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### Differences in Pathogenicity, Response to Vaccination, and Innate Immune Responses in Different Types of Ducks Infected with a Virulent H5N1 Highly Pathogenic Avian Influenza Virus from Vietnam

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**SUMMARY.** In a previous study, we found clear differences in pathogenicity and response to vaccination against H5N1 highly pathogenic avian influenza (HPAI; HA clade 2.3.4) between Pekin (Anas platyrhynchos var. domestica) and Muscovy (Cairina moschata) ducks vaccinated using a commercial inactivated vaccine (Re-1). The objective of the present study was to further investigate the pathogenicity of H5N1 HPAI viruses in different species of ducks by examining clinical signs and innate immune responses to infection with different strains of H5N1 HPAI virus (HA clade 1) in two domestic ducks, Pekin and Muscovy, and one wild-type duck, mallard (Anas platyrhynchos). Protection conferred by vaccination using the Re-1 vaccine against infection with this virus was also compared between Pekin and Muscovy ducks. Differences in pathogenicity were observed among the virus-infected ducks, as the Muscovy ducks died 2 days earlier than did the Pekin and mallard ducks, and they presented more-severe neurologic signs. Conversely, the Pekin and mallard ducks had significantly higher body temperatures at 2 days postinfection (dpi) than did the Muscovy ducks, indicating possible differences in innate immune responses. However, similar expression of innate immune-related genes was found in the spleens of virus-infected ducks at this time point. In all three duck species, there was up-regulation of IFN-α, IFN-γ, IL-6, CCL19, RIG-I, and MHC class I and down-regulation of MHC class II, but variable expression of IL-18 and TLR7. As in our previous study, vaccinated Muscovy ducks showed less protection against virus infection than did Pekin ducks, as evidenced by the higher mortality and higher number of Muscovy ducks shedding virus when compared to Pekin ducks. In conclusion, infection with an H5N1 HPAI virus produced a systemic infection with high mortality in all three duck species; however, the disease was more severe in Muscovy ducks, which also had a poor response to vaccination. The differences in response to virus infection could not be explained by differences in the innate immune responses between the different types of ducks when examined at 2 days dpi, and earlier time points need to be evaluated.

**RESUMEN.** Diferencias en la patogenicidad, la respuesta a la vacunación, y la respuesta inmune innata en diferentes tipos de patos infectados con un virus de influenza aviar de alta patogenicidad virulento H5N1 en Vietnam.

En un estudio anterior, se encontraron claras diferencias en la patogenicidad y en la respuesta a la vacunación contra un virus de la influenza aviar altamente patógeno H5N1 (clado de hemoaglutinina 2.3.4) entre patos Pekín (Anas platyrhynchos var. domestica) y patos reales (Cairina moschata), que fueron vacunados con una vacuna comercial inactivada (Re-1). El objetivo del presente estudio fue investigar más a fondo la patogenicidad del virus de influenza aviar H5N1 en diferentes especies de patos mediante el examen de los signos clínicos y la respuesta inmune innata a la infección con una cepa diferente del virus de la influenza aviar altamente patógena H5N1 (subtipo HA 1) en dos patos domésticos, Pekín y pato real, y un pato silvestre, y mallard (Anas platyrhynchos). La protección conferida por la vacunación con la vacuna Re-1 contra la infección con este virus también fue comparada entre Pekín y los patos reales. Se observaron diferencias en la patogenicidad entre los patos infectados por el virus, como con los patos reales que murieron dos días antes en comparación con los patos Pekín y mallard, y presentaron signos neurologicos más severos. Por el contrario, los patos Pekín y reales mostraron temperaturas corporales significativamente superiores en los dos días posteriores a la infección en comparación con los patos reales, indicando las posibles diferencias en la respuesta inmune innata. Sin embargo, una expresión similar de los genes relacionados con las respuestas inmunes innatas se encontraron en los bazos de patos infectados por virus en este punto del tiempo. En las tres especies de patos, se observó regulación positiva de IFN-α, IFN-γ, IL-6, CCL19, RIG-I y MHC de clase I y regulación negativa de MHC de clase II, y expresión variable de IL-18 y TLR7. Al igual que en nuestro estudio anterior, los patos reales vacunados mostraron una menor protección contra la infección por el virus en comparación con los patos Pekín como lo demuestra la mayor mortalidad y el mayor número de patos reales eliminando el virus en comparación con los patos Pekín. En conclusión, la infección con un virus H5N1 de la influenza aviar altamente patógena produce una infección sistémica con una elevada mortalidad en las tres especies de patos, sin embargo, la enfermedad fue más grave en los patos reales, los cuales también mostraron una pobre respuesta a la vacunación. Las diferencias en la respuesta a la infección por el virus no pueden ser explicadas por diferencias en las respuestas inmunitarias innatas entre los diferentes tipos de patos cuando se examinaron a los dos días después de la infección, y puntos mas tempranos deben ser evaluados.

