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Protection from Clinical Disease Against Three Highly Virulent Strains of Newcastle Disease Virus After *In Ovo* Application of an Antibody–Antigen Complex Vaccine in Maternal Antibody–Positive Chickens

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SUMMARY. Worldwide, Newcastle disease (ND) remains one of the most economically important diseases of poultry. Current vaccination strategies for commercial poultry include the use of inactivated and live ND vaccines that typically induce protection against virulent field viruses. Here, we tested the efficacy of an antigen–antibody complex (AAC) ND vaccine delivered *in ovo*. Commercial maternal antibody–positive broiler chickens (*Gallus domesticus*) were vaccinated *in ovo* with an AAC vaccine composed of live B1-LaSota Newcastle disease virus (NDV) complexed with NDV-specific antiserum, and then they were challenged at weekly intervals after hatch. Challenge viruses included three exotic ND disease (END) viruses: the neurotropic strain Texas GB NDV-92-01 (TxGB) and two viscerotropic isolates, one isolate from the 2002–2003 outbreak in California (California 2002 isolate S212676 [CA]) and the other isolate from a 1997 END outbreak in South Korea (South Korea 94-147 [SK]). Results demonstrate that maternal antibody was able to provide approximately 50% protection in either vaccinated or control chickens at 7 days of age after TxGB challenge. However, with challenge at ≥14 days, most control birds died, whereas all AAC-vaccinated birds were protected. Challenge with the CA or SK viruses in chickens at 28 days of age resulted in 100% protection of vaccinated birds, whereas all control birds died. In addition, AAC-vaccinated birds displayed decreased incidence of viral shedding in oral and cloacal swabs than control birds. Antibody titers were significantly ($P < 0.05$) higher in vaccinated chickens, as determined by enzyme-linked immunosorbent assay and hemagglutinin-inhibition tests, than in nonvaccinated controls. Together, these results demonstrate the efficacy of AAC vaccines delivered *in ovo* to protect commercial poultry.

RESUMEN. Protección contra la enfermedad clínica inducida por tres cepas altamente virulentas del virus de la enfermedad de Newcastle después de la aplicación *in ovo* de una vacuna con base en complejos antígeno-anticuerpo en pollos con inmunidad materna.

La enfermedad de Newcastle sigue siendo una de las enfermedades económicamente más importantes en la avicultura a nivel mundial. Las estrategias actuales de vacunación para las aves comerciales incluyen el uso de vacunas inactivadas y vivas que normalmente inducen protección contra los virus de campo virulentos. En este trabajo, se analizó la eficacia de una vacuna aplicada *in ovo* basada en complejos antígeno-anticuerpo contra la enfermedad de Newcastle. Se vacunaron pollos de engorde (*Gallus domesticus*) por vía *in ovo* y que tenían anticuerpos maternos con una vacuna compuesta de complejos inmunes conteniendo un virus vivo de la enfermedad de Newcastle cepa B1-Sota adicionada con un antisuero específico, y luego fueron desafiados a intervalos semanales después de la eclosión. El desafío de los virus incluyó tres virus de la enfermedad de Newcastle exóticos: la cepa neurotrópica Texas GB NDV-92-01 (TxGB) y dos cepas viscerotrópicas, un aislamiento del brote en California durante loa años 2002–2003 (aislamiento de California 2002, S212676 [CA]) y otro aislamiento de un brote a finales de 1997 en Corea del Sur (Corea del Sur 94-147 [SK]). Los resultados demuestran que los anticuerpos maternos fueron capaces de proporcionar aproximadamente el 50% de protección, ya sea en los pollos vacunados o en los controles a los 7 días de edad después de la exposición con la cepa TxGB. Sin embargo, con los desafíos los ≥ 14 días, la mayoría de las aves del grupo control murieron, mientras que todas las aves vacunadas con la vacuna con complejos inmunes estuvieron protegidas. El desafío con las cepas CA o SK en los pollos a los 28 días de edad indujo una protección del 100% en las aves vacunadas, mientras que todas las aves de control murieron. Además de las aves vacunadas con la vacuna de complejos inmunes mostraron menor incidencia para la excreción del virus en las muestras orales y cloacales en comparación con las aves del grupo control. Los títulos de anticuerpos fueron significativamente ($P < 0.05$) mayores en los pollos vacunados, según lo determinado por el ensayo de inmunoabsorción con enzimas ligadas y por las pruebas de inhibición de la hemaglutinación, en comparación con los controles no vacunados. En conjunto, estos resultados demuestran la eficacia de las vacunas por complejos inmunes aplicadas *in ovo* para proteger aves de corral comerciales.

