AVIAN DISEASES 47:627–632, 2003

Attenuation, Safety, and Efficacy of an Infectious Bronchitis Virus GA98 Serotype Vaccine

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Received 8 November 2002

SUMMARY. In 1998, novel strains of infectious bronchitis virus (IBV) were identified in chickens from the southeastern United States and classified as a new serotype designated Georgia 98 (GA98). Because of the widespread nature of the GA98 virus in the southeastern United States and the lack of adequate protection with the DE072 vaccine, we developed a specific vaccine for the GA98 serotype. The GA98/0470/98 isolate of IBV was passaged in embryonating chicken eggs 70 times, and attenuation of the virus was determined in specific-pathogen-free chicks. Pass 70 of the GA98/0470/98 strain of IBV when given at 1 day of age by coarse spray and at 14 days of age in the drinking water at $1 \times 10^{4.5}$ 50% embryo infectious dose/bird protected against the homologous GA98 challenge as well as provided good protection against the DE072-type virus. In addition, the vaccine was shown to be adequately attenuated and safe at a 10× dosage.

RESUMEN. Atenuación, seguridad y eficacia de una vacuna con el virus de bronquitis infecciosa serotipo GA98.

En 1998 se identificaron cepas nuevas del virus de bronquitis infecciosa en pollos del Sudeste de los Estado Unidos que fueron clasificadas como un serotipo nuevo denominado Georgia 98 (GA98). Debido a la amplia difusión del virus en el Sudeste de los Estados Unidos y a la carencia de una protección adecuada al emplear la vacuna DE072, se desarrolló una vacuna específica para el serotipo GA98. El aislamiento del virus de bronquitis infecciosa GA98/0470/98 se pasó 70 veces en embriones de pollo y se determinó su grado de atenuación en pollos libres de patógenos específicos. Al inocular el pasaje 70 del virus de bronquitis infecciosa GA98/0470/98 al día de edad por aspersión y a los 14 días en el agua de bebida a una dosis infectiva del 50% en embrión de pollo de $10^{4.5}$ por ave, se observó protección contra el desafío con el virus homólogo GA98 y contra el virus tipo DE072. La vacuna mostró una atenuación adecuada y segura al ser empleada a una dosis 10 veces mayor.

Key words: infectious bronchitis virus, Georgia 98 serotype vaccine

Abbreviations: CAS = chorioallantoic sac; %CV = percent coefficient of variance; EID_{50} = 50% embryo infective dose; ELISA = enzyme-linked immunosorbent assay; GA98 = GA/CWL0470/98; GMT = geometric mean titer; HI = hemagglutination inhibition; IBV = infectious bronchitis virus; PBS = phosphate-buffered saline; RT-PCR/RFLP = reverse transcriptase–polymerase chain reaction/restriction fragment length polymorphism; SPF = specific-pathogen free

Recently, a new serotype of infectious bronchitis virus (IBV) was identified in chickens from the southeastern United States and designated Georgia 98 (GA98) (9,10). The first isolate of that new serotype (GA98/0470/98) was obtained in 1998 from commercial broilers in Georgia. Genetic characterization of that isolate and similar more recent isolates indicated that they are related to the DE072 serotype of IBV but constitute a separate group (10). Cross virus-neutralization testing in embryonating eggs and protection studies in specific-pathogen-free (SPF) chickens clearly showed that the GA98 group of viruses were serologically distinct from DE072 as well as all other currently recognized serotypes of IBV in the United States (9).

It is well known that little or no cross protection occurs between different serotypes of IBV (3). Thus, it is not surprising that attempts to vaccinate commercial chickens with currently available vaccines, in areas of the southeast where the GA98 virus

was identified, have been generally unsuccessful. The GA98 viruses continue to be a problem in the southeastern United States, and vaccination with both the DE072 and Arkansas serotypes has helped somewhat, but significant economic losses still occur when that combination of vaccines is used (Dr. John Smith, Fieldale Farms Corporation, Baldwin, GA, pers. comm.).

