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Source: Avian Diseases, 53(2) : 175-183

Published By: American Association of Avian Pathologists

URL: https://doi.org/10.1637/8465-090308-Reg.1
Infectious Bronchitis Virus Field Vaccination Coverage and Persistence of
Arkansas-Type Viruses in Commercial Broilers

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SUMMARY. To determine the coverage of infectious bronchitis virus (IBV) vaccine field boost in commercial broilers, estimate the relative amount of vaccine virus in the trachea, and follow the clearance of the vaccine, we collected approximately 100 tracheal swabs at various times postvaccination from 10 different flocks and used real-time reverse transcriptase–PCR (RT-PCR) to detect the virus. This allowed us to detect vaccine virus in as few as 3% of the birds in a flock of 20,000 birds with a 95% confidence level. We found that the number of birds positive for IBV vaccine following vaccination in the field resembled a parabolic-shaped curve that peaked around 14 days postvaccination, or it resembled a sinusoidal-type wave with a frequency of about 2 wk. The patterns did not appear to correlate with water or spray vaccination methods, nor did they correlate with the type of backpack sprayer used. The highest number of positive birds in a flock ranged from 66% to 100%. The viral genome copies in the tracheal swabs, as determined by real-time RT-PCR, ranged from $1 \times 10^{2.6}$/ml to $1 \times 10^{3.2}$/ml and, in most studies, had a positive correlation with the number of birds positive for vaccine virus in the flock. On the last sample day of each study, 21, 28, or 35 days postvaccination, from 12% to 66% of the birds were still positive for vaccine virus, and although different IBV vaccine types were used in each study, only Arkansas vaccine virus was identified in selected samples on those days. Arkansas vaccine virus was also the only virus identified in selected samples at 1, 3, and 5 days postvaccination, clearly indicating that Arkansas vaccine virus is persisting in the birds. Protection studies conducted on birds vaccinated with Arkansas- and Delaware-type vaccines and removed from the field at 21 days postvaccination showed complete protection against challenge with Delaware (except for one bird), whereas protection against Arkansas challenge was between 37.5% and 62.5%. Our findings show that introduction of IBV vaccines into a commercial broiler flock do not necessarily follow a seemingly logical pattern of a high number of birds infected followed by clearance from the trachea, but resembled either a parabolic curve or a sinusoidal-type wave. In addition, Arkansas vaccine viruses are clearly persisting in commercial broilers, which may be because of incomplete protection observed for that IBV type.

RESUMEN. Cobertura de la vacunación a nivel de campo contra el virus de la bronquitis infecciosa y persistencia de virus del tipo Arkansas en pollos de engorde comerciales.

Para determinar la cobertura de la vacunación de refuerzo contra el virus de la bronquitis infecciosa aplicada en el campo, para estimar la cantidad relativa de virus vacunal en la tráquea y para registrar el proceso de desaparición del virus vacunal, se recolectaron aproximadamente 100 hisopos traqueales de diez diferentes parvadas en tiempos diferentes después de la vacunación. Dichas muestras fueron analizadas para detectar al virus mediante una técnica en tiempo real de transcripción reversa-reacción en cadena por la polimerasa (de las siglas en Inglés RT-PCR). Este procedimiento permitió detectar al virus vacunal por lo menos en 3% de las aves en una parvada de 20,000 con un nivel de confianza del 95%. Se encontró que el número de aves positivas para la presencia de la vacuna de bronquitis infecciosa después de la vacunación en el campo, se asemeja a una curva parabólica con su nivel máximo alrededor de los 14 días después de la vacunación, o también se presentaba como una onda de tipo sinusoidal con una frecuencia de aproximadamente dos semanas. Los patrones no parecen tener correlación con los métodos de protección utilizados. El número más alto de aves positivas en una parvada, tuvo un rango de 66% a 100%. Las copias de genoma viral en los hisopos traqueales determinadas por RT-PCR en tiempo real mostraron un rango de $1 \times 10^{2.6}$/ml a $1 \times 10^{3.2}$/ml y en la mayoría de los estudios, se observó una correlación positiva con el número de aves positivas a la presencia del virus vacunal dentro de la parvada. En el último día de cada estudio, 21, 28 ó 35 días después de la vacunación, el 12% al 66% de las aves aún fueron positivas a la presencia del virus vacunal y aunque se utilizaron diferentes vacunas en cada estudio, solo la vacuna con el virus Arkansas fue identificada en las muestras seleccionadas en esos días. El virus vacunal Arkansas fue también el único virus identificado en las muestras seleccionadas a los días uno, tres y cinco después de la vacunación, indicando claramente que la vacuna con virus Arkansas persistió en las aves. Mediante estudios de protección conducidos en aves vacunadas con virus Arkansas y Delaware, que fueron removidas del campo al día 21 después de la vacunación, se demostró protección completa contra el desafío con el virus Delaware (excepto por una ave), mientras que la protección contra el desafío con Arkansas fue de 37.5% y 62.5%. Estos resultados muestran que la introducción de vacunas de la bronquitis infecciosa en las parvadas comerciales de pollo de engorda, no necesariamente sigue un patrón lógico en el cual un número alto de aves se infectan seguido de la eliminación viral en la tráquea, sino el comportamiento se asemeja a una curva parabólica o una onda sinusoidal. Además, las vacunas con virus Arkansas claramente persisten en las parvadas de pollo de engorde comerciales, lo cual puede ser debido a una protección incompleta observada contra dicho tipo viral.

