
</tr>
</tbody>
</table>

⁴ Week post vaccination.
⁵ Week post challenge.
⁶ Number of birds/positive/number tested.
⁷ Mean OD value ± standard deviation.
⁸ Mean fluorescent intensity ± standard deviation.
to HA-dominated antigenic competition [33]. At 2 wpc of young turkeys with killed vaccine, 50% and 0% were N2 antibody positive in reassortant rH3N4 and rH3N3-Nsdel virus vaccinated groups by N2-ELISA. In similar studies in chickens, the NI titers continue to increase until 4 weeks post-challenge [34], suggesting further investigations with a higher number of samples taken at different time-points should be undertaken to validate the sensitivity of N2-ELISA. It appears that the NI assay in our study produced false-positive reactions in young turkeys maybe due to sterical hindrance by homologous H3 HA-specific antibodies [35,36]. The use of an antigen with a heterologous HA subtype (H1N2) eliminated the false positive results, but also had lower sensitivity compared to the N2 ELISA test. Based on the result from this study, it is reasonable to conclude that currently available ELISA-based DIVA tests will be useful for flock monitoring but not for diagnosis of individual birds.

In our study, both ELISA and FMIA serological tests were used to monitor immune response to NS1 in turkeys vaccinated with inactivated as well as live vaccines. Both NS1-ELISA and -FMIA assays detected anti-NS1 antibodies in turkeys experimentally infected with TR H3N2 virus but not in the majority of turkeys vaccinated with either killed or live vaccines in line with previous studies [15–17,37]. However, when monitoring infection in both killed and live vaccine groups, the results of both NS1-ELISA and -FMIA showed that only low percentage of birds seroconverted in killed vaccine groups of wild type and rH3N4 as well as live rTK/OH/04-Dsdel-pc4 (H3N2) vaccine group after homologous A/turkey/OH/31305/03 (H3N2) virus challenge, although all turkeys from each group were positive for NP antibodies by NP-FMIA. There are couple explanations for this result. First, unlike highly pathogenic avian influenza (HPAI) virus, low pathogenic avian influenza (LPAI) virus normally replicates in a limited area and does not cause systemic spread in naïve birds. In the case of vaccinated birds, the replication of a LPAI virus could be much lower than in unvaccinated controls; therefore, LPAI infection in vaccinated birds may produce a very weak or undetectable immune response against NS1 protein as previously reported [38]. Second, the duration of the anti-NS1 immune response is variable among different subtypes of avian influenza virus and different species [28,39]. It remains to be determined duration for detectable anti-NS1 antibodies in both naïve and vaccinated turkeys with TR H3N2 infection. Previous studies in chickens have shown that the NS1 response continues to rise until 3 weeks post-challenge, and more birds may have seroconverted in this experiment if samples were taken later [38].

Several DIVA strategies including heterologous NA and NS1 have been proposed to differentiate naturally infected from vaccinated birds [40]. To our knowledge, our study is the first to simultaneously evaluate two different DIVA vaccine strategies, heterologous NA and NS1. Regarding NS1 as a DIVA marker, several previous studies have proven this concept by only showing its ability to differentiate infection from vaccination [15–17,37]. Two studies have recently determined whether NS1 could be used to define infection in both naïve and previously vaccinated chickens [38,41]. Compared with those earlier studies, unexpectedly, it was found that none or low percentage of naïve chickens infected with different subtypes of LPAI viruses have anti-NS1 antibody. Furthermore, antibody against NS1 was detected only in extremely low percentage of vaccinated chickens after challenge with LPAI viruses [38]. In the present study, it was demonstrated that NS1 could be used as a marker to distinguish infection between infected and vaccinated turkeys. Although the result of identifying infection in vaccinated turkeys is similar to that in chickens [38], our study is first to determine whether NS1 could be used to define TR H3N2 infection in vaccinated turkey species, and also adds evidence to that NS1 could have limited ability as a DIVA vaccine marker to identify LPAI virus infection in immunized poultry. On the other hand, the data from our study and previous field evidence [40] support that heterologous NA-based DIVA vaccine could be more practical than NS1-based DIVA vaccine used to control avian influenza in poultry.

In conclusion, we developed heterologous NA-based and truncated NS1-based DIVA influenza vaccines to protect against turkey TR H3N2 and demonstrated that the vaccines could effectively reduce virus in the oviduct of breeder turkeys and as well as trachea and cloaca. Thus, proper vaccination can effectively prevent drop in egg production and potential viral contamination of eggs in infected turkeys in the fields. With further optimization and validation, N2-ELISA and NS1-FMIA could be used as useful diagnostic assays to distinguish between infected and vaccinated birds to control TR H3N2 influenza in turkeys.

Acknowledgements

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