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Evaluation of the Protection Conferred by Commercial Vaccines Against the California 99 Isolate of Infectious Bronchitis Virus

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SUMMARY. An infectious bronchitis virus (IBV) was isolated from commercial broilers from the state of California exhibiting respiratory distress, inflamed tracheas, airsaculitis, and edematous lungs. After reverse transcriptase–polymerase chain reaction (RT-PCR), the California isolate exhibited an identical restriction fragment length polymorphism (RFLP) pattern to some isolates obtained from California, known as California 99 isolates. Commercial Mass–Conn and Mass–Ark vaccines were used to vaccinate commercial broiler chickens via eye drop once at 1 or 10 days of age or twice at 1 and 10 days of age. At 27 days of age the birds were challenged via eye drop with the isolated IBV California 99 strain. Protection was measured by failure to reisolate the challenge virus from tracheas 5 days postchallenge and complemented with the tracheal and epithelium thickness scores. When the Mass–Ark vaccine was included in the vaccination programs, there was protection against challenge with the IBV California 99 isolate. The Mass–Conn vaccine conferred protection when used once at 1 day of age and twice at 1 and 10 days of age. However, no total protection was achieved when used as the only vaccine at 10 days of age, since one of the replicates was positive for virus isolation. Significant differences ($P < 0.05$) in the epithelium thickness and tracheal scores were observed between the unvaccinated–unchallenged group and the groups vaccinated once or twice with the Mass–Conn vaccine. Based on these results, all chickens were protected against the California 99 isolate when the IBV Arkansas type was used as a vaccine.

RESUMEN. Evaluación de la protección proporcionada por vacunas comerciales del virus de bronquitis infecciosa contra el aislado conocido como California 99.

Se aisló un virus de bronquitis infecciosa a partir de pollos de engorde del estado de California. Los pollos presentaban problemas respiratorios con inflamación en las tráqueas, aerosaculitis y edema pulmonar. Mediante la prueba de la transcriptasa reversa-reacción en cadena por la polimerasa y el análisis de la longitud de los fragmentos de restricción, se observó un patrón similar entre este aislado y otros aislados obtenidos en el estado de California, conocidos como California 99. Se vacunaron pollos de engorde a la edad de 1 y 10 días o únicamente a los 10 días empleando vacunas comerciales Massachusetts-Connecticut y Massachusetts-Arkansas. Se desafiaron las aves a los 27 días por vía ocular con el aislado del virus de bronquitis infecciosa clasificado como California 99. Se evaluó la protección ofrecida por las vacunas comerciales mediante la incapacidad para aislar el virus California 99 a partir de la tráquea 5 días después del desafío y mediante valores obtenidos en el análisis histopatológico de la tráquea. Se observó protección contra el aislamiento California 99 en las aves que recibieron la vacuna Massachusetts-Arkansas, lo mismo que en las aves que recibieron la vacuna Massachusetts-Connecticut al día de edad y al día 1 y 10. Sin embargo, no se observó una protección completa en las aves que recibieron esta vacuna únicamente a los 10 días de edad, aislándose el virus de desafío en una de las réplicas del grupo. Se observó una diferencia significativa ($P < 0.05$) en los valores de los análisis histopatológicos de la tráquea entre el grupo no vacunado no desafiado y los grupos vacunados una o dos veces con la vacuna Massachusetts-Connecticut. Con base en estos resultados, se observó protección contra el aislamiento California 99 en los pollos de engorde vacunados con el serotipo Arkansas del virus de bronquitis infecciosa.

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Key words: avian infectious bronchitis virus, California 99, Nebraska 95, Massachusetts–Connecticut vaccine, Massachusetts–Arkansas vaccine

Abbreviations: *Bst*YI = *Bacillus stearothermophilus* Y 406; CV = coefficient of variation; ELISA = enzyme-linked immunosorbent assay; GMT = geometric mean titer; *Haem*III = *Haemophilus aegypticus*; H&E = hematoxilin-eosin staining; HI = hemagglutination inhibition; IB = infectious bronchitis; IBV = infectious bronchitis virus; S1 = spike glycoprotein 1; PDRC = Poultry Diagnostic and Research Center; PES = polyethersulfone; RFLP = restriction fragment length polymorphism; RT-PCR = reverse transcriptase–polymerase chain reaction; SNK = student–Newman–Keuls; SPF = specific pathogen free; TPB = tryptose phosphate broth; *Xcm*I = *Xantomonas campestris*