Key words: infectious bronchitis virus, field boost vaccination, vaccine coverage, spray vaccination, water vaccination, protection, vaccination, challenge

Abbreviations: Ct = cycle threshold; EID$_{50}$ = 50% egg infective dose; ELISA = enzyme-linked immunosorbent assay; IBV = infectious bronchitis virus; ILTV = infectious laryngotracheitis virus; Mass = Massachusetts; NDV = Newcastle disease virus; PBS = phosphate-buffered saline; RT-PCR = reverse transcriptase–PCR; SPF = specific-pathogen-free

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Infectious bronchitis virus (IBV) is worldwide in distribution, highly infectious, and extremely difficult to control because it has extensive genetic diversity, a short generation time, and a high mutation rate. It continues to cause disease in chickens, even in vaccinated birds because the virus is constantly changing and evolving to avoid the immune response. Infectious bronchitis virus, a group III coronavirus, is an enveloped, single-stranded, positive-sense RNA virus (14). The RNA genome codes for the viral RNA-dependent RNA polymerase, four structural proteins (spike, envelope, membrane, and nucleocapsid), and numerous regulatory proteins. The most studied structural protein in coronaviruses is the spike glycoprotein (S, S1, S2), which form club-shaped projections on the surface of the virus particles. The S1 subunit forms the outer portion of spike and is anchored in the envelope of the virus by the S2. Spike mediates cell attachment and virus-cell membrane fusion, and plays an important role in host-cell specificity. In addition, neutralizing antibodies are directed against spike.

Although IBV causes a mild upper-respiratory tract disease in chickens, the virus replicates in the ciliated columnar epithelium, which compromises the mucociliary apparatus, predisposing diseased birds to potentially lethal secondary pathogens (5). Thus, control of the disease is extremely important and modified live vaccines are commonly used. To obtain locally protective antibodies in the upper respiratory tract, two vaccinations, a prime and a boost, are given to broilers; the prime is given in the hatchery at 1 day of age, and the boost in the field, typically between 14 and 18 days of age. Mass delivery of the boost vaccine to 20,000 or more commercial broilers in a flock poses some unique challenges. Typically the field boost is given in either the water or via a backpack sprayer. Water vaccination is accomplished using a proportioner or by the pump-and-barrel method in which vaccine is mixed in a barrel and pumped through the water lines in the house. Vaccination by the spray method uses either a Stihl 420 backpack sprayer (Stihl, Virginia Beach, VA), which is a blower-type sprayer that delivers vaccine to high-velocity air from a gasoline-powered blower, or a SoloVac backpack sprayer (Merial, Athens, GA), which uses a battery-powered pump to force vaccine through a small orifice in a wand assembly to create a fine mist.

It is important to vaccinate chickens with the type of IBV causing the disease because different serotypes do not cross-protect (5). Different types of IBV include multiple serotypes and variants of the virus, which arise due to mutations, insertions, deletions, and recombination events during viral RNA replication. Compounding this situation is the ability of IBV to rapidly change and adapt to the host. Thus, it is extremely important not only to identify the IBV type causing disease, but also to choose an appropriate vaccine. There are approximately six different serotypes of IBV vaccine used in the United States. And, although Arkansas, Connecticut (Conn), and Massachusetts (Mass) are three of the most common types used (5), the most frequently detected IBV type in commercial broilers in the United States is Arkansas (9,17).

Identifying IBV vaccine viruses isolated from birds is key to understanding the dynamics of field vaccination for IBV and to elucidating the source of persisting Arkansas IBV–type viruses in commercial broilers. Until recently, only in vivo pathogenicity studies conducted in susceptible birds could be used to determine if isolates of IBV from commercial chickens were reisolated vaccines or pathogenic field viruses. Now, nucleotide sequences for the commercially available vaccines used in the United States, as well as for those vaccines reisolated from experimentally vaccinated birds, are available, making it possible to genetically identify vaccine viruses isolated from commercial chickens (15,19). In this study, we sought to determine the coverage of IBV vaccine field boost in broilers, determine the relative amount of vaccine virus in the trachea, and follow the clearance of the vaccine. In addition, we examined the nature of persisting Arkansas viruses in the flocks and tested the immune status of the vaccinated birds to IBV by challenging with pathogenic viruses homologous to the vaccines.

**MATERIALS AND METHODS**

**Experimental design.** To examine the number of birds with IBV vaccine virus in their trachea and follow the clearance of the vaccines, we swabbed the tracheas of 100 birds per house, unless otherwise indicated, at various times postvaccination. In general we collected samples until the flock went to processing but specific sample collection days are indicated for each study and are presented in Table 1. The swabs were placed in 1 ml of ice-cold phosphate-buffered saline (PBS; pH 7.4), transported to the laboratory on ice, then frozen at −80°C until used for RNA extraction. A total of 10 broiler houses on six different farms were studied over a 2-yr period, and all the IBV vaccines were given at the manufacturer’s recommended dose.

The relative amount of IBV vaccine given in the field boost was determined by isolation of nonhemagglutinating virus in 10-day-old embryonating eggs (studies 1 and 3 only) by previously published methods (18) and by real-time reverse transcriptase-PCR (RT-PCR; see below). To isolate the vaccine in the water, approximately 1 ml of the vaccine in the barrel, at the nipple drinkers, and at the end of the water line was collected. When spray vaccination was conducted, approximately 1 ml of the vaccine in the backpack tank and from the spray nozzle was collected. In addition, 12 × 75-mm sterile polystyrene culture test tubes (Fisher Healthcare, Houston, TX) were placed at the floor level of the chicken house to collect vaccine from the sprayer. Antibody titers to IBV were detected using a commercial enzyme-linked immunosorbent assay (ELISA) test (IDEXX Laboratories, Inc., Westbrook, ME). A total of six different studies were conducted.