Key words: Newcastle disease, antigen–antibody complex, vaccine

Abbreviations: AAC = antigen–antibody complex; Ag = agriculture; BSL = biosafety level; CA = California 2002 isolate S212676; EID = egg infective dose; ELISA = enzyme-linked immunosorbent assay; END = exotic Newcastle disease; HI = hemagglutination inhibition; ICPI = intracerebral pathogenicity index; ND = Newcastle disease; NDV = Newcastle disease virus; pc = postchallenge; SK = South Korea 94-147; TxGB = Texas GB NDV-92-01

Newcastle disease (ND) is a disease of chickens (*Gallus domesticus*) of economic importance throughout the world, impacting foreign trade. The highly virulent form of the virus is endemic in numerous countries and produces a lethal disease characterized by severe respiratory illness, conjunctivitis, neuropathy, enteritis, or a

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combination. Although ND is readily controlled by low-virulence live vaccines in countries where the field viruses are mildly virulent, these live vaccines are often not sufficient to induce full protection against a highly virulent challenge (15).

ND virus (NDV) belongs in the *Avulavirus* genus, within the *Paramyxoviridae* family. NDV isolates can be classified by pathogenicity as lentogenic, mesogenic, or velogenic, depending on the severity of disease produced by the isolate in chickens (1). The occurrence of virulent NDV infections because of mesogenic or velogenic strains is recognized as ND, a notifiable disease reportable to the Office of International Epizooties (21). The term exotic ND (END) has been used to describe the notifiable disease in the United States. Virulent NDV isolates have entered the United States via illegal importation of birds, including psittacine birds (9,22,28), the causal agents of the last two major outbreaks in the United States in southern California during 1971–1973 and 2002–2003 (18,19,20,23,26,29). Outbreaks of virulent NDV have been reported from turkeys (*Meleagris gallopavo*) in North Dakota during 1992 (16), cormorants (*Phalacrocorax* spp.) in the north central United States (3,25,27), and game birds in California during 1998, underscoring the threat of virulent NDV to commercial U.S. poultry production (6).

Commercial vaccination programs for NDV in North America and western Europe include the use of low-virulent live-virus vaccines and inactivated vaccines designed to control against endemic, low-virulence field strains. For broilers, the goal of these vaccination programs is to induce protective immunity without affecting production efficacy. Immunization of poultry breeders is used to elicit maternal antibodies in the offspring. Although protecting chicks during the crucial early weeks of life, maternal antibodies also may interfere with acquisition of immunity to vaccination (7,8,15,30).

In countries where chickens are highly likely to encounter the virulent forms of NDV, it is not uncommon for poultry producers to vaccinate each chick with a killed vaccine, often in combination with a live vaccine either in the hatchery or as a boost in the field (5). Such vaccination programs can induce high antibody titers and lasting protection. The disadvantage of this type of vaccination regimen is the cost of the vaccine and the effort required to handle each chick and ensure it receives its vaccine dose. Thus, it would be preferred if a highly effective vaccine could be easily mass delivered at the hatchery.

The Embrex® Inovoject® delivery device (Pfizer Animal Health, Durham, NC) offers a method to administer vaccines to the chicken egg before hatch, ensuring that each egg receives a uniform dose of vaccine. Marek's disease vaccine, infectious bursal disease vaccine, and fowlpox vaccines are routinely administered via the Embrex Inovoject device. NDV, however, is highly lethal when administered into the egg; therefore, conventional live ND vaccines cannot be administered via the Embrex Inovoject device. The vaccine used in this study uses antibody–antigen complex (AAC) technology (12,13,31) to delay the release of virus until after hatch when the immune system is sufficiently developed to keep the virus in check, thus allowing *in ovo* administration.

In ND outbreak situations, controlling clinical disease and reducing the shed of virus from infected birds to susceptible cohorts are critical to controlling spread of disease. The objectives of the present study were to extend the knowledge of protection against highly virulent NDV by *in ovo* vaccination of commercial maternal antibody–positive chickens. An important factor for any vaccine is its ability to provide protection against a variety of challenge strains to which the bird may be exposed in the field. This study evaluated protection against three strains of highly virulent NDV in broilers vaccinated *in ovo* with an AAC NDV.

