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Research Note—

Evaluation of the Protection Conferred by a Disinfectant Against Clinical Disease Caused by *Avibacterium paragallinarum* Serovars A, B, and C from Argentina

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SUMMARY. This work evaluates the efficiency of the administration of the disinfectant N-alkyl dimethyl benzyl ammonium chloride (TIMSEN™) in the prevention of the horizontal transmission of serovars A, B, and C of *Avibacterium paragallinarum*, the causative agent of avian infectious coryza. This disinfectant was administered in drinking water (50 ppm) and once or twice per day by coarse spray (800 ppm, 8 ml per m^3 during 3 seconds). In three trials conducted with vaccinated birds, the disinfectant reduced the clinical signs of infectious coryza significantly ($P < 0.05$). There was no significant effect when the product was used in a fourth trial with unvaccinated birds. Furthermore, the application of only one daily environmental spraying was sufficient to significantly reduce clinical signs. According to these results, in order to diminish the clinical signs of infectious coryza in birds vaccinated against *A. paragallinarum*, it is recommended to administer this disinfectant in drinking water and by environmental spraying.

RESUMEN. *Nota de Investigación*—Evaluación de la protección conferida mediante el empleo de un desinfectante contra los signos clínicos de coriza infecciosa causada por *Avibacterium paragallinarum* serovariedades A, B y C de la Argentina.

Se evaluó la eficiencia de la administración del desinfectante N-alquil dimetil benzilo cloruro de amonio (TIMSEN™) para prevenir la transmisión horizontal de serovariedades A, B y C de *Avibacterium paragallinarum*, el agente causal de la coriza infecciosa de las aves. El desinfectante fue administrado en el agua de bebida (50 ppm) y mediante aspersión por gota gruesa (800 ppm, 8 ml por m^3 durante 3 segundos), una o dos veces por día. En tres ensayos realizados con aves vacunadas, el uso del desinfectante contribuyó significativamente a reducir los signos clínicos de la coriza infecciosa ($P < 0.05$). En un cuarto ensayo, no se observó ningún efecto significativo al aplicarlo en las aves que no habían sido vacunadas. Además, la utilización de un único asperjado diario fue suficiente para disminuir los signos clínicos. De acuerdo con estos resultados, para disminuir los signos clínicos de coriza infecciosa en aves vacunadas contra *A. paragallinarum*, es recomendable el empleo de este desinfectante administrado en el agua de bebida y mediante aspersión.

Key words: *Avibacterium paragallinarum*, disinfectant, *Haemophilus paragallinarum*, infectious coryza, N-alkyl dimethyl benzyl ammonium chloride, TIMSEN

Abbreviations: BHI = brain–heart infusion; CFU = colony-forming units; CLBA = Columbia agar base plus 7% equine hemolyzed blood; NADH = β-nicotinamide adenine dinucleotide hydrate; PI = postinoculation; SPF = specific-pathogen-free

Avibacterium (formerly *Haemophilus*) *paragallinarum* is the causative agent of avian infectious coryza (1,3). Infectious coryza is a highly contagious acute respiratory disease. The most common symptom is acute inflammation of the head and eyes, occasionally with inflammation of the wattles (16); septicemia and arthritis are less common (14). This disease remains a serious problem for the poultry industry in many countries all over the world and affects mainly laying hens, though there are descriptions in broilers (14,16). The loss of egg production can reach as high as 40%, especially in multiage farms (3).

Avibacterium paragallinarum is classified into three serovars, named A, B, and C by serological tests using the Page scheme (11). This scheme was amplified by Kume *et al.* (9) and later by Blackall *et al.* (2) using hemagglutination-inhibition tests.

Vaccination is the most commonly used method for the prevention of infectious coryza (3,20). During recent years, experimental models were developed to investigate pathogenicity, horizontal infection, and invasiveness of *A. paragallinarum* (15). These models have demonstrated that a proper vaccination may provide adequate protection against infection with *A. paragallinarum* (20,22). However, some commercial vaccines have been shown to give insufficient protection against regional strains of *A. paragallinarum*, possibly because some regional strains might possess antigens that are not present in the international standard vaccine strains (8,22). As a result, and due to the absence of cross-protection between some strains that are classified within the same Page serovar (17,23), there is a need to find additional management tools to support the efficacy of infectious coryza vaccines. Application of strict measures of biosecurity supported by efficient disinfectants may diminish the possibility of disease outbreaks in vaccinated birds (5), particularly in those flocks exposed to variant strains.