**Key words:** highly pathogenic avian influenza, H5N1 type, ducks, pathogenicity, innate immunity, vaccination

**Abbreviations:** AI = avian influenza; BHI = brain heart infusion; CCL19 = chemokine ligand 19; dpi = days postinfection; ECE = embryonating chicken eggs; EI50 = 50% egg infective dose; HEPA = high efficiency particulate air; HI = hemagglutination inhibition; HPAI = highly pathogenic avian influenza; IFN-α = interferon alpha; IFN-γ = interferon gamma; IL-1β = interleukin 1 beta; IL-6 = interleukin 6; IL-18 = interleukin 18; MDT = mean death time; MHC-I = major histocompatibility complex class I; MHC-II = major histocompatibility complex class II; MP3b = macrophage inflammatory protein 3 beta; PG = prostaglandin; qRT-PCR = quantitative real-time reverse transcriptase-PCR; RIG-I = retinoic acid-inducible gene 1; SPF = specific-pathogen-free; TLR7 = toll-like receptor 7

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The natural host species and reservoir for avian influenza (AI) viruses are wild aquatic birds, especially those of the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (shorebirds, gulls, terns, and auks) (51,55,60). Normally, ducks naturally or experimentally infected with H5 and H7 highly pathogenic AI (HPAI) viruses develop only subclinical to mild disease (2,14,41,46). However, the pathobiology of HPAI viruses has changed, as many Asian lineage H5N1 HPAI viruses can replicate systemically and produce neurologic dysfunction and death in ducks. H5N1 HPAI viruses are currently widespread in poultry in Asia and have also spread to countries in the Middle East, Europe, and Africa, causing great losses to the poultry industry. The pathogenicity of H5N1 HPAI viruses in ducks can vary depending on the virus and on the age and species of the ducks tested (6,21,40,42,44,57). Asymptomatic infected migratory ducks are suspected of contributing to the spread of H5N1 HPAI viruses westward from Qinghai Lake, China in 2005 to Europe, Africa, India, and the Middle East (11,25,26). On the other hand, domestic ducks have a higher likelihood of perpetuating H5N1 HPAI viruses. Free-range, as well as backyard domestic ducks, have been associated with the spread of H5N1 HPAI viruses (19,22,26,48). Because of this, reducing the risk of H5N1 HPAI virus infection in ducks is important for controlling the continuing circulation and spread of H5N1 HPAI viruses (12,23,28,54).

Vaccination has proven effective in protecting ducks against H5N1 HPAI and is being used in several countries to help control the disease (47). However, vaccinated ducks may still shed virus into the environment, which may result in possible transmission to other hosts and contribute to an endemic situation (10). Several studies have been conducted to determine vaccine efficacy in ducks against HPAI lethal challenge (5,7,16,27,37,43,52,59,61), and differences in response to vaccination have been reported between domestic duck species (52,53). Differences in virus pathogenicity among duck species have also been described (21,25,31). In a recent study, mallard and Muscovy ducks infected with different H5N1 HPAI viruses (HA clades 1, 2.3.2, and 2.3.4) showed clear differences in response to infection, with the Muscovy ducks presenting a higher mortality than did mallards, regardless of the virus given (44). Distinctions between species are not necessarily due to domestic versus wild duck species. A study comparing morbidity, mortality, and viral shedding in five species of wild North American ducks with two different H5N1 HPAI viruses found that only one of the five duck species became sick or died with either virus (8).

Host immune responses most likely play a role in the differences observed in pathogenicity and response to vaccination, but little is known about the immune response in ducks to AI virus infection. Some studies have addressed individual innate immune gene expression in duck-origin cells infected with AI viruses by the use of reverse-transcriptase–PCR (RT-PCR) assays (1,24,29,49), but studies exploring host gene expression in ducks infected with AI viruses have been very limited (4,18,45). One of these immune related genes, retinoic acid-inducible gene 1 (RIG-I), is present in ducks and plays a critical role in the innate immune response to influenza (4,34). Up-regulation of RIG-I in ducks after infection with H5N1 HPAI viruses has been previously reported (4). There is also little information available about fever in birds, although there is evidence that suggests that functional characteristics of avian fever are similar to those found in mammals (20). Prostaglandins (PGs), as in mammalian fevers, seem to play a role in the generation of fever in birds (3,20,33). A role for cytokines, including interleukin 1 beta (IL-1β) and interleukin 6 (IL-6), has also been suggested in the signaling pathways that lead to PG synthesis (33). In a recent study investigating the role for pro-inflammatory cytokines in duck fever, it was concluded that interleukins IL-1β and IL-6 might mediate fever by instigating an accelerated synthesis of brain-derived PG. IL-6 was also suggested to serve as one of the terminal mediators of the avian febrile response (35).

In our previous study, we compared the response to vaccination with a commercially available H5N1 inactivated vaccine, Re-1, between Pekin (Anas platyrhynchos var. domestica) and Muscovy (Cairina moschata) ducks, and found that Muscovy ducks developed lower viral antibody titers induced by vaccination than did Pekin ducks, and they presented with higher morbidity and mortality after challenge with an HA clade 2.3.4 H5N1 HPAI virus from Vietnam (9). When comparing the response to virus infection in nonvaccinated ducks, differences were also observed, with infected Muscovy ducks presenting a more-severe disease than did Pekin ducks. Up-regulation of interferon alpha (IFN-α), RIG-I, IL-6, and major histocompatibility complex class II (MHC-II), and down regulation of MHC-II, was found in both types of ducks with significant differences in expression of RIG-I, IL-6, and MHC-II genes (9).