MATERIALS AND METHODS

Vaccine. Newplex™ vaccine (Lohmann Animal Health International, Enterprise, AL) is a live ND vaccine composed of a B1-LaSota NDV complexed with NDV-specific hyperimmune serum (note that this vaccine is no longer commercially manufactured). The lyophilized vaccine was reconstituted with sterile Marek's vaccine diluent (Merial Select, Duluth, GA) to give 1 dose/100 μl. The reconstituted vaccine was administered *in ovo* on day 18 of incubation at 100 μl/egg. Control eggs were injected with 100 μL of Marek's vaccine diluent.

Challenge viruses. Challenge viruses were the virulent NDV strains Texas GB NDV-92-01 (TxGB), California 2002 isolate S212676 (CA), and South Korea 94-147 (SK). The TxGB virus was obtained from the U.S. Department of Agriculture Center for Veterinary Biologics (Ames, IA). It has an intracerebral pathogenicity index (ICPI) of 1.75 and was used at a challenge dose of $10^{4.3}$ egg infective dose $(EID)_{50}$ per bird, applied by intramuscular injection in 0.1 ml. The CA virus was a chicken isolate from the California END outbreak of 2002 with an ICPI of 1.79. The CA challenge dose used was $10^{5.7}$ EID_{50}/bird. The SK isolate was a 1997 isolate from chickens in South Korea. It has an ICPI of 1.78 and was administered at $10^{5.9}$ EID_{50}/bird. Dilutions of CA and SK challenge virus were made in tryptose phosphate broth (Sigma Chemical Co., St. Louis, MO), with challenges administered intraocularly in 100-μl volumes split into each eye (50 μl each).

Study design and birds. Ross × Hubbard fertile broiler eggs (175) were inoculated *in ovo* at 18 days of embryonation with AAC vaccine. Matched diluent-injected eggs served as controls. Hatched chicks of each group were placed in isolated floor pens for rearing, with feed and water provided *ad libitum*. At 1, 7, 14, 21, 28, and 35 days of age, birds were taken from the floor pens and bled for antibody titers against NDV. At 7, 14, 21, 28, and 35 days of age, 11 control and 22 vaccinated birds were placed into isolator cages and challenged as described above with virulent TxGB in biosafety level (BSL)-2 plus Agriculture (Ag) conditions. Clinical signs and mortality of challenged birds were monitored daily for 14 days. Surviving birds were bled and euthanatized by CO_2 inhalation at 14 days postchallenge (pc). Oral and cloacal swabs were collected before challenge (day 0) and at 4 days pc for NDV reisolation.

At 26 days of age, 25 control (sham-vaccinated) birds and 40 vaccinated (Newplex) birds were randomly selected and shipped by ground to Southeast Poultry Research Laboratory (Athens, GA) for challenge in BSL-3 Ag facilities with the CA and SK virulent NDV isolates. Upon arrival, birds were assigned to treatment groups as follows: control unchallenged (five birds), control SK challenged (10 birds), control CA challenged (10 birds), vaccinated SK challenged (20 birds), and vaccinated CA challenged (20 birds). Birds were housed in isolation units (five birds/unit) and allowed to rest and acclimate for 2 days. Challenges were then administered on day 28 of age.

Serology. Both hemagglutination inhibition (HI) assays and enzyme-linked immunosorbent assay (ELISA) were performed on individual serum samples. HI was performed by standard microtiter plate methods (2) using four HA units per well of a LaSota strain NDV. The HI geometric mean titers were expressed as reciprocal $\log_2$. Antibody to NDV also was measured by ELISA using a commercial test kit (IDEXX FlockChek®; IDEXX Laboratories, Westbrook, ME) in accordance with the manufacturer's directions.

Incidence of virus shedding. Cloacal and pharyngeal swabs were used for detection of challenge virus at day 4 pc. Swabs were hydrated in tryptose phosphate broth and inoculated into specific-pathogen-free eggs at 9–11 days of incubation and then tested for NDV as described previously (15).

Statistical analysis. Data were analyzed with SigmaStat 2.0.3 (SPSS Inc., Chicago, IL). ANOVA using pairwise comparisons with Tukey's method was used to compare HI and ELISA values. Mortality and frequency of virus isolation were analyzed for significance by Fisher exact test. All tests were performed with a 5% level of significance.

