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Cross-Protection by Infectious Bronchitis Viruses Under Controlled Experimental Conditions

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SUMMARY. Infectious bronchitis virus (IBV) cross-protection trials were performed in healthy chickens maintained under controlled environmental conditions. Chickens primed or primed and boosted with a Massachusetts (Mass)-type attenuated vaccine were subsequently challenged with either IBV Arkansas (Ark) or GA13-type virulent strains. In addition, Ark-vaccinated chickens were challenged with IBV GA13. Spike protein 1 (S1) amino acid identities between IBV vaccine and challenge strains varied from 76.0% to 77.3%. Contrary to expectations, assessments of clinical signs, viral load, and histopathology indicated a significant level of cross-protection among these antigenically distant IBV strains. Moreover, prime and booster vaccination with Mass protected against GA13 and improved protection against Ark when compared with Mass single vaccination. These results emphasize the need to include both single vaccination control groups and control groups primed and boosted with a single serotype when testing the efficacy of IBV protectotypes and/or novel IBV vaccine combinations against heterologous serotypes under controlled experimental conditions. Such controls are of distinct importance in experiments supporting the introduction of attenuated IBV vaccine strains exotic to regions, since these exotic strains may provide new genetic material for recombination and emergence of novel IBV strains.

RESUMEN. Protección cruzada entre virus de la bronquitis infecciosa de las aves bajo condiciones experimentales controladas. Se realizaron pruebas de protección cruzada para el virus de la bronquitis infecciosa (IBV) en pollos sanos mantenidos bajo condiciones ambientales controladas. Los pollos primo-vacunados o primo-vacunados y revacunados con una vacuna atenuada con el serotipo Massachusetts (Mass) fueron posteriormente desafíados con el serotipo Arkansas (Ark), o cepas virulentas del tipo GA13. Además, los pollos vacunados con el serotipo Arkansas fueron desafíados con el tipo GA13. Las identidades genéticas de los aminoácidos de la proteína S1 entre las cepas vacunales y de desafío fueron desde 76.0% a 77.3%. Contrariamente a lo esperado, las evaluaciones de los signos clínicos, la carga viral y la histopatología indicaron un nivel significativo de protección cruzada entre estas cepas del virus de la bronquitis infecciosa que son antigenéticamente distantes entre sí. Por otra parte, la primo-vacunación y revacunación con el serotipo Massachusetts protegió contra el tipo GA13 y mejoró la protección contra el serotipo Arkansas cuando se comparó con la vacunación con una sola aplicación de Massachusetts. Estos resultados enfatizan la necesidad de incluir grupos control con una sola vacunación y con-primo-vacunación y revacunación utilizando un solo serotipo, cuando se prueba la eficacia de protectotipos o de combinaciones nuevas de vacunas contra serotipos heterólogos bajo condiciones experimentales controladas. Tales controles son de suma importancia en los experimentos que apoyan la introducción de cepas atenuadas de vacunas que son exóticas en una región, ya que estas cepas exóticas pueden proporcionar nuevo material genético para la recombinación y la aparición de nuevas cepas del virus de la bronquitis infecciosa.

Key words: infectious bronchitis virus, coronavirus, genetic variation, IBV Arkansas, vaccine, GA13

Abbreviations: Ark = Arkansas; DPI = Delmarva Poultry Industry; EID₅₀ = 50% embryo infectious dose; FRET = fluorescence resonance energy transfer; IB = infectious bronchitis; IBV = infectious bronchitis virus; Mass = Massachusetts; qRT = quantitative reverse transcriptase; S = spike protein; SPF = specific pathogen free