Infectious bronchitis (IB) is one of the most important infectious diseases present on commercial poultry farms. IB produces severe economic losses due to drops in egg production with poor internal and external quality of the eggs in layers. In broilers, IB affects weight gain and feed efficiency, and, when complicated with bacterial infections like *E. coli* or *S. aureus*, mortality and increased condemnations result (1,10). Characteristic clinical signs of IB are coughing, gasping, tracheal rales, and nasal discharge. Inflammation of the eyes and sinuses may also be present (1).

IB is produced by a pleomorphic virus that belongs to the *Coronaviridae* family, genus *Coronavirus* (1). Infectious bronchitis virus (IBV) replicates in the upper respiratory tract, mainly in the trachea disseminating via the blood stream to different tissues like intestines, ovary, and kidneys, remaining in these tissues for long periods of time (1,10). During the viral replication process, in which point mutations and recombinations may be present, changes in the genomic nucleotide sequence are common. As a consequence, a variation in the amino acid sequence of the expressed proteins (especially in the S1 glycoprotein) could make the already present IBV antibodies unable to recognize the new protein with no neutralization of the virus, which may allow induction of disease (2).

Vaccination programs against IB rely on the use of vaccines containing virus antigenically similar to the virus present in the field. However, the presence of multiple serotypes or appearance of new serotypes or variants can make establishment of an adequate vaccination program difficult. Sometimes, this requires application of multiple vaccines (3,5). Cross protection among different serotypes may be unpredictable (5). For this reason, the characterization of new isolates present in the field is very important (5). IBV has been isolated from trachea, cecal tonsils, lungs, kidneys, and oviduct (1). Processed tissues are inoculated into specific pathogen free (SPF) embryonated chicken eggs, and IBV is detected in allantoic fluid by reverse transcriptase–polymerase chain reaction (RT-PCR) using primers specific for the S1 gene. Further characterization by restriction fragment length polymorphism (RFLP) is possible (4,6).

Broiler flocks in California have shown respiratory problems with inflamed tracheas, airsaculitis, and edematous hemorrhagic lungs. Several tracheal and cecal tonsil samples from a 3.5 week-old broiler flock were submitted to the diagnostic laboratory of the Poultry Diagnostic and Research Center (PDRC) for virus isolation. An IBV was isolated from cecal tonsils.

This paper reports the results obtained with this IBV isolate and the level of protection conferred by commercial IBV vaccines against it under laboratory conditions.

MATERIALS AND METHODS

Virus isolation and detection. Samples of trachea and cecal tonsils from a 5.5-week-old broiler breeder flock from California were received at the diagnostic laboratory of the Poultry Diagnostic and Research Center (PDRC) of the University of Georgia for virus isolation and characterization. Different tissue samples were processed separately as follows. The samples were frozen at −70 C and thawed three times. Small pieces of the tracheas and cecal tonsils were minced separately in 10 ml of a solution of 500 ml of tryptose phosphate broth (TPB) with 1% penicillin (10,000 IU/ml)–streptomycin (10,000 μg/ml) (Sigma Chemical Co., St. Louis, MO) and 1% amphotericin B (250 μg/ml) (Sigma Chemical Co.) and centrifuged at 7600 × *g* for 10 min. The supernatant was filtered through a sterile 0.22 μm polyethersulfone (PES) syringe filter (Whatman Inc., Clifton, NJ) into a sterile vial.

Nine-day-old leghorn type specific pathogen free (SPF) embryos (SPAFAS Inc., Norwich, CT) were inoculated by the chorioallantoic sac route with 0.1 ml of the filtered supernatant. Forty-eight hours later, the allantoic fluid was harvested. The presence of

hemagglutinating virus (Newcastle and influenza) was determined by direct hemagglutination of 5% red blood cells. The presence of IBV was determined via two methodologies.