RESULTS

Maternal antibody and onset of immunity. Because commercial layers were the source of birds used in these studies, maternal antibody

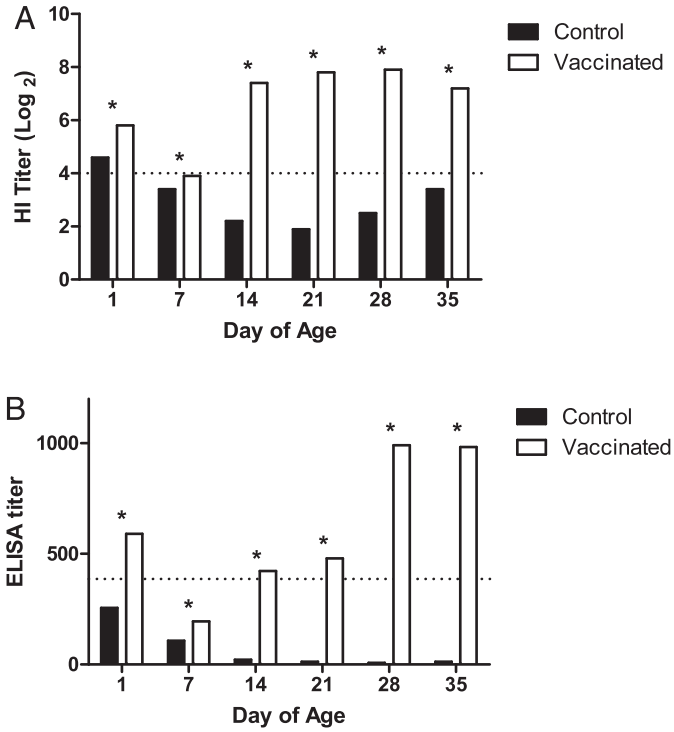

Fig. 1. Onset of antibody responses in maternal antibody–positive chickens receiving *in ovo* NDV vaccination determined by HI (A) and ELISA titer (B) at different days of age. Groups of chickens received mock (control) or antibody-complexed NDV (vaccinated) *in ovo* vaccination at 18 days of embryonation. At 1, 7, 14, 21, 28, and 35 days posthatch, serum was collected from birds in each group for testing. ELISA titers >396 are considered positive. Asterisk (*) indicates vaccinated birds significantly different from controls on that day by ANOVA ($P \leq 0.05$).