Infectious bronchitis (IB) virus (IBV) is highly prevalent in the world’s poultry industry. IBV commonly isolated from outbreaks of disease in the United States belongs to the types Arkansas (Ark), Connecticut, DE072, Massachusetts (Mass), Georgia variants, and California variants, as well as additional variant profiles that do not fully match reference viruses (14,21). Multiple further types of IBV have been identified in other regions of the world (13). IBV evolves by natural selection, i.e., generation of genetic diversity by mutation and recombination events followed by selection of most fit virus populations (20). The fact that distinct IBV strains emerge and become predominant in different regions of the world likely results from evolutionary events occurring independently. An exception to this general rule is, for example, strains belonging to the Massachusetts serotype, which are endemic worldwide from introduction as attenuated vaccines (8,16). Unfortunately, the introduction of exotic wild or live-attenuated IBV vaccine strains into regions free of those serotypes provides novel genetic material suitable for recombination and creates a risk of emergence of novel strains with unpredictable consequences. An example of this adverse effect is provided by the widespread use of attenuated IBV vaccine DE072. Severe outbreaks of disease were attributed to a novel IBV strain designated GA98, which after molecular and serotypic characterizations of the virus was shown to have originated from IBV DE072 (15).

The evaluation of IBV vaccine efficacy is usually performed in experiments under controlled environmental conditions in which vaccinated chickens (Gallus gallus domesticus) are subjected to homologous or heterologous challenge. Cook and de Wit (4) recently reviewed and discussed European and U.S. guidelines on different methods used to assess protection by live-attenuated IBV vaccines against the effects of IBV challenge. According to these authors, the variables to be considered include the age, breed and type of chicken, and the dose and route of application of both vaccine and challenge viruses. In addition, they addressed the relevant question of when and how protection against challenge is assessed (4).

The aim of the current study was to assess cross-protection among different IBV types in vaccination-challenge trials conducted in specific-pathogen-free (SPF) healthy chickens maintained under...
controlled environmental conditions. These evaluations were distinctively relevant because many studies used to support introduction of attenuated IBV vaccines strains into regions without history of presence of those types have failed to consider this aspect. To explore this issue, we experimentally vaccinated SPF chickens and subsequently challenged them with heterologous IBV.

**MATERIALS AND METHODS**

**Chickens.** White leghorn chickens hatched from SPF fertile eggs (Sunrise Farms, Catskill, NY) were used in all experiments. Chickens were maintained in Horsfall-type isolators in biosafety level 2 facilities. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care–accredited institution.

**Vaccine viruses.** Commercially available Mass and Arkansas Delmarva Poultry Industry (ArkDPI)-type vaccines were used. Vaccines were delivered via the ocular and nasal routes using the dose recommended by the vaccine manufacturer.

**IBV challenge viruses.** IBV virulent strains belonging to two different types were used for heterologous challenge in vaccinated chickens: 1) virulent IBV Ark-type (GenBank accession JN861120) previously described (10); 2) virulent IBV GA13-type obtained from the kidneys of Georgia broiler chickens in 2013 kindly provided by Dr. M. Burleson (Wayne Farms). The Spike 1 (S1) percentage amino acid identities between the vaccine and challenge strains are shown in Table 1. As seen in this table, the S1 amino acid identities between IBV vaccine and challenge strains varied from 76.0% to 77.3%, indicating substantial antigenic distance among the strains used. Challenge was performed via the ocular and nasal routes, with a total volume of 100 μl (by inoculating 25 μl into each nostril and each eye) of virus stock.

**Trial 1: Mass vaccine vs. Ark challenge.** Two groups of 20 chickens each were vaccinated as follows: 1) Mass vaccine at 1 day old and Mass boost at 12 days old, 2) Mass vaccine at 12 days old. Control Group 3 included 10 unvaccinated/challenged chickens, and Control 4 included 10 unvaccinated/not challenged chickens. Groups 1–3 were challenged 23 days after vaccination with 10^5.0 50% embryo infectious dose (EID_{50}) per bird of virulent IBV Ark. Five days after challenge all chickens were evaluated blind for respiratory rales (nasal and/or tracheal) by closely listening to each bird (bringing the head of each bird near the examiner’s ear). Signs were scored on a 0 (absent), 1 (mild), 2 (moderate), or 3 (severe) viral load was determined by fluorescence resonance energy transfer (FRET) quantitative reverse transcriptase (qRT) PCR quantitating IBV RNA (nucleocapsid gene) in lachrymal fluids as previously described (23), and tracheal samples were obtained for histopathology. Formalin-fixed longitudinal sections of the cranial third of the tracheal mucosa were embedded in paraffin, sectioned at 4 to 6 μm, and stained with hematoxylin and eosin. Deciliation in the tracheal mucosa was evaluated blindly and scored 1 through 5 based on severity (i.e., normal, mild, moderate, marked, severe lesion). Values for each chicken group were analyzed by one-way ANOVA followed by Tukey multiple comparisons test. Differences were considered significant with P values of <0.05.