In order to better understand the differences observed in pathogenicity between different species of ducks, in this study we compared the outcomes of infection with another H5N1 HPAI virus (HA clade 1) in Pekin, Muscovy, and mallards ducks. Innate immune responses were also examined by determining the expression of several immune related genes including IFN-α, interferon gamma (IFN-γ), interleukin 18 (IL-18), IL-6, toll-like receptor 7 (TLR7), chemokine ligand 19 (CCL19), RIG-I, major histocompatibility complex class 1 (MHC-I), and MHC-II. To confirm our previous findings, we also studied the response of Pekin and Muscovy ducks to vaccination against avian influenza, using the same previously used vaccine, and determined protection against challenge with the HA clade 1 H5N1 HPAI virus.

**MATERIALS AND METHODS**

**Virus and vaccine.** The H5N1 HPAI virus A/Dk/NauGiang/NCVD07-12/07, belonging to HA clade 1, was obtained from the National Center for Veterinary Diagnosis, Hanoi, Vietnam. The virus was propagated in embryonating chicken eggs (ECE), and virus 50% egg infective dose (EID50) was determined as previously described (38). Allantoic fluid was diluted in brain heart infusion (BHI) medium (BD Bioscience, Sparks, MD) in order to obtain an inoculum with 105 EID50 per 0.1 ml per bird. A sham inoculum was made using sterile allantoic fluid diluted 1:300 in BHI medium. The commercial inactivated Re-1 vaccine (Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, People’s Republic of China) (58) was used to vaccinate the ducks. This vaccine was produced by reverse genetics and derived its HA and NA genes from A/Goose/Guangdong/96; the virus was attenuated by removing the multiple basic amino acids at the HA cleavage site. The six internal genes of this recombinant virus were derived from the high-growth A/Puerto Rico/8/34 (PR8) virus. All experiments using H5N1 HPAI viruses, including work with animals, were performed in biosafety level-3 enhanced facilities at the Southeast Poultry Research Laboratory (SEPRL), Agricultural Research Service, United States Department of Agriculture (USDA); all personnel were required to wear a powered air-protection respirator with a high efficiency particulate air (HEPA)-filtered air supply (3M™, St. Paul, MN).

**Duck experiment.** Pekin ducks, Muscovy ducks, and mallard ducks (Anas platyrhynchos) were obtained at day of age from commercial farms. Serum samples were collected from 15 ducks of each species to ensure that the birds were serologically negative for AI virus as determined by an ELISA test using IDEXX FlockChek AI MultiS-Screen (Idexx Laboratories, Westbrook, ME). Ducks were cared for and housed in accordance with protocols approved by SEPRL’s Institutional Animal Care and Use Committee.
Table 1. Morbidity and mortality. Pekin, Muscovy, and mallard ducks were challenged intranasally at 30 days of age with $10^7$ EID$_{50}$ of A/Dk/NauGiang/NCVD07-12/07 (HA clade 1) H5N1 HPAI virus. A group of Pekin and Muscovy ducks was also vaccinated at 14 days of age with inactivated Re-1 AI vaccine.

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of ducks</th>
<th>No. of ducks euthanatized at 2 dpi</th>
<th>Body temperatures at 2 dpi (F)</th>
<th>No. of ducks with neurologic signs/total (day of onset)</th>
<th>Mortality/total (MDT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pekin, nonchallenged controls</td>
<td>5</td>
<td>5</td>
<td>107.2 ± 0.18$^b$</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Pekin, challenged</td>
<td>8</td>
<td>5</td>
<td>108.9 ± 0.26$^a$</td>
<td>3/3 (3 dpi)</td>
<td>3/3 (4.5)</td>
</tr>
<tr>
<td>Pekin, vaccinated, challenged</td>
<td>10</td>
<td>0</td>
<td>107.3 ± 0.32$^b$</td>
<td>1/10 (5 dpi)</td>
<td>1/10 (6)</td>
</tr>
<tr>
<td>Muscovy, nonchallenged controls</td>
<td>10</td>
<td>5</td>
<td>106.3 ± 0.20$^b$</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Muscovy, challenged</td>
<td>11</td>
<td>5</td>
<td>106.5 ± 0.62$^b$</td>
<td>6/6 (1 dpi)</td>
<td>6/6 (2.3)</td>
</tr>
<tr>
<td>Muscovy, vaccinated, challenged</td>
<td>10</td>
<td>0</td>
<td>107.4 ± 0.28$^a$</td>
<td>4/10 (3 dpi)</td>
<td>4/10 (3.5)</td>
</tr>
<tr>
<td>Mallard, nonchallenged controls</td>
<td>5</td>
<td>5</td>
<td>106.8 ± 0.14$^b$</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Mallard, challenged</td>
<td>10</td>
<td>5</td>
<td>109.9 ± 0.26$^a$</td>
<td>5/5 (3–4 dpi)</td>
<td>5/5 (5)</td>
</tr>
</tbody>
</table>

$^a$Mean ± SD. Groups with different lowercase letters are significantly different ($P < 0.05$).