Because of the widespread nature of the GA98-type viruses in the southeast and the inability to control losses with commercially available vaccines, we attempted to develop a vaccine specific for the GA98 serotype. In this study, we passaged the GA98/CWL0470/98 strain of the virus in embryonating eggs and tested its attenuation, safety, and efficacy according to the requirements outlined in Title 9 of the Code of Federal Regulations (1).

MATERIALS AND METHODS

Viruses. The GA98/CWL0470/98 strain of IBV (pass 8, titer = 1.0×10^6 50% embryo infectious dose [EID_{50}]/ml) was previously characterized (9, 10) and used in this study. In addition, the DE/072/92 strain (pass 7, titer = 1.06×10^8 EID_{50}/ml), a pathogenic isolate of the Delaware serotype of IBV (5), was used in the vaccine-challenge studies.

Attenuation. The GA98/CWL0470/98 strain was passaged by inoculating 9-to-11-day-old embryonating chicken eggs (Charles Rivers Spafas, North Franklin, CT) by the chorioallantoic sac (CAS) route (6). Inoculated eggs were incubated for 48 hr at 37 C, and the chorioallantoic fluid was harvested for subsequent passage. At every 10th passage, starting with pass 20, the virus was examined for viability by incubation of two to three additional eggs for 7 days and observation of the embryos for clinical signs consistent with IBV infection. In addition, selected passages were examined by electron microscopy (EM Laboratory, College of Veterinary Medicine, University of Georgia, Athens, GA) for the presence of coronavirus and by reverse transcriptase–polymerase chain reaction/restriction fragment length polymorphism (RT-PCR/RFLP) (8) to verify the type of the virus.

Purity and virus identity tests were conducted as described in section 113.300 of Title 9 of the Code of Federal Regulations (1). Virus stocks of embryonating egg pass 50 and pass 70 were prepared from the previous pass in at least 30 embryonating eggs and tested for purity from bacteria, fungi, mycoplasma, and extraneous viruses including chicken anemia virus, hemagglutinating viruses, and avian leukosis virus (15). The viruses were titrated by inoculating 10-fold serial dilutions of the virus stocks into the CAS of 10-day-old embryonating eggs, and the titer was calculated by the method of Reed and Muench (11),

The virus stocks were tested for attenuation in one-day-old SPF leghorn chicks (Charles Rivers Spafas) according to the procedures in section 113.327 of Title 9 of the Code of Federal Regulations (1). Chicks were randomly separated into three groups of 10 birds each and housed in positive-pressure Horsfal isolation units. The birds were given feed and water *ad libitum* and examined twice daily throughout the experiment. The birds in the first treatment group were given at least 1×10^4 EID_{50} of pass 50, and those in the second group were given 1×10^4 EID_{50} of pass 70 of the GA98/CWL0470/98 virus by coarse spray at 1 day of age. Birds in group 3 were not exposed and served as negative controls. The birds were examined for clinical signs daily. Five birds per group were killed and necropsied at 5 and at ten days postexposure. At necropsy, the birds were weighed, and tracheal swabs were placed in 3 ml of ice cold phosphate-buffered saline (PBS), filtered through a 0.2-μm syringe filter (Gelman Laboratory, Ann Arbor, MI), and inoculated into 9-to-11-day-old embryonating eggs for virus isolation. Sera were collected, diluted 1:8 in PBS, and tested for antibodies to IBV by commercial enzyme-linked immunosorbent assay (ELISA) (IDEXX, Westbrook, Maine). For histopathologic examination, the lower halves of tracheas (below the area swabbed) were collected, fixed in 10% neutral buffered formalin, and routinely processed into paraffin, and 5-μm sections were cut for hematoxylin and eosin staining. Epithelial hyperplasia, lymphocyte infiltration, and the severity of epithelial deciliation were scored for each trachea from 1 to 4 with 1 = normal, 2 = focal, 3 = multifocal, and 4 = diffuse. The least significant difference of the means was statistically calculated with the Student *t*-test for each pair and JMP Statistical Discovery Software (SAS Institute, Inc., Cary, NC).