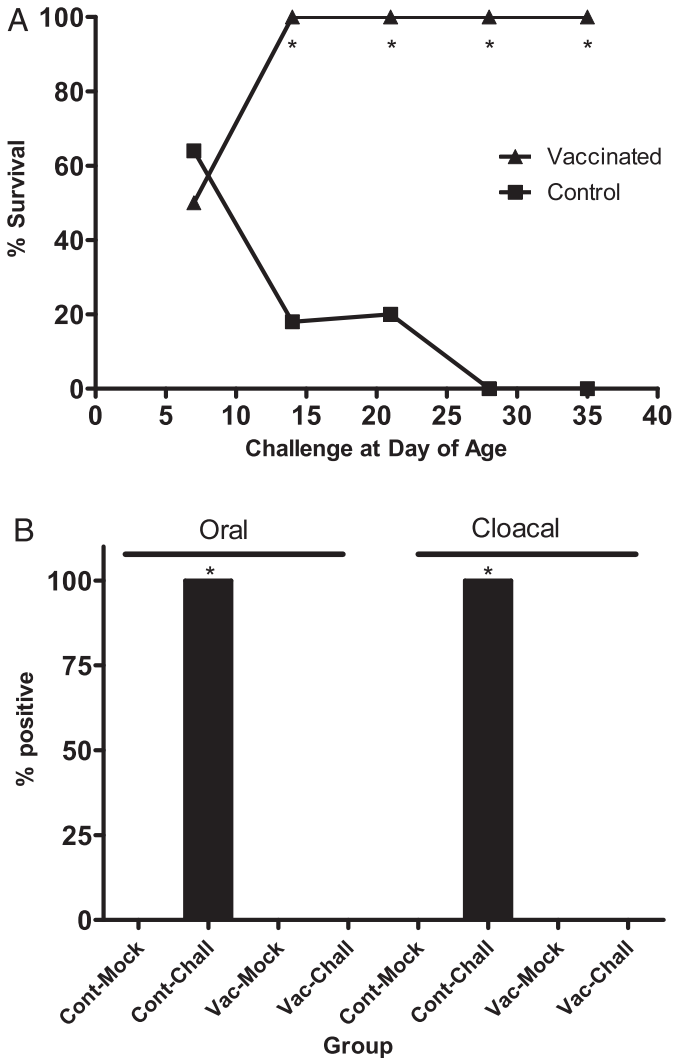

Fig. 2. Protection of maternally antibody–positive chickens after *in ovo* NDV vaccination against NDV TxGB challenge at different days of age. Groups of chickens received mock (control) or AAC NDV (vaccinated) by *in ovo* vaccination at 18 days of embryonation. (A) At 7, 14, 21, 28, and 35 days posthatch, birds ($n = 11$ and 22 for control and vaccinated groups, respectively) were challenged with $10^{4.3}$ EID_{50} TxGB via intramuscular route, and survival was monitored over a 14-day period. (B) Incidence of viral shedding in birds challenged at day 28 posthatch. Results shown are from oral and cloacal swabs taken 4 days pc. Asterisk (*) indicates vaccinated birds significantly different from controls on that day by Fisher exact test ($P \leq 0.05$).

levels against NDV were expected to be present. Both groups of birds had maternal antibodies to NDV at 1 day of age (Fig. 1). The HI and ELISA titers for both treatment groups dropped from day 1 to day 7 of age, indicating a decrease in maternal antibody. After day of hatch, the anti-NDV serum antibody levels in unchallenged control birds declined to <4 ($\log_2$) as measured by HI, demonstrating a typical antibody decay pattern, and levels remained there throughout the study. In contrast, HI titers in vaccinated birds rose to >6 ($\log_2$) between 14 and 35 days of age, demonstrating a strong humoral immune response after *in ovo* vaccination. ELISA titers of control birds displayed similar antibody decay as observed in HI titers, with the highest ELISA titers found immediately after hatch and dropping thereafter (Fig. 1B). As stated by the manufacturer, titers >396 are considered positive. Birds in the vaccinated group demonstrated increasing ELISA titers from day 14 (405) through day 28, reaching a titer of approximately 1000 (Fig. 1B).

Response to TxGB challenge. When challenged with TxGB at 7 days of age, 64% of control birds survived challenge, indicating the protective potential of maternal antibody (Fig. 2A). Among the ACC-vaccinated birds, only 50% were protected from clinical signs and mortality, despite significantly higher antibody levels (Fig. 1A). At 14 and 21 days of age, only 18% and 20% of control birds survived challenge without clinical symptoms, probably due to waning maternal antibody. After 21 days of age, all control birds succumbed to challenge. In contrast, 100% of vaccinated birds challenged at 14, 21, 28, or 35 days of age survived challenge, a level that was statistically different from that of the control group.

Experiments to determine incidence of viral shedding at 4 days pc demonstrated that 100% of control birds were shedding virus in both oral and cloacal swabs (Fig. 2B). In contrast, no NDV-positive swabs were recovered from vaccinated or unchallenged groups.

Response to END challenge with isolates from California and South Korea. To determine cross-protection against other END isolates, birds were challenged at 28 days of age with either isolates from outbreaks in California (2002) or Korea (1997). All unvaccinated control birds succumbed to challenge after inoculation with CA and SK strains of NDV (Fig. 3A). In contrast, *in ovo*–vaccinated birds were 100% protected against both CA and SK virus challenges.

Comparison of the prechallenge (day 0) and pc (day 14) HI titers demonstrated that vaccinated birds had protective antibody titers (≥4) before challenge (Fig. 3B). An anamnestic response was observed in these groups to END challenge at day 14. No detectable