**Study 1.** Arkansas and GA98 IBV vaccines were given to the birds in the hatchery. At 18 days of age, birds were spray-vaccinated with a Stihl sprayer containing Arkansas and GA98 IBV vaccines and Newcastle disease virus (NDV) C2 vaccine (Intervet, Schering-Plough Animal Health, Millisboro, DE). Tracheal swabs were collected at 3, 7, 14, 21, and 28 days postvaccination. Sera were collected from 20 birds on the last sample day, 28 days postvaccination.

**Study 2.** Arkansas and Mass IBV vaccines were given to the birds in the hatchery. At 22 days of age, birds were vaccinated in the water by the barrel/pump method with Arkansas and Mass IBV vaccines. NDV B1 vaccine (Merial-Select, Gainesville, GA), DE072 (Intervet, Schering-Plough), and infectious laryngotracheitis virus (ILTV) vaccine (Merial-Select). Tracheal swabs were collected at 1 (day of vaccination, 20 swabs taken approximately 1 hr after vaccination), 7, 14, 21, 28, and 35 days postvaccination.

**Study 3.** Arkansas and DE072 IBV vaccines were given to the birds in the hatchery. For this study, two different houses on the same farm were examined. At 18 days of age, house A was water-vaccinated by the barrel/pump method and house B was spray-vaccinated with a Stihl sprayer. Both houses were given Arkansas (Merial-Select) and DE072 IBV vaccine, NDV C2 vaccine and ILTV vaccine (Intervet, Schering-Plough). Tracheal swabs were collected at 3, 7, 14, 21, and 28 days postvaccination.

**Study 4.** The Mass and Conn IBV vaccines were given to the birds in the hatchery. At 18 days of age, a Stihl sprayer was used to vaccinate birds in two different houses (houses A and B) on the same farm. The birds received Mass and Conn IBV vaccines (Intervet, Schering-Plough) and swabs were collected at 1 (day of vaccination, 25 swabs taken approximately 1 hr after vaccination), 3, 7, 14, and 21 days postvaccination.

**Study 5.** Arkansas, Mass, and DE072 vaccines were given to the birds in the hatchery. At 22 days of age, birds were vaccinated by spray with
To determine if the birds in Study 6 were indeed

Table 1. IBV real-time RT-PCR average Ct values ± SD for tracheal swabs taken following field boost vaccination in commercial broilers.

<table>
<thead>
<tr>
<th>Study</th>
<th>Day</th>
<th>Spray (Stihl)</th>
<th>Spray (SoloVac)</th>
<th>Water</th>
<th>Spray (Stihl)</th>
<th>Spray (SoloVac)</th>
<th>Water</th>
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<tr>
<td>1</td>
<td>1</td>
<td>ND</td>
<td>32.48 ± 0.73</td>
<td>ND</td>
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Average Ct values were calculated only from the positive samples. Larger numbers equal less viral RNA present in the tracheal swabs.

Protection study. To determine if the birds in Study 6 were indeed protected against challenge with IBV, we removed 24 birds from each of the three houses at 21 days postvaccination, transported them to the Poultry Diagnostic and Research Center (Athens, GA), and placed them into positive-pressure Horsfal isolation units. Because the birds had been vaccinated with Arkansas and Delaware IBV vaccines at 1 and 17 days of age, we separated the birds from each house into three groups of eight birds each and challenged them intraocularly and intranasally with Arkansas or Delaware (DE072). Birds in group 1 were challenged with 3.2 \times 10^4 50% egg infectious dose (EID\textsubscript{50})/bird of pathogenic Arkansas virus; birds in group 2 were challenged with 2.0 \times 10^5 EID\textsubscript{50}/bird of pathogenic DE072 virus, and birds in group 3 were held as negative control birds and were not challenged. In addition, we challenged six 2-wk old specific-pathogen-free (SPF) chickens (Charles River, SPAFAS, Inc., North Franklin, CT) with Arkansas virus (same dose given the broilers) and five SPF birds with DE072 (same dose given the broilers), which served as positive control groups. All of the birds were killed and necropsied at 5 days postchallenge. At necropsy, sera were collected, tracheal swabs were placed in ice-cold PBS (pH 7.4) for real-time RT-PCR, and the lower halves of tracheas (below the swabbed area) and kidneys were collected and fixed in 10% neutral buffered formalin for histopathologic analysis. Tissues were routinely processed and stained, and tracheal tissues were scored as described previously (7).