First, a rapid plate hemagglutination assay was performed directly from fresh allantoic fluid treated with 1 U/ml of commercial neuraminidase (Sigma Chemical Co.) as described by Ruano *et al.* (9).

Second, RT-PCR was used to amplify the S1 gene of IBV as follows. The IBV RNA was extracted from infected allantoic fluid. Briefly, 0.25 ml of allantoic fluid was mixed with 0.75 ml of Trizol LS (Life Technologies Inc., Grand Island, NY), vortexed, and incubated at room temperature for 5 min. After the addition of 0.2 ml of chloroform, the sample was vortexed, incubated at room temperature for 8 min, and centrifuged at 11,750 × *g* for 15 min. Then, the supernatant aqueous phase was transferred to a new DNAse/RNAse free microfuge tube, and 0.5 ml of isopropyl alcohol and 5 μl of DNA/RNA polyacryl carrier (Molecular Research Center Inc., Cincinnati, OH) were added. After 10 min of incubation at room temperature, the sample was centrifuged at 11,750 × *g* for 10 min at 4 C. Then, the supernatant was removed and the pellet was washed with 1 ml of 75% ethanol, vortexed, and centrifuged at 6610 × *g* for 5 min at 4 C. Finally, the pellet was resuspended in DNAse/RNAse free water and stored at −80 C.

RT-PCR. A commercial one tube RT-PCR kit (Roche Diagnostics Corp., Indianapolis, IN) was used. All of the reagents but the primers are provided by the commercial kit. Briefly, 25 μl of DNAse/RNAse free water, 2.5 μl of DTT (100 mM), 4 μl of dNTP mix (10 mM), 1 μl (1.0 μM) of forward primer 5′-TGAAACT-GAACAAAAGAC-3′, 1 μl (1.0 μM) of reverse primer 5′-CCATAAGTAACATAAGGRCRA-3′, 1 μl of RNAse inhibitor (5 U/μl), 10 μl of 5 × PCR buffer, 2 μl of $MgCl_2$ (25 mM), and 1 μl of enzyme mix were used per 2 μl of RNA sample. The RT-PCR was conducted by incubation for 1 hr at 60 C, heating at 94 C for 2 min and 35 cycles of denaturation at 94 C for 30 sec, annealing at 55 C for 30 sec, and polymerization at 68 C for 80 sec with a final elongation step of 7 min at 68 C for the last cycle with a GeneAmp PCR system 2400 thermal cycler (Perkin-Elmer, Norwalk, CT). The amplification products were analyzed in a 1.5% agarose gel with ethidium bromide.

RFLP analysis was performed following the technique described by Kwon *et al.* (6). The RT-PCR products were digested using the commercial restriction enzymes *Bst*YI, *Hae*III, and *Xcm*I (New England Biolabs Inc., Beverly, MA). RFLP was observed following electrophoresis (100 V constant voltage) in a 1.5% agarose gel with ethidium bromide.

Experimental design. Two hundred, 1-day-old commercial broiler chickens were obtained from a local hatchery. One hundred eighty of the commercial broilers were divided into nine treatment groups with 20 birds per group. Each group was subdivided into two replicates with 10 birds per replicate. Each replicate was kept in one Horsfall unit under positive pressure during the study. The birds from group 1 through 7 were vaccinated via eye drop at 1, 10, or 1 and 10 days of age (Table 1). At 27 days of age, all the birds in groups 1 through 8 were challenged by eye drop with $10^{5.0}$ EID_{50}/0.1 ml of the California 99 isolate. The eighth group was used as a positive control. The ninth group was left unchallenged as a negative control.

Table 1. Vaccines and vaccination schedules used to test the protection against challenge with the IBV California 99 isolate.