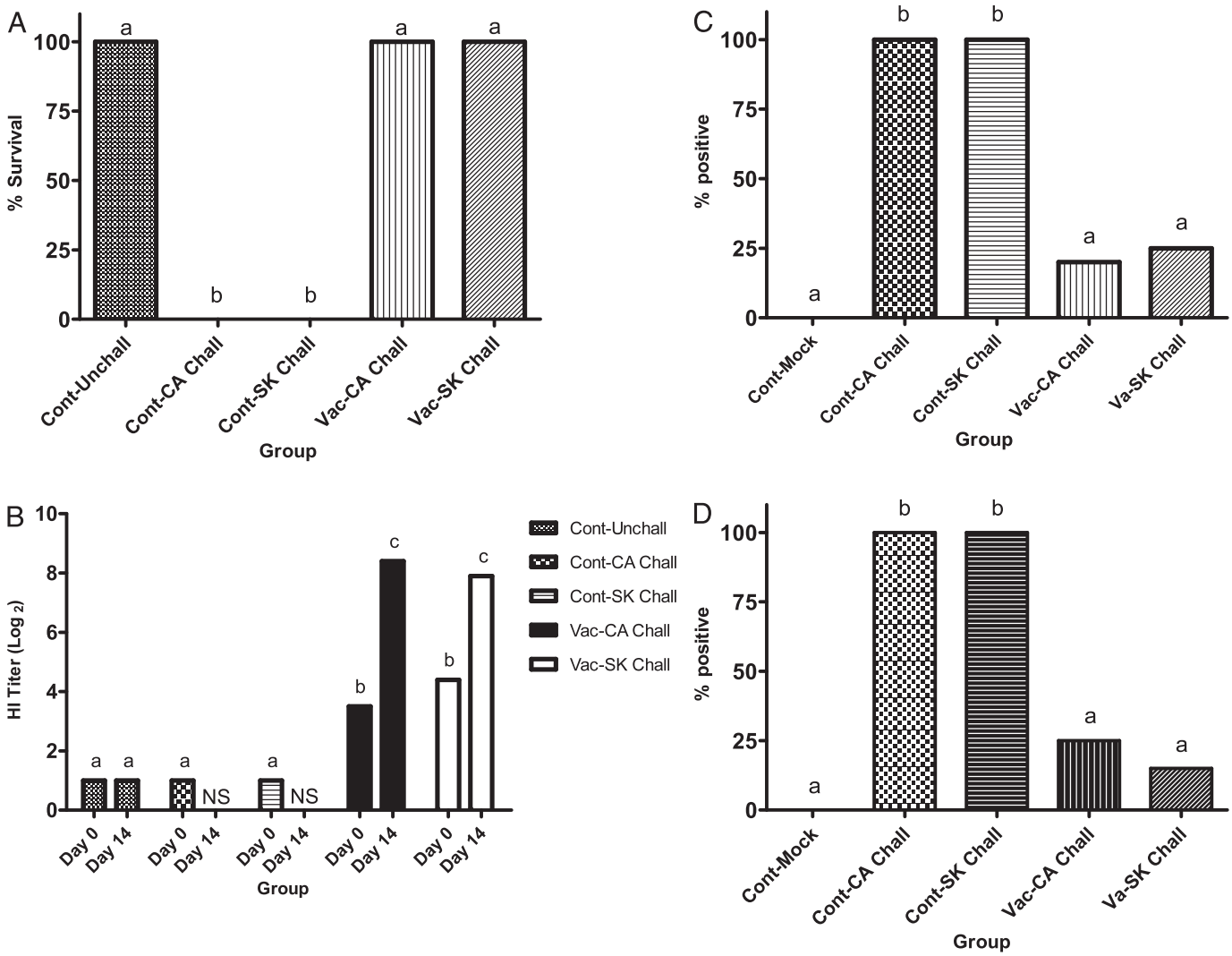

Fig. 3. Response of vaccinated birds to challenge with virulent NDV strain CA or SK at 28 days of age. Groups of maternal antibody–positive chickens received mock (control) or antibody-complexed NDV (vaccinated) *in ovo* vaccination at 18 days of embryonation. At 28 days posthatch, birds were challenged with approximately 10^6 EID_{50} CA or SK via intraocular route, and survival was monitored over 14-day period. (A) Protection after END challenge. (B) At 0 and 14 days pc, serum was collected from birds in each group for HI testing. Incidence of viral shedding from oral (C) and cloacal (D) swabs in groups of birds at day 4 pc. NS = no surviving birds. Differences in lowercase letters indicates significant difference ($P \leq 0.05$) between groups by Fisher exact test for survival and ANOVA for HI and viral shedding.

antibody titers were observed in unvaccinated birds before challenge, indicating maternal antibody was no longer present in these groups.

No virus was recovered from oral or cloacal swabs before challenge (data not shown). After challenge, virus was recovered from 100% of nonvaccinated control birds in both oral and cloacal swabs at 4 days pc (Fig. 3C, D). In contrast, virus was identified in 15%–25% of swabs from vaccinated birds on day 4 pc.