**Determination of virus shedding.** Oropharyngeal and cloacal swab samples collected from vaccinated ducks were suspended in 2 ml sterile BHI and frozen at −70°C until RNA extraction. RNA was extracted using a previously described combination of Trizol LS reagent (Invitrogen, Inc., Carlsbad, CA) and the MagMax AI/ND RNA isolation kit (Ambion, Inc., Austin, TX) (15). qRT-PCR targeting the influenza M gene (50) was performed using the SmartCycler® 2 (Cepheid Inc., Sunnyvale, CA) and the OneStep RT-PCR kit (Qiagen, Valencia, CA) in accordance with the U.S. National Veterinary Services Laboratories protocol AVSOP1521.01. A standard curve for virus quantification was established with RNA extracted from dilutions of the same titrated stock of the challenge virus, and results were reported as EID$_{50}$/ml equivalents (43). The calculated qRT-PCR detection limit was $10^2$ EID$_{50}$/ml per reaction.

**qRT-PCR for immune related genes.** Differences in expression of innate immune-related genes was determined in spleen samples collected from five nonchallenged and five virus-challenged ducks normalized by weight and homogenized in BHI medium. The spleen was chosen because it is an immune organ in birds which is also affected by systemic infection with H5N1 HPAI viruses, as previously demonstrated (9,21,42). Total RNA was extracted from spleen homogenates using Trizol following the manufacturer’s instructions (Invitrogen), followed by purification with the Qiagen RNeasy Midiprep Kit (Qiagen). The RNA concentrations of all samples were determined using a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Wilmington, DE). One-hundred micrograms of RNA from each sample were additionally treated with a one-column DNase digest (RNase-free DNase Set; Qiagen) for 15 min at room temperature. DNase digest efficacy was determined in random samples by PCR amplification using MHC-II primers (9). RNA integrity numbers were measured with the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and were between 8 and 10. The relative quantitation in gene expression following infection was performed using several sets of primers (Table 2). β-actin was used as the endogenous control. MHC-I and MHC-II, interleukin 18 (IL-18), and CCL19, also known as macrophage inflammatory protein 3 beta (MIP3b), primers were based on published sequences and designed using DNASTAR Lasergene primer select software (Madison, WI). Primers for IFN-γ, IFN-β, IL-1β, IL-6, TLR7, and RIG-I have been previously published (1,4) (Table 2). Primer pair specificity among duck species was determined by dissociation curves. Primers for IL-1β could not be optimized to avoid primer dimers in circumstances when target presence was low and, therefore, was only used for qualitative comparison. All reactions were run in triplicate with the ABI Prism® 7500 (Applied Biosystems, Carlsbad, CA) using Power SYBR® Green RNA-to-C$^TM$ 1-Step Kit (Applied Biosystems). qRT-PCR conditions and cycling parameters were the same for all primer pairs used and were as follows: 50 ng RNA per reaction on cycle at 50°C for 30 min, 1 cycle at 95°C for 15 min, 40 cycles of 95°C for 15 sec, and 58°C for 32 sec, followed by dissociation curve determination of the products with 1 cycle at 95°C for 15 sec, 55°C for 20 sec, 99°C for 15 sec, and 60°C for 15 sec. In order to confirm correct target amplification, PCR products were homogenized to a 10% (wt/vol) final concentration. Tenfold dilutions of the 10% homogenates (100 μl) were inoculated into 10-day-old ECEs and virus titers as log$_{10}$ EID$_{50}$/gram of tissue were calculated. The minimal detectable titer was 10$^{1.95}$ EID$_{50}$/g.

**Serology.** Hemagglutination inhibition (HI) assays were performed with serum collected from vaccinated ducks immediately before virus challenge and 12 days after challenge to quantify antibody response to vaccination, as previously described (43). Beta-propiolactone-inactivated antigen (Ag) made from either a virus with a similar HA as the recombinant vaccine virus (Goose/Hong Kong/437-6/99) or with the challenge virus (A/ Dk/VN/88/07) was used in the test. HI titers were reported as log$_{2}$ titters, with 3 log$_{2}$ titters being the minimum positive titer.

**Virus titers in tissues.** Titers of infectious virus from tissues collected at 2 dpi were determined as previously described (39). In brief, tissues were normalized by weight and homogenized in BHI medium, and clarified homogenates were titrated for virus infectivity in eggs. Tissues were homogenized to a 10% (wt/vol) final concentration. Tenfold dilutions of the 10% homogenates (100 μl) were inoculated into 10-day-old ECEs and virus titers as log$_{10}$ EID$_{50}$/gram of tissue were calculated. The minimal detectable titer was 10$^{1.95}$ EID$_{50}$/g.
were cloned into pCR-TOPO2.1 sub-cloning vectors (Invitrogen) and sequenced using M13 forward and M13 reverse.

Calculations and statistical analysis. A one-way ANOVA with Tukey's post-test was used to analyze the ducks’ body temperatures and virus titers in tissues using Prism v.5.01 software (GraphPad Software Inc., San Diego, CA). For statistical purposes, any tissue sample in which virus was not isolated was given a numeric value of 10⁻⁶ EID₅₀/g. Statistical significance was set at P < 0.05. Cytokine gene expression fold change was determined by the 2⁻ΔΔCt method (30) using β-actin as the endogenous reference gene to normalize the level of target gene expression. A one-way ANOVA with Tukey’s multiple comparison tests (GraphPad Software Inc.) was used to determine the statistical significance between fold change values of the three duck species. Statistical significance was set at P < 0.05.