RNA extraction and real-time RT-PCR. Viral RNA was extracted from 50 μl of the PBS from the tracheal swab using the MagMAX\textsuperscript{TM}-96 RNA Isolation Kit (Ambion Inc., Austin TX) according to the manufacturer’s protocol on a KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA). Real-time RT-PCR was conducted using a Smart Cycler II (Cepheid, Sunnyvale, CA) and the AgPath-ID\textsuperscript{TM} One-Step RT-PCR kit (Ambion Inc.) according to the manufacturer’s recommendations. Primers and probe for the real-time RT-PCR were previously published (2) and consist of a forward primer, IBV5/GU391 (5’-GCTTTTGAGCCCTAGGT-3’); a reverse primer, IBV5/GL533 (5’-GCCATGGTTTGACTGTCATTT-3’); and a Taqman\textsuperscript{TM} dual-labeled probe IBV5/G probe (5’-FAM-CACCACCCAGACCTGTA CCTC-BHQ1-3’). The primers were obtained from Integrated DNA Technologies (Coralville, IA) and the Taqman\textsuperscript{TM} probe was synthesized by BioSearch Technologies (Novato, CA). Real-time RT-PCR components and thermocycler parameters were conducted as previously described, and a standard curve for the assay, which was previously published, was used to calculate the approximate genome copy number for each sample (2). Any sample below the calculated level of detection (100 genome copies) was considered negative.

Controls were meticulously included in the RNA extraction and the real-time RT-PCR assay to ensure that no field or laboratory contamination of the samples occurred. Negative control swab samples, which were swabs placed in buffer while in the chicken house at approximately every 25th tracheal swab sample taken, were included in the RNA extraction and real-time RT-PCR assay. Controls also included known negative and positive samples for RNA isolation and for real-
time RT-PCR at every 16th sample analyzed. Allantoic fluid from noninoculated SPF eggs was used for the RNA isolation negative controls, and the isolated material was carried forward to the real-time RT-PCR assay. An additional negative control for the real-time RT-PCR assay consisted of the reaction mixture without an RNA template. A positive control consisting of allantoic fluid containing Mass-41 type IBV whole virus \( (1 \times 10^{4.0} \text{ EID}_{50}/\text{ml}) \) was included for the RNA extraction and carried forward to the real-time RT-PCR assay. In addition, known positive (from previous real-time RT-PCR positive samples) IBV RNA from the Mass-41 strain of IBV was used as template in a positive control reaction for the real-time RT-PCR. The experimental samples and controls were restested if any of the controls were not as expected. Routinely, the negative controls do not yield any detectable fluorescence in the real-time RT-PCR assay after 40 cycles and the positive controls have an average cycle threshold (CT) value of approximately 28.

**Molecular characterization.** The S1 glycoprotein gene was RT-PCR amplified following previously published methods and the amplified product was sequenced (12,13). Primers based on sequence data for the spike gene were used to amplify an approximately 450-bp sequence in the hypervariable region in spike and the amplified products were purified using GenElute\textsuperscript{TM} spin columns (Supelco, Bellefonte, PA) and concentrated using Microcon\textsuperscript{TM} 30 columns (Amicon, Beverly, MA). The \(^3\prime\) primer designated Ag0723 \((5\text{\prime}-\text{GACCAGGCTGTTCAACATC}-3\text{\prime})\) and a \(^5\prime\) primer designated NEWS1079S \((5\text{\prime}-\text{TAAAGCT-}

**RESULTS**

We collected approximately 100 tracheal swabs at each sampling time because we wanted our data to be as accurate as possible. Based on the formula by Cannon and Roe, collecting tracheal swabs from 100 birds per house per day calculates to detecting vaccine virus in as few as 3% of the birds in a 20,000 bird flock (approximate average for all studies) with a 95% confidence level (3). We did not observe clinical signs consistent with an outbreak of infectious bronchitis in any of the studies, nor did we detect any variant infectious bronchitis-type viruses.

**Study 1.** Birds in this study were spray vaccinated at 18 days of age with a Stihl backpack sprayer containing \(1 \times 10^{6.75} \text{ EID}_{50}/\text{ml of vaccine in the sprayer tank. We detected } 1 \times 10^{6.37} \text{ EID}_{50}/\text{ml of IBV vaccine at the sprayer nozzle and } 1 \times 10^{6.75} \text{ EID}_{50}/\text{ml of IBV vaccine at the floor. The percentage of birds positive for IBV by real-time RT-PCR roughly followed a parabolic-shaped curve in which 35% of the birds were positive at 3 days postvaccination and percentage of IBV-positive birds peaked at 14 days postvaccination (92%) then fell to 26% at 21 days postvaccination (Fig. 1A). At 28 days post-vaccination, which was only a few days before the birds went to the processing plant, 21% were still positive for IBV. The average Ct values (Table 1) correlated with the number of birds positive for virus in the trachea with the lowest Ct values (highest amount of virus) coinciding with the highest number of birds positive for virus at 14 days postvaccination. Based on the previously published standard curve for this real-time RT-PCR assay (2) the calculated IBV genome copy number in the tracheal swabs for positive samples only are reported in Fig. 1, and ranged from \(1 \times 10^{3.4} \) to \(1 \times 10^{5.1} \) copies/ml. Birds were bled at 28 days postvaccination, the last sample day, and eight of 20 were positive for IBV antibodies by commercial ELISA (IDEXX) with an average titer of 1838. The type of IBV vaccine was examined from selected tracheal swabs at 28 days postvaccination and Arkansas vaccine was identified in the birds. No other IBV types were detected.