**Results**

The results of Trial 1 are shown in Fig. 1. Mass vaccination significantly reduced respiratory signs (Fig. 1A), tracheal deciliation (Fig. 1B), and viral load in the lachrymal fluids (Fig. 1C) in Ark-challenged chickens compared with unvaccinated challenged controls. Prime and booster vaccination with a Mass vaccine showed a clear tendency to improve the protection both for respiratory signs and damage to the tracheal ciliary mucosa compared with single Mass vaccination. Indeed, while tracheal deciliation in single Mass-vaccinated chickens was significantly greater than in uninfected controls, in chickens immunized with Mass followed by Mass boost it was not.

The results of Trial 2 are shown in Fig. 2. Ark vaccination provided protection against virulent GA13 challenge as determined by significant reduction of respiratory signs (Fig. 2A) and viral loads both in the lachrymal fluids (Fig. 2B) and the larynx (Fig. 2C) of challenged chickens compared with unvaccinated challenged controls.

The results of Trial 3 are shown in Fig. 3. Mass prime and booster vaccination provided protection against virulent GA13 challenge as determined from a significant reduction of respiratory signs (Fig. 3A), viral load in the lachrymal fluids (Fig. 3B), and tracheal mucosal thickness (Fig. 3C) of challenged chickens compared with unvaccinated challenged controls.

**discussion**

Control of IB has been largely based on a multiplicity of type-specific IBV attenuated vaccines. In general IBV strains belonging

### Table 1. Percentage amino acid identity among IBV vaccine and challenge strains used.

<table>
<thead>
<tr>
<th>Vaccine Type</th>
<th>Mass Vaccine</th>
<th>Ark Vaccine</th>
<th>Ark Challenge</th>
<th>GA13 Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass vaccine</td>
<td>100</td>
<td>76.7</td>
<td>76.5</td>
<td>76.0</td>
</tr>
<tr>
<td>Ark vaccine</td>
<td>100</td>
<td>95.8</td>
<td>77.3</td>
<td>78.2</td>
</tr>
<tr>
<td>Ark Challenge</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

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to different serotypes do not cross-protect under field conditions (2). The protective efficacy of IBV-attenuated vaccines is usually performed in healthy, immunocompetent SPF chickens maintained in isolation under controlled environmental conditions. As discussed by Cook and de Wit (4) protection against IBV challenge can be measured by different methods, and results vary between chickens of different ages. Thus, in order to obtain representative results, the present experimental trials included vaccination at different ages. IBV vaccine efficacy in the United States is evaluated by detection of the challenge virus in the trachea (3). However, we have demonstrated that qRT-PCR of RNA isolated from tears is more sensitive for IBV detection than qRT-PCR of RNA isolated from trachea (23) and is able to establish significant differences among protection resulting from different vaccination strategies (22). Therefore protection was determined in all trials by measurement of relative viral loads in lachrymal fluids 5 days postchallenge. Moreover, in all trials we blindly assessed respiratory signs as described (21). Other methods of evaluation of protection against challenge were also used in some experiments, including determination of viral RNA in the larynx, and the more definitive, but also labor intensive, tracheal histopathology and histomorphometry. The results of the current research demonstrate that significant cross-protection among antigenically distant IBV strains can be achieved when vaccine protection efficacy is evaluated under experimental conditions. Indeed, our results in Trial 1 showed that single Mass vaccination protects against Ark challenge, and in Trial 2 Ark vaccination protects against GA13. Moreover, prime and booster vaccination with Mass protects against GA13 (Trial 3) and improves protection against Ark (Trial 1) when compared with Mass single vaccination. These results emphasize the need to include both single vaccination and prime and boost (with a single serotype) control groups when testing the efficacy of IBV protectotypes and/or novel IBV vaccine combinations under