RESULTS

Clinical signs and mortality. All nonvaccinated ducks challenged with the H5N1 HPAI virus presented neurologic signs and died in less than 5 days (Table 1); however, Muscovy ducks showed earlier and more-severe neurologic signs and had a lower mean death time (MDT) than did the Pekin and mallard ducks. Neurologic signs included whole body tremors, uncontrollable shaking, marked loss of balance, tilted head, seizures, loss of vision, and paralysis. Ducks from these groups were also depressed and had anorexia and watery, greenish diarrhea. Nine of 10 vaccinated Pekin ducks and six of 10 vaccinated Muscovy ducks survived the virus challenge, but all presented mild clinical signs consisting of conjunctivitis and anorexia, and three ducks from each group had fever at 2 dpi (>108.0°C). Four of 10 vaccinated Muscovy ducks and one of 10 vaccinated Pekin ducks had mild to severe neurologic signs and died. By 12 dpi, all surviving ducks, except one Muscovy duck, had recuperated. The surviving Muscovy duck had mild torticolis. The nonvaccinated Pekin and mallard ducks challenged with the H5N1 HPAI virus had significantly higher body temperatures than the Muscovy ducks (Table 1). The body temperatures of the vaccinated Pekin and Muscovy ducks challenged with virus were not significantly different from the nonchallenged controls and the virus-challenged Muscovy ducks.

Hemagglutination inhibition titers and virus shedding in vaccinated Pekin and Muscovy ducks. Results are presented in Table 3. Mean titers are given for only the positive ducks. Five of 10 vaccinated Pekin ducks had positive prechallenge HI titers when using the vaccine-like virus antigen in the HI assay; however, only one of 10 was positive when using the challenge virus. Two of 10 Muscovy ducks were seropositive when using the vaccine-like virus in the test, and the titers were lower than the titers in Pekin ducks. No titers were detected when using the challenge virus. This is a common occurrence when HI tests are done using a virus different than what the ducks were initially exposed to; in this case, the vaccine. Moderate to high postchallenge HI titers were observed in the vaccinated ducks when using the vaccine-like virus (>7 log₂) or the challenge virus (>3 log₂) in the test, with Muscovy ducks showing higher titers than the Pekin ducks.

There were clear differences between Pekin and Muscovy ducks in regard to the number of ducks shedding virus after challenge and in the duration of virus shedding (Table 3). Only one or two of 10 Pekin ducks shed virus at 3 and 5 dpi. Most Muscovy ducks shed viruses from the oropharyngeal route at both 3 and 5 dpi, and half shed virus through the cloacal route at 3 dpi.

Virus titers in tissues collected from Pekin, Muscovy, and mallard ducks challenged with the H5N1 HPAI virus. Moderate to high virus titers (log₁₀ 5.1–7.8 EID₅₀) were present in the lung, brain, heart, spleen, and muscle of virus-challenged ducks (Fig. 1). There were significantly higher virus titers in the hearts of the Muscovy ducks than in the Pekin and mallard ducks, but no significant difference in titers was found between the other tissues, although the titers were higher in the Muscovy duck tissues compared to the Pekin ducks.

Table 2. Primers sequences used in real-time RT-PCR.

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Size (bp)</th>
<th>Source reference</th>
</tr>
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<tr>
<td>IFN-α</td>
<td>ggccccggcagcaaccta</td>
<td>cttgaggggrgctgccagaa</td>
<td>76</td>
<td>(8)</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>aaatccctctccatggcaagaa</td>
<td>agctgcgggtggatttcctga</td>
<td>90</td>
<td>Adams (pers. comm.)</td>
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<tr>
<td>IL-1β</td>
<td>tctctctctggtggcaggt</td>
<td>cttgaggggrgcttggtta</td>
<td>151</td>
<td>Adams (pers. comm.)</td>
</tr>
<tr>
<td>IL-6</td>
<td>ttggaggtggctggagtctct</td>
<td>cctgcctcagggctggtttg</td>
<td>150</td>
<td>(1)</td>
</tr>
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<td>TLR7</td>
<td>cctgccagagcgactcattca</td>
<td>tcaagagaatacagataatccatca</td>
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<td>MHC-I</td>
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<td>(8)</td>
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<td>(8)</td>
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<td>β-actin</td>
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<td>gggtggtcctcaggggctactctcac</td>
<td>228</td>
<td>(45)</td>
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</table>

Table 3. Hemagglutination inhibition titers and virus shedding. Pekin and Muscovy ducks were vaccinated with an inactivated commercial vaccine at 15 days of age and challenged intranasally at 30 days of age with an H5N1 HPAI virus. Serum samples were taken at the day of challenge and 12 days postchallenge. HI titers were obtained using a virus similar to the vaccine or the challenge virus as antigen in the test. Swab samples were taken from all birds remaining at 3 and 5 dpi. Mean titers are given for only the positive values.