Group	Vaccine (1 day)	Vaccine (10 days)
1	Mass–Conn	—
2	Mass–Conn	Mass–Conn
3	—	Mass–Conn
4	Mass–Ark	—
5	Mass–Ark	Mass–Ark
6	—	Mass–Ark
7	Mass–Conn	Mass–Ark
8	Unvaccinated–challenged	
9	Unvaccinated–unchallenged	

Vaccines. Two different vaccines were used. One was a commercial Massachusetts–Connecticut vaccine (Merial-Select Laboratories Inc., Gainesville, GA) and the other was a Massachusetts–Arkansas vaccine obtained by mixing the separate Massachusetts Mildvac-Ma5 and Arkansas Mildvac-Ark vaccines (Intervet Inc., Millsboro, DE). The vaccines were diluted following the recommendations of the manufacturers. The birds were vaccinated with $10^{4.1}$ EID_{50}/0.1 ml of the Massachusetts–Connecticut vaccine and with $10^{4.4}$ EID_{50}/0.1 ml of the mixed Massachusetts–Arkansas vaccine by the ocular route.

Virus titration. The vaccine and challenge viruses were titrated using SPF embryonated eggs following the method of Reed and Müench (11). The Massachusetts–Connecticut, the Massachusetts Mildvac-Ma5, and the Arkansas Mildvac-Ark vaccines had titers of $10^{5.1}$ EID_{50}/ml, $10^{5.4}$ EID_{50}/ml, and $10^{5.6}$ EID_{50}/ml, respectively. The IBV California 99 isolate had a titer of $10^{6.0}$ EID_{50}/ml.

Antibody levels. IBV antibodies were detected by using a commercial enzyme-linked immunosorbent assay (ELISA) (IDEXX Laboratories Inc., Westbrook, ME), following the recommendation of the manufacturer. Twenty of the commercial broilers were bled at 1 day of age, and serum was collected to establish the presence of maternal antibody titers. At 27 and 32 days of age, serum samples from the remaining 180 birds were collected and the levels of antibodies were measured.

Virus reisolation and detection. Five days after challenge, birds were sacrificed and tracheal

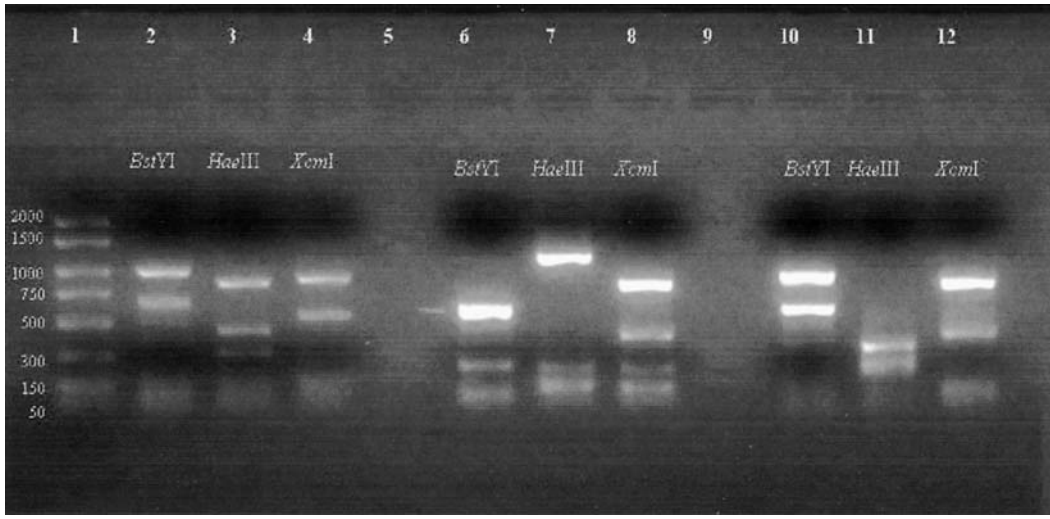

Fig. 1. RFLP patterns of the PCR amplified S1 glycoprotein gene from IBV strains digested with the *Bst*YI, *Hae*III, and *Xcm*I restriction enzymes, respectively. Lane 1 = molecular-weight marker made from a mixture of defined double strand DNA markers exhibiting the following molecular weights; 2000, 1500, 1000, 750, 500, 300, 150, and 50 bp; Lanes 2, 3, and 4 = RFLP pattern of a Massachusetts (Mildvac-Ma5 strain) IBV strain; Lanes 6, 7, and 8 = RFLP pattern of an Arkansas (Mildvac-Ark strain) IBV strain; Lanes 10, 11, and 12 = RFLP pattern of the California 99 isolate.

samples from each replicate in every group were taken and processed for virus reisolation as previously described. Protection was defined as failure to reisolate the IBV challenge strain from the tracheas 5 days after challenge.