Although the disinfectant N-alkyl dimethyl benzyl ammonium chloride (TIMSEN™) has been shown to be effective in reducing the numbers of certain bacteria in broiler carcasses (13) and marine *Vibrio* populations (19), there are no data about its effect in preventing infectious coryza in chickens. Therefore, the objective of this work was to evaluate the effect of this disinfectant upon the horizontal transmission of different serovars of *A. paragallinarum*.

MATERIALS AND METHODS

Experimental animals. One-day-old chicks, *Salmonella*-free males of Gy-brown (Great yield) laying hens (La Mendocina,

Table 1. Comparison of mean scores of the clinical signs of vaccinated and unvaccinated cocks challenged with different *A. paragallinarum* serovars and treated with the disinfectant N-alkyl dimethyl benzyl ammonium chloride (TIMSEN™).

Group	*n*	Vaccination	Challenge strain (serovar)	Treatment with the disinfectant[A]		Mean scores of clinical signs [B]				
				Drinking water	Daily spraying	Days PI				
						2	4	6	8	10
Trial 1										
A	10	Yes	H23 (A)	No	No	0.56[a]	0.94[a]	0.89[a]	1.06[a]	0.61[a]
B	9	Yes	H23 (A)	Yes	Twice	0.35[a]	0.40[b]	0.20[b]	0.20[b]	0.20[b]
Trial 2										
C	10	Yes	H8 (B)	No	No	0.85[a]	0.85[a]	1.05[a]	1.05[a]	0.50[a]
D	10	Yes	H8 (B)	Yes	Once	0.20[b]	0.35[b]	0.55[b]	0.56[b]	0.11[a]
E	10	Yes	H8 (B)	Yes	Twice	0.10[b]	0.30[b]	0.30[b]	0.50[b]	0.30[a]
Trial 3										
F	10	No	H8 (B)	No	No	1.05[a]	1.90[a]	2.20[a]	1.65[a]	0.70[a]
G	8	No	H8 (B)	Yes	Once	0.69[a]	1.56[a]	1.86[a]	1.86[a]	0.71[a]
H	10	No	H8 (B)	Yes	Twice	0.20[b]	1.90[a]	1.95[a]	1.15[a]	0.75[a]
Trial 4										
I	10	Yes	H32 (C)	No	No	0.70[a]	0.70[a]	1.10[a]	0.70[a]	0.35[a]
J	10	Yes	H32 (C)	Yes	Twice	0.20[b]	0.20[b]	0.20[b]	0.31[b]	0.06[b]

[A]Administration of the disinfectant in drinking water (50 ppm) and by coarse spray (800 ppm, 8 ml per m^3 during 3 seconds).
[B]Mean scores in the same column of each trial with different superscripts are significantly different ($P < 0.05$).

Argentina), were reared in cages until 26 wk old. All of these chicks were reared under high biosecurity measures in installations that permitted conditions of total isolation. *Salmonella* spp.-free status was confirmed by cloacal swabbing every 2 wk. The animals received balanced food free from *Salmonella* spp., verified by bacteriological analysis (12) consisting of lactose broth, tetrationate broth, Xylose-Lysine-Desoxycholate-Tergitol 4, and MacConkey agar plates followed by characterization with biochemical tests. Furthermore, the food was prepared without the addition of antibiotics or coccidiostats and was free from meat or fishmeal. Food and water were given *ad libitum* throughout the trials. Under these aforementioned strict biosecurity measures, the animals did not show any symptoms of illness, and received neither vaccination nor treatment.

***Avibacterium paragallinarum* strains.** Strains H23, H8, and H32 (serovars A, B, and C, respectively) were used in this work (22). The three strains were passed through a specific-pathogen-free (SPF) chicken before using. Each strain was retrieved from liquid nitrogen, streaked onto Columbia agar base plates plus 7% equine hemolyzed blood (CLBA), and prepared as previously described (21). The plates were incubated for 48 hr in a candle jar. The growth was used to inoculate a biphasic CLBA slope containing Balcarce broth, prepared as previously described (22). After overnight incubation at 37 C, 0.2 ml of each broth culture was injected into the right infraorbital sinus of one SPF laying hen. At the second day postinoculation (PI) the hens were sacrificed; their heads were removed and kept frozen at −20 C. Before the beginning of the trials, these heads were defrosted at 37 C. The skin of the infraorbital area was cauterized and an incision was made onto the right infraorbital sinus (15). The skin was separated and a sterile swab, previously moistened in sterile brain–heart infusion (BHI) broth, was introduced. This swab was immediately streaked onto plates containing CLBA and CLBA with antibiotics: bacitracin (5 IU/ml, SIGMA B-0125), cloxacillin sodium salt (6.5 μg/ml, SIGMA C-9393) and vancomycin hydrochloride (10 μg/ml, SIGMA V-2002).