Table 4. Virus shedding no. of positive ducks/total ducks (mean titers ± SEM)⁴

<table>
<thead>
<tr>
<th>Groups</th>
<th>Prechallenge (vaccine Ag)</th>
<th>Prechallenge (challenge Ag)</th>
<th>Postchallenge (vaccine Ag)</th>
<th>Postchallenge (challenge Ag)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pekin</td>
<td>1/10 (2.8 ± 0.49)</td>
<td>1/10 (0.4)</td>
<td>9/9 (7.3 ± 0.42)</td>
<td>9/9 (3.8 ± 0.53)</td>
</tr>
<tr>
<td>Muscovy</td>
<td>2/10 (1.1 ± 0.43)</td>
<td>0/10</td>
<td>6/6 (9.16 ± 0.31)</td>
<td>6/6 (5.5 ± 0.50)</td>
</tr>
<tr>
<td></td>
<td>1/10 (2.08 ± 0.35)</td>
<td>0/10</td>
<td>1/10 (3.0)</td>
<td>1/10 (3.1)</td>
</tr>
<tr>
<td></td>
<td>6/8 (3.5 ± 0.37)</td>
<td>4/8 (3 ± 0.71)</td>
<td>6/7 (2.7 ± 0.14)</td>
<td>1/7 (3.2)</td>
</tr>
</tbody>
</table>

⁴Log₁₀ EID₅₀ equivalents were determined using qRRT-PCR specific for type A avian influenza matrix gene. Numbers in parentheses are averages of viral titers shed from birds in each group.
H5N1 highly pathogenic avian influenza in different types of ducks

Fig. 1. Virus titers in tissues collected from ducks infected with the A/Dk/NauGiang/NCVD07-12/07 H5N1 HPAI virus. Tissues were collected at 2 dpi from three ducks. Values are the mean ± standard deviation. The threshold of detection was 10^1.97 EID_{50}g. There was a significant difference in virus titers in the heart of the Muscovy versus the Pekin and mallard ducks. No significant difference was found between the other tissues.

when compared to the Pekin and mallards titers, except for in the spleen.

**Gross and microscopic lesions and viral antigen distribution in tissues.** The virus-challenged Pekin, Muscovy, and mallard ducks presented characteristic lesions of infection with H5N1 HPAI viruses including dehydration, empty intestines, splenomegaly, thymic atrophy, dilated and flaccid hearts with increased pericardial fluid, and congested malacic brains (40). The presence of microscopic lesions and virus antigen in tissues was studied in order to determine differences in virus tissue tropism in the three types of ducks. Microscopic lesions were widespread in tissues from all ducks examined and have been previously described for virulent H5N1 HPAI viruses isolated in Vietnam (42). Viral antigen staining was present in multiple tissues of all ducks infected with the virus, indicating systemic infection (Table 4). Viral antigen was observed in the pancreatic acinar epithelium, neurons and glial cells of the brain, trachea epithelium, alveolar epithelium, fragmented cardiac and skeletal myofibers, adrenal corticotrophic cells, Harderian gland epithelia, and tongue epithelia. In lymphoid organs, viral antigen was only identified in resident and infiltrating phagocytes but not in apoptotic lymphocytes. Viral antigen was also identified in the glandular epithelium of the proventriculus, in hepatocytes and Kupfer cells in the liver, smooth muscle of the ventriculus, autonomic ganglia of the enteric tract, and feather epidermal cells. Virus infection in Muscovy ducks resulted in more widespread viral antigen staining in tissues compared to Pekin and mallard ducks (Table 4). Virus staining was found in the enteric tract, kidney, and skeletal muscle of the Muscovy ducks but not in the Pekin and mallard ducks.

**Innate immune-related host genes expression.** RNA extracted from spleens was used to investigate differences in the innate immune response among the three duck species by examining gene expression patterns of various cytokines and other innate immune-response genes (Fig. 2). Although there were differences in the sequences of the cytokines examined between the different duck species, the amplified products were >98% similar. A similar trend of up- or down-regulation of innate immune genes was observed in spleens when examined at 2 dpi (Fig. 2). All three duck species showed up-regulation of IFN-α, IFN-γ, IL-6, RIG-I, CCL19, and MHC-I. Anti-viral type I IFN-α gene expression was significantly higher in Pekin and mallard ducks when compared to Muscovy ducks. IFN-γ gene expression was up-regulated in all three duck

Table 4. Distribution of viral antigen in tissues collected from ducks intranasally inoculated with the A/Dk/NauGiang/NCVD07-12/07 H5N1 HPAI virus. Tissues were collected from three ducks at 2 days postchallenge.

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Pekin</th>
<th>Muscovy</th>
<th>Mallard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trachea</td>
<td>+/+/+/+^A</td>
<td>++/+/+/+</td>
<td>+/+/+/+</td>
</tr>
<tr>
<td>Lung</td>
<td>++/+/+/+</td>
<td>+/+/+/+/+</td>
<td>+/+/+/+</td>
</tr>
<tr>
<td>Heart</td>
<td>++/+/+/+</td>
<td>++/+/+/+/+</td>
<td>++/+/+/+</td>
</tr>
<tr>
<td>Brain</td>
<td>+++/+/+</td>
<td>+++/+/+/+</td>
<td>+++/+/+</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>++/+/+</td>
<td>+++/+/+/+</td>
<td>++/+/+</td>
</tr>
<tr>
<td>Duodenum</td>
<td>−/−/−</td>
<td>+/+/+</td>
<td>−/−/−</td>
</tr>
<tr>
<td>Pancreas</td>
<td>+/+/+/+</td>
<td>+/+/+/+/+</td>
<td>+/+/+/+</td>
</tr>
<tr>
<td>Liver</td>
<td>+/+/+/+</td>
<td>+/+/+/+/+</td>
<td>+/+/+/+</td>
</tr>
<tr>
<td>Kidney</td>
<td>−/−/−</td>
<td>−/+</td>
<td>−/−/−</td>
</tr>
<tr>
<td>Spleen</td>
<td>++/+/+</td>
<td>++/+/+/+</td>
<td>++/+/+</td>
</tr>
<tr>
<td>Bursa</td>
<td>++/+/+</td>
<td>+/+/+/+</td>
<td>++/+/+</td>
</tr>
<tr>
<td>Thymus</td>
<td>+++/+/+/+</td>
<td>+++/+/+/+</td>
<td>+++/+/+/+</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>+/+/+−</td>
<td>−/+</td>
<td>+/+/+−</td>
</tr>
</tbody>
</table>