DISCUSSION

Overall, the *in ovo*–administered ND vaccine provided full protection against all three virulent isolates of the virus when challenged at 28 days of age. Maternal antibodies alone were able to provide partial protection against TxGB up to 21 days of age, although *in ovo* vaccination clearly enhanced protection. We have demonstrated previously the protection achieved from TxGB challenge after *in ovo* vaccination with an AAC vaccine (10). Because TxGB is the virulent reference challenge strain for licensing vaccines in the United States, all commercially licensed NDV vaccines are expected to provide protection against it. As new viral strains emerge, however, it is important to determine vaccine protection against them. The END challenge strains CA and SK are not closely related phylogenetically to TxGB and offered an opportunity to determine efficacy against distant END isolates (17,23). As demonstrated here, *in ovo* vaccination with AAC vaccines can induce protective levels of immunity in commercial chickens containing maternal antibody against more phylogenetically distant strains.

The rapid decline in antibody titers between 1 and 7 days of age indicates only a modest amount of maternal antibody at hatch. Nevertheless, survival of 64% of control birds to TxGB challenge at day 7 is indicative of the protection afforded by maternal antibody. Unlike inactivated or live NDV vaccines applied at day of age, the maternal antibody did not interfere with acquisition of protective immunity in *in ovo*–vaccinated birds. The HI titers of vaccinated birds reached fully protective levels by day 14, corresponding with 100% protection to TxGB challenge. Protection remained constant through 35 days of age.

An interesting observation was that ELISA titers developed more slowly than HI titers. This is in contrast with ELISA profiles for broilers vaccinated with conventional live virus vaccines at hatch and may be a feature of the immune-complexed ND vaccine (15). The earlier development of HI than ELISA titers also was observed under field conditions (11). HI titers, however, are more predictive of protection to virulent challenge (24).

Although *in ovo*–vaccinated birds were fully protected from challenge against all three virulent NDV strains, viral shedding was not eliminated. Although no virus was recovered from TxGB-challenged birds, virus was isolated from both oral and cloacal swabs of a few of the birds in the CA and SK groups 4 days pc, and this isolation may indicate decreased antigenic homology of the vaccine virus (B1-LaSota) to the challenge viruses. Decreased suppression of the challenge virus after CA and SK challenge also may be due to the slightly higher pathogenicity, as indicated by higher ICPI values of 1.79 and 1.78, respectively, than for TxGB strain (1.75).

Viral shedding after challenge may indicate the degree of local immunity in the respiratory system and digestive tract. Kapczynski and King (15) demonstrated that viral shedding in chickens challenged with CA strain virulent NDV was lower in birds vaccinated with a live vaccine by intraocular inoculation than in those vaccinated subcutaneously with an inactivated oil adjuvant ND vaccine (15). It is worth noting that the live vaccines used in our previous study (NEWHATCH-C2®; Merck Animal Health, Summit, NJ) differed from the vaccine used in these studies (B1-LaSota). The frequency of viral reisolation after CA challenge in the present study using *in ovo* vaccination is comparable to the published results for the live vaccine and is consistent with the presence of live virus in the AAC vaccine and indicates that the mechanism of protection may not be affected by complexing the virus with antibody. The role of local immunity to NDV in the respiratory tract has been demonstrated and is important for field protection against aerosol challenge (4). The challenges used in this study also mimic this route (administered intraocular) so as to match the desired conditions as well as possible. The *in ovo* route of vaccination presents antigen to the mucosal surfaces of the respiratory tract and digestive tract (14). This route favors development of mucosal immunity. *In ovo* vaccination for ND also elicits sufficient humoral immunity to afford protection against intramuscular challenge with TxGB as early as day 14 (10).

In summary, *in ovo* vaccination for ND broadens the options available to poultry producers and may offer a more efficient, cost-effective vaccination strategy. Whether *in ovo* NDV vaccination affects growth of broilers remains to be determined. Because full immunity against phylogenetically distant virulent viruses can develop at an early age in maternal antibody–positive chickens, this type of vaccine has an advantage when applied to commercial poultry. Finally, *in ovo* vaccination seems to stimulate both local and humoral immunity to NDV and may potentially enhance cell-mediated immunity, although that is the subject of future research.

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