Fig. 1. (A) Respiratory signs, (B) tracheal deciliation (boxes, 25th percentile, median, 75th percentile; whiskers, min and max.) and (C) viral load in lachrymal fluids (IBV N gene by FRET qRT-PCR) detected 5 days postchallenge in chickens that were primed at 1 day of age or primed at 1 and boosted at 12 days with a commercially available attenuated Mass-type vaccine and subsequently challenged with a virulent IBV Ark strain (ARK) at 35 days of age. In A and C individual values, average and SD are shown. N/N = unvaccinated/not challenged. Different letters indicate significant differences ($P < 0.05$).

Fig. 2. (A) Respiratory signs, (B) IBV RNA in lachrymal fluids and IBV RNA in larynx (after one passage in embryonated eggs) detected by Taqman qRT-PCR 5 days postchallenge in chickens that were vaccinated at 21 days of age with a commercially available attenuated ArkDPI-type vaccine and subsequently challenged with a virulent IBV GA13 strain at 37 days of age. Individual values, average and SD are shown. N/N = unvaccinated/not challenged (not shown in B and C). Different letters indicate significant differences ($P < 0.05$).
controlled experimental conditions. These results are particularly relevant because several studies that have been performed in recent years lack these important controls. Specifically, in more recent years the concept has been introduced that when a live IB vaccine from one serotype is followed by vaccination with an IB variant from another serotype, birds develop cross-reacting antibodies to other IB serotypes in addition to immunity to the serotypes in both vaccines (5). Based on this concept, a protocol using a Mass vaccine on day 1 followed by booster vaccination with IBV vaccines produced from a European IBV type designated 4/91 or 793B originally identified in the United Kingdom (12) has been introduced in several countries. Several communications supporting these beneficial effects have allowed introduction of 4/91-type vaccine strains into regions without previous history of the presence of this IBV type. For example, the introduction of IBV 4/91 vaccine in Chile was supported by studies by de Wit (6), which indicated that priming with Mass followed by 4/91 booster provided protection against challenge with Chilean IBV variants. The 51 percentage identity between the Chilean isolates and the IBV vaccines 4/91 and Mass is 79% and 77%, respectively. Even though the two-vaccine concept may be promising, this study lacked both Mass prime and boost and 4/91 prime and boost control groups. Without these control groups, it is not possible to determine that priming and boosting with different serotypes provides better protection than priming and boosting with a single serotype.

Several other studies are also missing these controls (18) and/or even lack control groups of single immunization with Mass or 4/91 (7,17,19). Thus, it is difficult to confirm that the “two IBV vaccine concept” indeed has strong scientific support. For instance, the control of regional serotypes may not be the consequence of broad immune responses elicited by the vaccine combination but perhaps the result of displacement of regional wild strains by a very aggressive 4/91 strain. It deserves attention that IBV 4/91 became undetectable in Northern Italy after 4/91 vaccine application was discontinued (9).

The introduction of proper controls in experiments that support the result of displacement of regional wild strains by a very aggressive 4/91 strain. It deserves attention that IBV 4/91 became undetectable in Northern Italy after homologous vaccine application was

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Fig. 3. (A) Respiratory signs, (B) IBV RNA in lachrymal fluids (individual values, average, and SD) detected by Taqman qRT-PCR and (C) tracheal mucosal thickness (each value is the average of five histomorphometric measurements performed in the trachea of each chicken, average, and SD) detected 5 days postchallenge in chickens that were primed at 3 days of age and boosted at 17 days with a commercially available attenuated Mass-type vaccine and subsequently challenged with a virulent IBV GA13 strain at 30 days of age. N/N = unvaccinated/not challenged (not shown in A and B). Different letters indicate significant differences (P < 0.05).


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