^A Duck 1/duck 2/duck 3; (−) no virus antigen staining; (+) infrequent; (+) common; (+++) widespread.
species with a significant difference in expression between Pekin and mallard ducks. Expression of IL-18 gene, a signal cytokine which stimulates the production of IFN-γ, was also increased (less than 2-fold) in Muscovy ducks and was statistically different from Pekin ducks, in which it was decreased. A significant difference in expression of the pro-inflammatory cytokine IL-6 gene was found between Muscovy and mallard ducks, increasing 15-fold in Muscovy ducks, while induction in mallard and Pekin was 11- and 4-fold, respectively. Pro-inflammatory signal mediator TLR7 expression in Pekin ducks was statistically different compared to mallard ducks, increasing in expression by 2-fold in Pekin and Muscovy ducks but down-regulated 1.4-fold in mallards. Chemokine CCL19 gene expression was up-regulated more than 9-fold in all three types of ducks with no significant difference in expression between types of ducks. RIG-I, an innate immunity response gene in ducks (4), was also up-regulated 8- to 11-fold in all three duck species, but expression was very variable and not statistically different. Expression of the MHC-II molecule was minimally decreased in all three duck types. IL-1β gene expression (qualitative comparison) decreased in all three duck species (data not shown).

**DISCUSSION**

Until 2002, H5N1 HPAI viruses caused only mild or no clinical disease in ducks. Since then, many H5N1 HPAI viruses have shown to be pathogenic in ducks (40). Nevertheless, the pathogenicity of H5N1 HPAI viruses in ducks varies depending on viral strain, the age of infected ducks and, as this and other studies demonstrates, the duck species. Based on the results of this study, there is a clear difference in response to H5N1 HPAI virus infection between the domestic Muscovy ducks when compared to the domestic Pekin and the closely related wild mallard ducks. Muscovy ducks belong to a different avian species than the Pekin and mallard ducks and seem to be more closely related to geese than ducks. This could explain the differences observed in pathogenicity and response to vaccination among these different types of ducks.

All three types of ducks presented 100% mortality after challenged with the Vietnam H5N1 HPAI HA clade 1 virus used. Similar results were obtained in Pekin ducks inoculated with other virulent viruses from Vietnam (9,37,42,43,63). The high mortality observed in the mallard ducks was not expected, based on previous research showing that mallards were less susceptible to disease produced by H5N1 HPAI viruses than were other duck species (8,17,25,36,44). The Muscovy ducks MDT was half that of the Pekin or mallard ducks, and they showed earlier and more severe neurologic signs, similar to what Phoung et al. (44) reported when comparing Muscovy and mallard ducks. Interestingly, Muscovy ducks did not mount as strong a feverish response to virus infection as the Pekin and the mallard ducks did, which was also previously found when comparing infections between Muscovy and Pekin ducks infected with another H5N1 HPAI virus (9). Furthermore, a larger number of vaccinated, challenged Muscovy ducks showed neurologic signs and died earlier than the Pekin ducks. In addition, 75% of the vaccinated Muscovy ducks shed virus, mostly via the oropharyngeal route, versus only 20% of the vaccinated Pekin ducks, and a larger number of Muscovy ducks overall (85%) shed virus until 5 dpi. Together, these results indicate Muscovy ducks are more susceptible to H5N1 HPAI infection than are Pekin and mallard ducks.

Virus distribution in tissues from the nonvaccinated, virus-challenged ducks examined at 2 dpi indicated some differences between the different types of ducks. Muscovy ducks had viral
antigen present in the enteric tract, kidney, and skeletal muscle that was not seen in Pekin and mallard ducks. In addition, Muscovy ducks had widespread viral staining in the heart, adrenal glands, and liver when compared to the lesser virus staining observed in tissues from Pekin and mallard ducks. However, no statistical difference in virus titers was found in the brain, lung, spleen, and muscle in the three types of ducks. This corroborates the severe systemic infection of this particular H5N1 HPAI virus in ducks. The significantly higher virus titers found in the hearts of the Muscovy ducks might explain the earlier deaths observed in this species. Mortality in domestic ducks has been associated with heart failure resulting from increased H5N1 HPAI viral cardiotoxicity (56,62). The immune system of the Pekin and mallard ducks could also have more-efficiently controlled virus replication and spread, thus prolonging the time of death of the ducks.