**Study 2.** Birds in this study were vaccinated via the water at 22 days of age. No IBV vaccine virus was detected in the barrel containing the working stock of vaccine by real-time RT-PCR and an average Ct value of 39.02, which is below the calculated level of detection, was observed for 3/8 samples taken from the waterlines. We did not attempt vaccine virus isolation using embryonating eggs. The percent of birds positive for IBV vaccine virus in the trachea following vaccination showed a sinusoidal-type wave pattern (Fig. 1B). Similar to Study 1, the average Ct values (Table 1) correlated with the number of birds positive for virus in the trachea with the lowest Ct values (highest amount of virus) coinciding with the highest number of birds positive for virus at 1 and 21 days postvaccination. Based on the previously published standard curve for this real-time RT-PCR assay (2) the calculated IBV genome copy number in the tracheal swabs for positive samples only are reported in Fig. 1, and ranged from \(1 \times 10^{3.4} \) to \(1 \times 10^{5.5} \) copies/ml. The type of IBV vaccine was examined from selected tracheal swabs at 1 and 35 days postvaccination and Arkansas vaccine was identified in the birds. No other IBV types were detected.

**Study 3.** Birds in this study were vaccinated at 17 days of age and two houses were monitored. Birds in house A were vaccinated using a Stihl sprayer and birds in house B were vaccinated via the water. The Stihl backpack sprayer contained \(1 \times 10^{6.4} \text{ EID}_{50}/\text{ml of IBV vaccine in the sprayer tank, } 1 \times 10^{4} \text{ EID}_{50}/\text{ml of IBV vaccine was detected at the sprayer nozzle, and no IBV vaccine was detected at the floor by isolation in embryonating eggs. By real-time RT-PCR the average Ct values for the tank and nozzle were 31.2 and 31.9, respectively. For house B, no IBV vaccine virus was detected in the water barrel containing the working stock of vaccine or in the waterlines by isolation in embryonating eggs, but 4/6 samples from the barrel were positive (average Ct \(=36.25\)) and 5/6 samples from the water lines were positive (average Ct \(=36.19\)) by real-time RT-PCR. The percentage of birds positive for IBV by real-time RT-PCR roughly followed a parabolic-shaped curve that peaked at 14 days postvaccination with 91% of the birds vaccinated by spray in house A and 95% of birds vaccinated via water in house B positive for IBV (Fig. 1C). At 28 days postvaccination, which was only a few days before the birds went to the processing plant, 12% and 37% of the birds in houses A and B, respectively, were still positive for IBV. The average Ct values (Table 1) correlated with the number of birds positive for virus in the trachea with the lowest Ct values (highest amount of virus) coinciding with the highest number of birds positive for virus at 14 and 21 days postvaccination in house A vaccinated by spray. For house B, vaccinated by water, the lowest average Ct values (highest amount of virus) correlated with the highest number of birds positive for virus in the trachea at 14 days postvaccination (Table 1). Based on the previously published standard curve for this real-time RT-PCR assay (2) the calculated IBV genome copy number in the tracheal swabs for positive samples only are reported in Fig. 1, and ranged from \(1 \times 10^{2.6} \) to \(1 \times 10^{4.8} \) copies/ml for the spray-vaccinated house A, and from \(1 \times 10^{2.9} \) to 1
The type of IBV vaccine was examined from selected tracheal swabs at 3 and 28 days postvaccination and Arkansas vaccine was identified in the birds. No other IBV types were detected.

Study 4. Birds in this study were vaccinated at 18 days of age and two houses were monitored. Birds in houses A and B were vaccinated with Mass and Conn IBV vaccines using a Stihl sprayer. We did not determine the titer of the vaccine in the sprayer. In house A, the

$\times 10^{4.5}$ copies/ml for the water-vaccinated house B. The type of IBV vaccine was examined from selected tracheal swabs at 3 and 28 days postvaccination and Arkansas vaccine was identified in the birds. No other IBV types were detected.

**Fig. 1.** Percentage of birds positive for IBV in the trachea by real-time RT-PCR following field vaccination. Tracheal swabs from 100 birds were taken at each time point, except for samples taken at day 1 (day of vaccination, see text for details) and tested individually. (A) Study 1: birds vaccinated with Arkansas and GA98 using a Stihl sprayer at 18 days of age. (B) Study 2: birds vaccinated with Arkansas and Mass in the drinking water at 22 days of age. (C) Study 3: Birds in two different houses on the same farm vaccinated with Arkansas and Delaware using a Stihl sprayer (black bars), and in the drinking water (gray bars) at 17 days of age. (D) Study 4: birds in two different houses, house A (black bars) and house B (gray bars), on the same farm vaccinated with Mass and Conn using a Stihl sprayer at 18 days of age. (E) Study 5: birds vaccinated with Arkansas, Delaware, and Mass using a SoloVac sprayer at 22 days of age. (F) Study 6: birds in three different houses on the same farm vaccinated with Arkansas and Delaware using a Stihl sprayer (black bars, house A; gray bars, house B) and a SoloVac sprayer (open bars, house C) at 17 days of age. Numbers in brackets above the bars represent the calculated average genome copy number/ml (2) in the positive tracheal swab samples detected by real-time RT-PCR.
percentage of birds positive for IBV showed a sinusoidal-type wave pattern. At 1 day postvaccination 56% of the birds were positive, which increased by 3 days postvaccination (76%) then decreased slightly at 7 days postvaccination (67%). The number of positive birds peaked at 14 days postvaccination (100%) then dropped again to 66% by day 21 postvaccination (Fig. 1D, black bars). In house B, the percentage of birds positive for IBV by real-time RT-PCR started out at 65% and roughly followed a parabolic-shaped curve with the maximum positives (90%) at 7 and 14 days postvaccination before falling to 58% at 21 days postvaccination (Fig. 1D, gray bars). The average Ct values (Table 1) correlated with the number of birds positive for virus in the trachea with the lowest Ct values (highest amount of virus) coinciding with the highest number of birds positive for IBV except at 7 days postvaccination in house A, where the Ct value dropped to 28.23 cycles indicating that more virus was present but the percentage of positive birds decreased. In addition, for house B on day 14 postvaccination, the Ct value increased to 34.44 indicating fewer virus particles present; however, the percentage of positive birds increased to 100%.