Safety. Safety testing was conducted according to section 113.327, d, 2, of Title 9 of the Code of Federal Regulations (1), which states that if five or fewer of 50 vaccinated birds show respiratory signs and/or death, the virus is considered to be safe. Fifty SPF chicks (Charles Rivers Spafas) were housed in positive-pressure Horsfal isolation units and given feed and water *ad libitum.* The chicks were vaccinated by eyedrop with 10 doses of the pass 70 GA98/CWL0470/98 virus at 5 days of age and

Table 1. Properties of the passage 70 GA98/CWL/0470/98[A] isolate of IBV in 1-day-old SPF chickens.

Day postexposure	Virus isolation		Average tracheal lesion scores		Average serum ELISA titers	
	Controls	Inoculated	Controls	Inoculated	Controls	Inoculated
Day 5	0/5	0/5	1.6	1.8	116.1	171.4
Day 10	0/5	0/5	2.4	1.8	92.8	117.5

[A]Birds were given 1×10^4 EID_{50} by coarse spray.

were examined twice daily for clinical signs for 21 days. All of the birds were killed and necropsied at 26 days of age. At necropsy, sera were collected for ELISA (IDEXX) and hemagglutination inhibition (HI) test (6), and tracheas were collected and processed for histopathology as described above.

Efficacy. SPF chicks were randomly separated into five groups and housed separately in positive-pressure Horsfal isolation units. The birds were given feed and water *ad libitum* and were examined twice daily throughout the experiment. Groups 1 and 2 had 24 birds each and were given the passaged GA98/CWL0470/98 virus ($1 \times 10^{4.5}$ EID_{50}/bird) by coarse spray at 1 day of age. At 2 wk of age, those birds were given a second immunization with the pass 70 GA98/CWL0470/98 virus ($1 \times 10^{4.5}$ EID_{50}/bird) administered in the drinking water. Groups 3 and 4 had 12 birds each and served as nonvaccinated challenge controls, and 12 additional birds in group 5 served as nonvaccinated, nonchallenged controls. Twenty-one days postvaccination, the birds were challenged by eyedrop (0.1 ml) with at least 1×10^4 EID_{50}/ml of pathogenic GA98 virus (groups 1 and 3) or DE/072/92 (groups 2 and 4). All of the birds were killed and necropsied at 5 days postchallenge. At necropsy, the birds were weighed, sera were collected, and tracheal swabs were placed in ice cold PBS for virus isolation as described above. Efficacy was based on not less than 90% of the controls positive for virus recovery and not less than 90% of the vaccinates negative for virus recovery. For histopathologic examination, the lower halves of tracheas (below the area swabbed) were collected, fixed, processed, stained, and scored as described above. The least significant difference of the means was statistically calculated with the Student *t*-test for each pair and JMP Statistical Discovery Software (SAS Institute, Inc., Cary, NC).

RESULTS

Attenuation. At every 10th passage, starting with pass 20, additional eggs were incubated for 7 days, and the embryos examined all had typical signs and lesions consistent with IBV infection (6). Coronavirus particles were observed in passes 25, 50, and 70 by electron microscopy, and RT-PCR/RFLP (8) analysis verified that the molecular type of the virus had not changed.

Virus stocks of passes 50 and 70 tested negative for bacteria, fungi, mycoplasma, and extraneous viruses including chicken anemia virus, hemagglutinating viruses, and avian leukosis virus (15). The viruses were titrated in 10-day-old embryonating eggs, and the titer was calculated to be 2×10^6 EID_{50}/ml for pass 50 and 7.3×10^7 EID_{50}/ml for pass 70.

One-day-old birds given 1×10^4 EID_{50} of the pass 50 GA98/CWL0470/98 virus by coarse spray had tracheal lesion scores of 2.9 compared with 1.1 for noninoculated controls, and virus was reisolated from 4 of 10 birds. Because attenuation of the pass 50 virus stock apparently was not complete, safety and efficacy studies were not conducted on that passage.