Avian influenza virus infection in naive hosts triggers a cascade of host defenses that are responsible for control and clearance of the virus and include innate and subsequent adaptive immune responses. Differences in the innate immune response between duck species is supported by the findings in this study, as virus-challenged Pekin and mallard ducks survived longer than the Muscovy ducks and presented higher body temperatures after virus infection. Body temperatures of Pekin ducks were also higher than in Muscovy ducks after infection with another H5N1 HPAI virus (9). Vaccinated Pekin ducks also mounted a better humoral immune response to vaccination, which conferred them better protection against the subsequent challenge with the H5N1 HPAI virus. This was shown by more vaccinated Pekin ducks presenting detectable antibodies prechallenge, as compared to Muscovy ducks, and less Pekin ducks shedding virus after challenge. Antibody titers after virus challenge where higher in the Muscovy ducks as a consequence of more-severe infection because of the lower levels of protection. This demonstrates that the humoral response also differs between Pekin and Muscovy ducks and supports our previous findings (9).

Differences in innate immune responses could have provided Pekin and mallard ducks an immunologic advantage in response to AI virus infection when compared to the Muscovy ducks. To examine the innate immune response to infection, expression of several innate immune-related genes was examined in spleens collected at the peak of virus infection at 2 dpi. Relative quantitative RT-PCR showed up-regulation of most innate immunity associated genes, with no significant differences observed between the three types of ducks with most cytokines, a consequence of the great variability in gene expression between the ducks in each group. Despite being more closely related and responding similarly to virus infection, no obvious gene expression pattern emerged that was shared by the domestic Pekin and mallard ducks but was different from that found in the domestic Muscovy ducks. In this study, we were unable to design primers for IL-2 that would work well for all three species, or quantitative primers for IL-1β, but could determine a decreased expression of IL-1β in all three species (data not shown). IL-6 up-regulation was previously shown to occur in both Pekin and Muscovy ducks after infection with another H5N1 HPAI virus, with IL-6 levels significantly higher in the Muscovy ducks (9). Similarly in this study, IL-6 gene expression in the Muscovy ducks was higher than in Pekin and mallard ducks, but the difference was not statistically significant between the Pekin and the Muscovy ducks as previously found. As in mammals, IL-6 has been associated with fever induction in ducks (35). Based on the higher body temperatures found in the virus-infected Pekin and mallard ducks, higher levels of IL-6 were expected when compared to the Muscovy ducks. It is not clear if this is a true difference in innate immune responses between the ducks or a consequence of the timing when the samples were taken. Ducks were sampled at 2 dpi, 2 days before any of the remaining Pekin and Muscovy ducks died. However, at this time point, four Muscovy ducks were found dead and the remaining two ducks died the day after. Consequently, the five Muscovy ducks sampled at 2 dpi, although still alive, could have been in different declining states, which might account for the lower body temperatures observed in these ducks. IFN-α and IFN-γ expression was up-regulated in all ducks, similar to what has been reported in vivo and in vitro in duck cells infected with other H5N1 HPAI viruses (1,24,29,45). IL-18, which stimulates the production of IFN-γ and thus facilitates Th1 immune responses (13), was not up-regulated in Pekin or mallard ducks but was in the Muscovy ducks. CCL19 is associated with modulation of inflammatory responses, so it is not surprising that it was up-regulated in all three duck species. Similar up-regulation of RIG-I was observed in all three types of ducks.

RIG-I, a cytoplasmic 5′-triphosphate RNA sensor found in ducks but not in chickens, initiates expression of other antiviral genes (4,32,64). RIG-I expression in 6-wk-old mallard ducks after infection with a H5N1 virus was greatly induced, but expression levels varied depending on the time after infection at which they were measured (4). We also found RIG-I up-regulation in Pekin and Muscovy ducks infected with another virulent H5N1 HPAI virus (9), but expression was higher in Pekin ducks, indicating that the virus strain might also affect cytokine gene expression. The expression of the TLR7 gene, a receptor implicated in the immune response to influenza virus, was up-regulated in Pekin and Muscovy ducks but down-regulated in the mallards. Finally, an interesting trend of up-regulated MHC-I occurring in tandem with down-regulated MHC-II was observed in this study and has been previously reported (1,9,45). The variability in immune-related gene expression among the ducks precludes us from making conclusions about differences in innate immune responses between the types of ducks studied. For this study, we chose to examine the innate immune response at the peak of virus infection, but a more-detailed time-course study would be required to show differences in responses to infection between the ducks. Differences in response are linked to the time and the magnitude of the innate immune response, with resistant animals typically producing an earlier induced innate immune response than do the more-susceptible animals.

In conclusion, different types of ducks respond differently to H5N1 HPAI virus infection and vaccination. Our findings clearly demonstrate a lower protection conferred by vaccination in Muscovy ducks to H5N1 HPAI virus infection when compared to Pekin ducks. This, and the earlier morbidity and mortality after infection, leads us to believe that in Muscovy ducks the innate immune response and, consequently, the adaptive immune response is less efficient in controlling virus infection. More studies examining the expression of more innate immune-related genes and at earlier time points should help determine this. The results from this study provide insight into the nature of immune responses in ducks to infection with H5N1 HPAI viruses.

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