Based on the previously published standard curve for this real-time RT-PCR assay (2) the calculated IBV genome copy number in the tracheal swabs for positive samples only are reported in Fig. 1, and ranged from $1 \times 10^{3.5}$ to $1 \times 10^{5.2}$ copies/ml for house A, and from $1 \times 10^{5.0}$ to $1 \times 10^{3.6}$ copies/ml for house B. The type of IBV vaccine was examined from selected tracheal swabs at 21 days postvaccination and Arkansas vaccine was identified in three different samples. No other IBV types were detected.

**Study 5.** In this study, one house was vaccinated by spray with the SoloVac sprayer at 22 days of age and the titer of the IBV vaccine in the sprayer tank and at the sprayer nozzle was determined to be approximately $1 \times 10^{3.2}$ genome copies/ml (average Ct = 29.24, n = 3) and approximately $1 \times 10^{3.1}$ genome copies/ml (average Ct = 29.50, n = 3), respectively. No IBV vaccine could be detected at the floor level by real-time RT-PCR. The percentage of birds positive for IBV vaccine virus in the trachea showed a sinusoidal-type wave pattern, in which 85% to 88% of the birds were positive for the first week after vaccination, dropping to 45% at 14 days postvaccination, cycling back up to 77% at 21 days postvaccination, and then dropping to 16% at 35 days postvaccination just prior to the birds going to the processing plant (Fig. 1E). The average Ct values (Table 1) correlated with the number of birds positive for virus in the trachea with the lowest Ct values (highest amount of virus) coinciding with the highest number of birds positive for virus between 1 and 7 days postvaccination. Based on the previously published standard curve for this real-time RT-PCR assay (2) the calculated IBV genome copy number in the tracheal swabs for positive samples only are reported in Fig. 1, and ranged from $1 \times 10^{2.9}$ to $1 \times 10^{4.6}$ copies/ml. Twenty-five birds were bled on the day of vaccination (22 days of age) and 10 birds were bled on the last sampling day (35 days postvaccination) and none of the samples were positive for IBV antibodies by ELISA (IDEXX). The type of IBV vaccine was examined from selected tracheal swabs at 3 and 35 days postvaccination and Arkansas vaccine was identified in the birds. No other IBV types were detected.

**Study 6.** At 17 days of age, the birds in houses A and B were vaccinated with a Stihl sprayer and the birds in house C were vaccinated with a SoloVac sprayer; only one sample from the sprayer tank used to vaccinate house B was positive by real-time RT-PCR (Ct = 38.77). All other samples from the sprayer tanks, nozzles, and floor were negative for IBV vaccine. The percentage of birds positive for IBV vaccine virus in the trachea showed a sinusoidal-type wave pattern for all three houses (Fig. 1F). Houses A and B, vaccinated with the Stihl sprayer, had a higher percentage of birds positive (72% and 69% respectively) on the day of vaccination compared to birds in house C (52%), vaccinated with the SoloVac sprayer. At 5 days postvaccination the percentage of positive birds dropped to 47%, 35%, and 36% for houses A, B, and C, respectively; then the percentage of birds with IBV vaccine in each of the houses rose above the initial percentage of coverage to peak at 14 days postvaccination. At 21 days postvaccination, the percentage of birds with IBV vaccine in houses A and B dropped (22% and 13%, respectively) then rose again at 28 days (45% and 64%, respectively) postvaccination, whereas the percentage of birds with IBV vaccine in house C, which was vaccinated with the SoloVac sprayer, decreased at 21 days postvaccination to 33% and at 28 days postvaccination to 20%. The lowest average Ct values (highest amount of virus) correlated with the most birds positive for virus in the trachea at 14 days postvaccination except for house C, which had similar average Ct values for days 0 and 14 (Table 1). Generally, higher Ct values (fewer viral genomes detected) were observed when a low percentage of birds were positive for vaccine in the flock. Based on the previous published standard curve for this real-time RT-PCR assay (2) the calculated IBV genome copy number in the tracheal swabs for positive samples only are reported in Fig. 1, and ranged from $1 \times 10^{3.5}$ to $1 \times 10^{5.1}$ copies/ml for house A, $1 \times 10^{5.0}$ to $1 \times 10^{6.6}$ copies/ml for house B, and $1 \times 10^{3.4}$ to $1 \times 10^{8.1}$ copies/ml for house C.

Fifteen birds from each house were bled 4 days postvaccination and only one bird was positive for IBV antibodies by ELISA in house A, with a titer of 952; none were positive in house B and one was positive in house C, with a titer of 420. At 14 days postvaccination, birds were bled from each house and nine of 15 were positive in house A with an average titer of 788, four of 12 were positive in house B with an average titer of 448, and three of 15 were positive in house C with an average titer of 833. Twelve birds from each house were bled 28 days postvaccination and nine in house A were positive with an average titer of 2123, eight were positive in house B with an average titer of 1326, and five were positive in house C with an average titer of 824.