Histopathology. Tracheal and kidney samples, taken 5 days after challenge (32 days of age), were fixed in buffered formalin (10%), processed, sectioned, and stained with hematoxylin and eosin (H&E) for histopathological evaluation of lesions produced by the challenge virus. The tracheal lesions were scored from 1 to 4 at 100× magnification based on the severity of the observed lesions, as follows: 1 = no lesions, 2 = mild epithelial hyperplasia and subepithelial lymphoid infiltrate, 3 = moderate epithelial hyperplasia and subepithelial lymphoid infiltrate, 4 = severe epithelial hyperplasia and subepithelial lymphoid infiltrate. For the epithelium thickness scores, the thickness was measured microscopically with one microscopic unit corresponding to 11 microns of epithelial thickness.

Statistical analysis. Statistical analyses were performed in the SAS system for Windows V8 (SAS institute Inc., Cary, NC) using tracheal lesion scores, epithelium thicknesses, and ELISA antibody titers as units. Means for each were analyzed separately using the Student–Newman–Keuls (SNK) test (7). The vaccinated and the positive control groups were compared with the negative control group by the Dunnett test. Significant differences in the scores were established at P values less than 0.05 ($P < 0.05$).

RESULTS

Virus isolation and detection. The cecal tonsil samples obtained from a 5.5-week-old broiler flock in California were positive for IBV after the second passage in 9-day-old SPF embryos. No IBV was isolated from tracheal samples. The isolated virus was detected by the rapid plate hemagglutination and RT-PCR assays and further characterized by RFLP as California 99 strain (Fig. 1).

Antibody levels. By the ELISA test (Table 2), the IBV maternal antibody levels ranged from 545 to 7636 with a geometric mean titer (GMT) of 2397 and a coefficient of variation (CV) of 69%. At 27 days of age, antibody titers were low in all groups, with the exception of the group vaccinated with the Mass–Conn vaccine at 10 days of age. For all vaccinated groups, the antibody titers detected after challenge (32 days of age) were statistically significantly higher ($P < 0.05$) than those obtained before challenge. Very low ELISA titers were observed in the unvaccinated–challenged and unvaccinated–unchallenged control groups, with no significant differences in the titers ($P < 0.05$) after challenge.

Protection against the California 99 isolate. The protection offered by commercial vaccines, established by the reisolation of the virus from tracheas 5 days postchallenge, was complete in the

Table 2. IBV geometric mean antibody titers detected by ELISA before and after challenge with the IBV California 99 isolate via ocular route.[A]

Vaccines	Vaccination age (days)	Prechallenge (27 days)	Postchallenge (32 days)
Mass–Conn	1	88[a]	2786[b]
	10	2852[a]	5169[b]
	1 and 10	15[a]	1971[b]
Mass–Ark	1	68[a]	607[b]
	10	142[a]	3546[b]
	1 and 10	223[a]	2817[b]
Mass–Conn/Mass–Ark	1 and 10	771[a]	3137[b]
Unvaccinated challenged	—	17	4
Unvaccinated unchallenged	—	132	34

[A]Significant differences ($P < 0.05$) between postchallenge and prechallenge antibody titers are denoted by different lowercase superscripts.

Massachusetts–Arkansas vaccinated groups and in the Massachusetts–Connecticut/Massachusetts–Arkansas vaccinated group. Complete protection was observed when the Massachusetts–Connecticut vaccine was used at 1 and 1 and 10 days of age. However, when this vaccine was used as the only vaccine at 10 days of age, no complete protection was obtained, since one of the replicates was positive for virus isolation. The challenge IBV was reisolated in the nonvaccinated challenged group, while no IBV was reisolated from the nonvaccinated unchallenged group. The challenge virus was detected by rapid plate hemagglutination and RT-PCR assays and further characterized by RFLP as California 99 strain.