Data on the attenuation of the pass 70 GA98/CWL0470/98 virus are presented in Table 1. One-day-old birds given $1 \times 10^{4.5}$ EID_{50} of the pass 70 GA98/CWL0470/98 virus by coarse spray had average tracheal lesion scores of 1.8 for both days 5 and 10 postinoculation, which was not statistically different from noninoculated controls that had score averages of 1.6 and 2.4 for days 5 and 10 postinoculation, respectively. No clinical sign was observed in any bird, and there were no statistical differences in body weights when inoculated birds were compared with noninoculated controls. No virus was isolated from the noninoculated control birds or from inoculated birds at 5 and 10 days postinoculation. The average ELISA titers for the inoculated birds were extremely low (day 5 = 171.4, day 10 = 117.5) and were not statistically different from the controls (day 5 = 116.1, day 10 = 92.8).

Safety. Fifty 5-day-old SPF chicks were given $1 \times 10^{5.5}$ EID_{50}/bird (10 doses) of the pass 70 virus, and no clinical sign was observed in any birds throughout the safety study. The average histopathology lesion score was 1.9. Nine serum samples collected prior to

Table 2. Efficacy of the passage 70 GA98/CWL0470/98 strain of IBV at day 5 after challenge with GA98 and DE072.

Group	Treatment	Clinical signs[A] (% protection)	Virus isolation[B] (% protection)	Histo-pathology[C]	ELISA titer[D]	Mean body weight (g)
1	GA98 vaccinated/GA98 challenged	4/24[E] (83%)	2/24 (92%)	2.5[a]	690	524.1
2	GA98 vaccinated/DE072 challenged	2/23 (91%)	2/23 (91%)	1.9[b]	9	521.3
3	GA98 challenged	7/12 (41%)	11/12 (8%)	3.5[c]	3	523.4
4	DE072 challenged	9/12 (25%)	9/12 (25%)	3.1[c]	1	511.2
5	Negative controls	0/12	0/12	1.0[d]	52	492.1

[A]Clinical signs were based on ocular and nasal discharge and tracheal rales.
[B]Individual tracheal swab material inoculated into 9-to-11-day-of-incubation embryonating eggs and examined for embryo lesions and for virus by the neurominidase test and by RT-PCR.
[C]Average tracheal lesion scores. Numbers with different lowercase superscripts are statistically different ($P \leq 0.05$).
[D]Geometric mean serum antibody titer.
[E]Number positive/total.

inoculation with the virus were negative for IBV antibodies in the ELISA test and had a geometric mean titers (GMTs) of 13 and 23 against the Mass 41 and DE072 antigens, respectively, in the HI test. The ELISA GMT for sera collected at 26 days of age was 49 with a coefficient of variance percentage (%CV) of 122.9. The GMTs for the HI test were 9 when the Mass 41 antigen was used and 18 with the DE072 antigen.

Efficacy. Results of the efficacy trial are presented in Table 2. One bird in group 2 died prior to challenge. That death was not related to vaccination. On the basis of virus recovery, 92% of the GA98-vaccinated birds were protected from GA98 challenge and 91% were protected from DE072 challenge. Ninety-two percent of the nonvaccinated GA98-challenged control birds were positive for virus recovery, indicating an adequate challenge, whereas only 75% of the nonvaccinated DE072-challenged control birds were positive for virus recovery, indicating a less severe challenge. No virus was recovered from the tracheas of negative control birds. Clinical signs observed at 5 days postchallenge correlated with virus recovery data for the DE072-challenged birds. However, 83% of the GA98-vaccinated birds and 41% of the nonvaccinated birds did not develop clinical signs after GA98 challenge.

Tracheal histopathology lesion scores for GA98-vaccinated birds challenged with GA98 and DE072 were 2.5 and 1.9, respectively. Those values were statistically different from each other and from GA98-challenged (average score = 3.5) and DE072-challenged (average score = 3.1) controls. All of the challenged birds had statistically higher lesion scores when compared with negative control birds (average score = 1.0).

The ELISA serum antibody titers measured 5 days postchallenge were essentially negative for all groups. The group of birds vaccinated with GA98 and then challenged with GA98 had a slightly elevated titer of 690, but the %CV was 105, indicating variable responses within that group. No statistical differences were observed in the average body weight recorded 5 days postchallenge.