The type of IBV vaccine was examined from selected tracheal swabs at 5 and 28 days postvaccination in all three houses and Arkansas vaccine was identified in the birds. No other IBV types were detected.

**Protection study.** To determine if the birds in Study 6 were indeed protected against IBV, we challenged 38 day-old birds from 3 different houses with Arkansas or DE072 (data is presented in Table 2). None of the nonchallenged negative control broilers had clinical signs associated with IBV challenge and challenge virus was not detected in tracheal swabs from those birds by real-time RT-PCR. Microscopic lesions in those birds were consistent with previous IBV vaccination and consisted of germinal centers and a mild lymphocytic trachitis. Kidney lesions were within normal limits and consistent with previous IBV vaccination. All of the positive control SPF leghorn birds that received either Arkansas or DE072 challenge virus had clinical signs and challenge virus was detected in tracheal swabs from five of six birds that received Arkansas and five of five birds that received DE072. Microscopic lesions in the trachea were typical of IBV infection and consisted of acute multifocal to diffuse necrotizing trachitis. Kidney lesions were within normal limits. Based on clinical signs and challenge virus detection, broilers challenged with Arkansas from houses A, B, and C were 62.5%, 37.5%, and 62.5% protected, respectively. Challenge virus was detected in each of the birds with clinical signs but not in the birds that were protected. The Ct values corresponded to approx-
imately $1 \times 10^4$-$5$, $1 \times 10^5$-$5$, and $1 \times 10^3$-$8$ genome copies/ml in birds from houses A, B, and C, respectively. Microscopic lesions in the trachea were typical of IBV infection and consisted of acute necrotizing tracheitis to multifocal lymphocytic tracheitis. Kidney lesions were within normal limits and consistent with previous IBV vaccination. Broilers challenged with DE072 from houses A and B were 100% protected from clinical signs and no virus was detected in tracheal swabs from those birds. Only one of eight birds from house C had clinical signs and approximately $1 \times 10^6$-$8$ genome copies/ml of challenge virus was detected in the tracheal swab from that bird. Microscopic lesions in the trachea consisted of germinal centers and a multifocal, mild to moderate, lymphocytic tracheitis. Kidney lesions were within normal limits and consistent with previous IBV vaccination.

The average serologic titers against IBV in broilers, determined by commercial ELISA test, ranged from 527.5 to 1266.4. No IBV antibodies were detected in the challenge control SPF leghorn birds by ELISA.

**DISCUSSION**

In this study, we examined IBV vaccination of commercial broilers in the field. The number of birds positive for vaccine virus, the amount of vaccine virus in the trachea, and the progression of the vaccine virus in the flock was followed in 10 different broiler houses on six different farms. Both water and spray vaccination were included in the study, and we tested birds from three different houses for protection against pathogenic viruses homologous to the vaccines used in the flocks. Using a KingFisher magnetic particle processor (Thermo Scientific) for automated RNA extraction and a Smart Cycler II (Cepheid) for real-time RT-PCR amplification of IBV in clinical samples, we obtained results from hundreds of tracheal swabs in a single day. With those tools, we were able to test approximately 100 tracheal swabs per house per sample day, which allowed us to detect vaccine virus in as few as 3% of the birds in a 20,000 bird flock at a 95% confidence level (3).

We observed two different patterns for the number of birds positive for vaccine virus following vaccination in the field. One pattern was a parabolic-shaped curve, where initially a low percentage of birds were positive for IBV vaccine in the trachea followed by a peak at approximately 14 days postvaccination with between 90% and 100% of the birds positive; then a decrease in the number of positive birds was observed but the vaccines did not completely clear from the flock. This pattern was observed in four of the 10 houses examined. The second pattern was similar to a sinusoidal-type wave that started with a high number of birds positive for IBV vaccine in the trachea, and cycled from peak to peak on approximately a 2-wk interval. Again, the vaccines did not clear from the flock prior to the birds going to processing. Six of 10 houses showed this type of pattern. The patterns did not correlate with the method of vaccination or the type of vaccine given. It should be recognized that vaccine virus may have been present in the trachea of some birds but was below the level of detection of the real-time RT-PCR test used in these studies.

The lower limit of detection for the real-time RT-PCR test used herein was previously measured to be $\geq 1 \times 10^2$ genome copies of IBV/ml (2). High vaccine virus genome copy numbers were detected in the working stocks from the backpack sprayer tanks except in Study 6 where the working stock in the sprayer used for house B was a weak positive and working stocks from the sprayers used for houses A and C were negative. Other than the presence of inhibitors of the RT-PCR test, it is not clear why the vaccine titers were low in those sprayers, but it appears an infectious dose of vaccine was delivered to the birds because vaccine virus was detected in the birds at all sample times postvaccination.

The minimum titer in a dose of IBV vaccine is approximately $1 \times 10^{3}$ EID$_{50}$ (Jackwood, unpubl. data). Vaccine in the storage tank of the backpack sprayers contains approximately 20,000 to 25,000 doses of vaccine in 20 liters of water, or a minimum of approximately $1 \times 10^{3}$ EID$_{50}$/ml and was readily detected by virus isolation and real-time RT-PCR (except in Study 6, see above). We observed up to half a log decrease in vaccine titer between the vaccine in the backpack storage tank and the vaccine collected at the sprayer nozzle, which is likely because of shearing forces breaking apart the virus particles. In Study 1, vaccine virus from the backpack sprayer was detected at the level of the floor but it is likely that the data are not accurate because technicians spraying the vaccine were aware of the location of the collection vesicles and sprayed directly into them, whereas no vaccine virus was detected at the level of the floor in Study 3 where the technicians did not know the location of the detection vesicles. This result was not unexpected because vaccine virus at the floor level from the backpack sprayers is likely below the detection limit of the real-time RT-PCR test.