Histopathology. The lowest mean tracheal epithelium thickness (4) was observed in the unvaccinated–unchallenged group, as expected (Table 3). In all vaccinated groups, lower mean tracheal epithelium thicknesses were present in groups vaccinated with bivalent vaccines containing the Arkansas strain. Higher mean tracheal epithelium thicknesses occurred in groups vaccinated with the Mass–Conn vaccine (Table 3). Significant differences ($P < 0.05$) in the mean epithelium thicknesses were observed between the negative control group and groups that received the Mass–Conn vaccine at 1, 10, or 1 and 10 days of age. Significant differences ($P < 0.05$) in the mean tracheal scores were observed between the negative control (unvaccinated–unchallenged) group and the unvaccinated–challenged, the Mass–Ark at 10 days, and all the Mass–Conn vaccinated groups. No lesions were observed in the kidneys in any group.

Table 3. Mean histopathology tracheal and epithelium thickness scores 5 days postchallenge with the IBV California 99 isolate via ocular route.

Vaccines	Age (days)	Mean tracheal score[A]	Mean tracheal epithelium thickness[B]
Mass–Conn	1	3[a]	10[b]
	10	3[a]	10[b]
	1 and 10	3[a]	8[b]
Mass–Ark	1	2	7
	10	3[a]	8
	1 and 10	2	6
Mass–Conn/Mass–Ark	1 and 10	3	7
Unvaccinated challenged	—	3[a]	8
Unvaccinated unchallenged	—	2	4

[A]Significant differences ($P < 0.05$) when compared with the unvaccinated–unchallenged group denoted by lowercase superscripts.

[B]Significant differences ($P < 0.05$) when compared with the unvaccinated–unchallenged group denoted by lowercase superscripts.

DISCUSSION

The protection offered by commercial vaccines against an isolate exhibiting a California 99 RFLP pattern was evaluated in commercial broilers under laboratory conditions. Maternal antibody levels at 1 day of age were similar to those frequently observed in progenies derived from parents that have been vaccinated several times with live vaccines and boosted with a killed vaccine before the onset of egg production. A significant increase ($P < 0.05$) in the antibody titers after challenge was observed in all groups but the controls, as expected, showing an antigenic stimulation of memory cells (Table 2). The vaccines induced protection in all but one of the vaccinated groups, where protection was partial, since the challenge virus was reisolated from one of the replicates. This group, vaccinated at 10 days of age with the Mass–Conn vaccine, exhibited the highest antibody titers before challenge. This finding may indicate lack of correlation between the serologic response and protection against challenge, as previously reported by Raggi and Lee (8). When compared with the unvaccinated–unchallenged group, significant differences ($P < 0.05$) in the mean tracheal epithelium thickness and tracheal scores were observed in the groups receiving the Mass–Conn vaccine at any age. Only one group receiving the Arkansas vaccine, Mass–Ark at 10 days, showed a significant difference ($P < 0.05$) in the tracheal scores. Based on the lack of reisolation of the challenge virus and lower damage of the trachea after challenge, protection against the California 99 was observed when the Arkansas type was included in the vaccine. The lower tracheal epithelium thickness scores observed in the groups vaccinated twice, compared with the groups receiving only one vaccine, could be due to the presence of higher levels of local immunity when challenged. From these results, it can be concluded that virus isolation and epithelium thickness scores are useful tools to evaluate protection conferred by IBV commercial vaccines against IBV challenge. The absence of histopathologic lesions in the kidneys, even in the unvaccinated–challenged group, may indicate lack of nephrotropism for this isolate.

The rapid plate hemagglutination test and RT-PCR showed a positive correlation (100%) for IBV detection as shown by Ruano *et al.* (9); however, even though less sensitive, the use of the rapid plate hemagglutination test for IBV detection should be considered because it is less expensive and easier to perform (9).

Modern intensive poultry production practices have contributed to the presence of new IBV variants, which can evade the immune response produced by commercial IBV vaccines and thus cause disease. Fortunately, these variants are usually restricted to specific areas, and their presence may in some cases be transient. Production of new commercial vaccines against these new variants is generally not attractive to biological companies due to the high cost and time required for their final approval. For this reason, protection conferred by commercially available IBV vaccines against new variants must be constantly evaluated. In this study, commercial IBV vaccines containing the Arkansas serotype provided an adequate level of protection against the California 99 isolate.

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