DISCUSSION

In this study, we passaged the GA98 strain of IBV in embryonating eggs and tested that passaged virus for attenuation, safety, and efficacy against the homologous virus and the DE072 virus in SPF chicks. On the basis of clinical signs, virus isolation, and tracheal lesion scores, pass 70, but not pass 50, was determined to be attenuated. When the pass 70 virus was given to 1-day-of-age SPF chicks, it was not recovered from the trachea at either 5 or 10 days postexposure, no clinical signs were observed, and the tracheal lesion scores were not statistically different from those of uninoculated controls. Serum antibody titers were slightly higher than those of uninoculated controls, but the numbers were extremely low and not statistically significant. On the basis of those findings, the pass 70 virus was tested for safety and efficacy.

The titer of the pass 70 GA98 virus was determined to be 7.3×10^7 EID_{50}/ml, and 10 doses ($1 \times 10^{5.5}$ EID_{50}/bird) were given to each of 50 SPF chicks. No clinical signs were observed after vaccination, tracheal lesion scores were considered to be low at 1.95, and ELISA and HI titers were essentially negative.

The pass 70 GA98 virus was efficacious on the basis of the criteria set forth in the Standard Requirements for IBV vaccines in Title 9 of the Code of Federal Regulations section 113.327 (1) when the homologous GA98 virus was used for challenge. When the DE072 virus was used to challenge the pass 70 GA98-vaccinated birds, 91% of the birds were protected on the basis of virus isolation. Note that the DE072 challenge virus was recovered from the tracheas of only 75% of the nonvaccinated DE072-challenge control birds. Standards for IBV vaccine licensing in Title 9 of the Code of Federal Regulations (1) require that not less than 90% of the challenge control birds test positive for virus isolation at 5 days postchallenge. Although our DE072 challenge did not meet that requirement, we did infect and observe clinical signs in 75% of the nonvaccinated challenge birds and observed histopathology scores that were statistically worse than those of vaccinated and challenged birds. As with any controlled laboratory study, it is possible that the pass 70 GA98 vaccine will not protect against IBV challenge in the field at the levels reported in this study.

Statistically, tracheal lesion scores for the vaccinated and challenged birds were significantly better than those for the nonvaccinated challenged birds but significantly worse than those for the negative controls. Some damage to the mucosal lining of the trachea is expected and most likely necessary for the development of a local immune response to IBV. In the efficacy study, as well as in the attenuation and safety studies, serum antibody titers were low or negative. This finding is typical of birds vaccinated for IBV and was not unexpected. Low serum antibody titers do not always correlate with lack of protection against IBV because local antibody responses and cell mediated immunity play a significant role in recovery from the disease (13).

In a previous study (9), we reported that the GA98 group of viruses were genetically similar but serologically distinct from DE072 type viruses. In that study, Archetti and Horsfall (2) relatedness values for cross virus-neutralization testing between GA98 and DE072 viruses in embryonating eggs were generally below 50%, indicating that the viruses were unrelated. In addition, birds vaccinated with the DE072 vaccine virus were less than 50% protected after GA98 challenge. In contrast, the data presented herein indicate that the GA98 virus can provide some cross protection against challenge with DE072. Some strains of IBV have been recognized to induce some limited cross-protection between closely related serotypes (7,12). It is possible that some IBV strains have antigens in common with other IBV types (4) or that some isolates are simply better at inducing an immune response. Particularly, inducing a strong cell mediated immune response may be important in cross protection because cross-reactive cytotoxic T lymphocytes can be found after immunization of chickens with a DNA vaccine against IBV (14).

Regardless of the mechanism of protection, apparently the pass 70 GA98 vaccine, when given at 1 day of age by coarse spray and at 14 days of age in the drinking water, can protect against a homologous GA98 challenge as well as provide good protection against the DE072 type viruses. In addition, the vaccine was adequately attenuated and safe at a 10× dosage. Although this vaccine was developed for commercial use, we have not at this time tested it experimentally in the field.

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ACKNOWLEDGMENTS

This study was supported by a grant from the U.S. Poultry and Egg Association.