Vaccine virus was also not detected in the water-vaccination barrel containing the working stock of vaccine or the water lines in Study 2 but some of the samples from the water-barrel vaccine working stocks and the water lines in Study 3 were positive by real-time RT-PCR. Virus isolation in embryonating eggs was attempted on the positive real-time RT-PCR barrel and water-line samples in Study 3 but they were all negative indicating that the real-time RT-PCR test...
for IBV detection was slightly more sensitive than virus isolation. Again, assuming the minimum titer of approximately $1 \times 10^3$ EID$_{50}$ per dose, when the vaccine is diluted to a working stock of 1 dose in 8–10 ml of drinking water, the virus titer is at the limit of the real-time RT-PCR IBV detection test used in these studies and likely explains why we could not detect vaccine virus in the water barrel containing vaccine working stocks.

Two vaccinations for IBV, a prime at 1 day of age and a field boost, typically at 14–18 days of age, are necessary to induce a protective local antibody response in the upper respiratory tract (4,5). Modified live vaccines used in the field boost can be administered in the drinking water or by spray, and conventional wisdom assumed that a high number of birds were initially infected, then the vaccine virus waned and finally cleared from the trachea of birds that were initially given a priming vaccination at 1 day of age. Evidence in the literature indicates that this is likely not the case when a single vaccination is given. In a study using 1-day-old broilers, Alvarado et al. (1) showed that Ark and Mass vaccines were detected in the trachea up to 14 and 28 days, respectively, following course spray vaccination. In addition, Naqi et al. (16) showed that leghorn chickens vaccinated at 1 day of age shed Mass-type vaccine virus in the trachea intermittently up to 77 days postvaccination. Based on molecular comparison with previously published IBV vaccine sequences (15), our findings showed that Arkansas-type vaccines were persisting in the vaccinated birds. In addition, the Arkansas vaccines identified were consistent with the sequence of the commercial vaccine used in the flock. For Study 4, the Arkansas sequences were consistent with vaccines being used in the area. Assuming that IBV vaccine viruses can be shed for an extended period of time after an initial exposure to the virus, it seems logical to suggest that persisting Arkansas vaccines in commercial broilers may be due to insufficient priming in the hatchery. We found that the birds vaccinated for Arkansas and Delaware in the field then brought to the laboratory and challenged at 21 days postvaccination were protected against Delaware but not adequately protected from Arkansas challenge virus. That observation is consistent with the suggestion that the birds were not adequately primed with Arkansas vaccine. The tracheal lesions and scores (Table 2) were more severe in the SPF challenged birds compared to the broilers, but the lesions and scores were similar between the IBV-challenged broilers and the nonchallenged broilers. This was because of the presence of germinal centers, which elevated the lesion scores of the nonchallenged broilers. Germinal centers in the trachea can result from IBV infection (11). Our challenge data indicate that the birds were adequately immunized against Delaware but not Arkansas type viruses. Because serologic differences between Delaware- and Arkansas-type viruses provide no cross-reactivity (6), it seems likely that the local immune response was predominately directed against Delaware-type viruses compared to Arkansas-type viruses; however, we did not directly measure local antibodies or cell-mediated immunity in the trachea.

Throughout our studies, other IBV vaccine types were not detected even though the methodology, which used RT-PCR primers that amplify IBV vaccine viruses, has been previously shown to detect all the vaccines used herein (8). It is not clear why Arkansas vaccine viruses were detected in birds in Study 4 because only Conn- and Mass-type vaccines were used in the flocks, but Arkansas vaccine was being used in the surrounding area at the time of sample collection.

In three of four flocks in which a parabolic curve was observed following field boost vaccination, the number of birds initially infected with the field boost vaccine was below 35%, whereas flocks exhibiting a sinusoidal-type wave had 50% to 90% of the birds initially infected with the field boost vaccine. Given that IBV vaccines can be shed for many days following vaccination of 1-day-old birds (1,16), it would be interesting to determine if IBV vaccine given in the hatchery is still present in birds at the time of field boost and if that affects the percentage of birds initially infected and the subsequent vaccine coverage following the boost.

In this work, we showed that infection with IBV vaccines administered to commercial broiler flocks in the field follows either a parabolic-type curve or a sinusoidal-type wave and in none of the studies did vaccine virus completely clear from the flock. In addition, the data showed that Arkansas-type vaccines were persisting in the birds and that adequate protection against Arkansas challenge was lacking, which suggests that locally protective antibodies against Arkansas were not present in the trachea.

**REFERENCES**


**ACKNOWLEDGMENTS**

This work was supported by a grant from the U.S. Poultry and Egg Association and by gifts from Merial-Select, Gainesville, GA, and Fieldale Farms, Baldwin, GA.

The authors would like to thank Lauren Byrd, Joshua Jackwood, Jamie Phillips, and Sharmi Thor for their help with collecting samples and technical assistance extracting RNA and running real-time RT-PCR assays. We would also like to thank the employees at Columbia Farms (Lavonia, GA), Foster Farms (Livingston, CA), and Merial-Select for help collecting samples.