For the preparation of the inoculation broth, growth from the CLBA plates, previously incubated for 48 hr as previously described (21) in microaerophilic atmosphere at 37 C, was suspended in BHI broth with the addition of 25 μg/ml of β-nicotinamide adenine dinucleotide (NADH) (SIGMA N-7004). Furthermore, biochemical tests (4,21) were performed to confirm the identity and purity of the bacteria: motility testing and 2,3,5-triphenyl-tetrazolium chloride reduction; nitrate reduction; urease, oxidase, and catalase activities; and carbohydrate (galactose, glucose, and trehalose) fermentation tests in phenol-red blood-agar base slopes.

Experimental Design. Four trials were simultaneously performed comprising 97 cocks, distributed in 10 experimental groups of eight to 10 cocks each (Table 1). Trial 1 (groups A and B), Trial 2 (C, D, and E), and Trial 4 (I and J) were designed using vaccinated cocks, whereas in Trial 3 (F, G, and H) all cocks remained unvaccinated. At 16 wk of age, the cocks of Trials 1, 2, and 4 were given an intramuscular dose (0.5 ml) of an oil-adjuvant trivalent commercial coryza vaccine (FDAH® GEL-3) together with a subcutaneous dose (0.5 ml) of an experimental vaccine, which included the corresponding challenge strains: H23 (Trial 1), H8 (Trials 2 and 3), or H32 (Trial 4). At 20 wk of age the same animals were similarly given another shot of both vaccines.

Experimental vaccines were grown overnight at 37 C in fermentors (22) containing BHI broth with 25 μg/ml of NADH. Each fermentor was seeded with a single CLBA colony of each corresponding challenge strain. The grown broth was inactivated by adding 0.5% formalin. After 24 hr of incubation at room temperature on CLBA plates, inactivation was confirmed, as no growth was observed. The inactivated broth was centrifuged at 24,424 × *g* for 10 min at 4 C (Sorvall RC5C) and the pellet was resuspended in the same supernatant broth to give a final concentration of 1×10^9 cells per dose, measured by spectrophotometer at 462 nm. Finally, 7% aluminum hydroxide gel, containing 2% aluminium oxide (22), was added as adjuvant.

Trial 1 and Trial 4 were designed to study the effect of the administration of the disinfectant at 50 ppm in drinking water, together with two daily environmental coarse sprayings administered over the heads of the animals at a concentration of 800 ppm (8 ml per m^3 during 3 seconds). In Trial 2 and Trial 3 an additional group of cocks was added to evaluate the effect of only one single daily environmental spraying administered in a similar way. Cocks in Trial 1 were challenged with serovar A (strain H23), cocks in Trial 2 and Trial 3 with serovar B (H8), and cocks in Trial 4 with serovar C (H32).

Challenge. At 26 wk old all cocks were transferred into special isolators distributing the animals into the aforementioned 10 experimental groups (Table 1). Each experimental group was housed in a separated isolator unit, previously washed and disinfected with chlorine. Each isolator was built with cement and was fitted with glass

front doors. Water was provided by nipples and food was given from the outside by specially adapted feeders. Air was forced inside 24 hr a day. The floor was made from elevated metallic nets that allowed the separation of the animals from the feces, which were removed daily by a special drainage system for the disposal of infectious materials.

A seeder-bird challenge system was used. On the day of transfer into the isolators, two additional unvaccinated cocks per experimental group were inoculated by injecting 0.2 ml of the aforementioned live *A. paragallinarum* culture into the right infraorbital sinus. These inoculated birds were immediately introduced into the isolators of each corresponding experimental group. The number of viable bacteria given to the seeder birds, enumerated by the method of Miles *et al.* (10), were as follows: Trial 1 (strain H23), 1.61×10^7 colony-forming units (CFU)/dose; Trials 2 and 3 (strain H8), 6.5×10^7 CFU/dose; Trial 4 (strain H32), 1.2×10^7 CFU/dose.

Evaluation of clinical signs. In order to evaluate the protection against clinical signs conferred by the disinfectant, a scoring method was applied (15,22). Clinical signs of each facial side were registered from the second day until the 10th day PI, according to the following key: 0, no clinical signs; 1, light conjunctivitis; 2, facial inflammation, infraorbital sinusitis, and/or conjunctivitis and/or tears; 3, edema and facial inflammation, infraorbital sinusitis, abundant nasal and eye secretion, and partially closed eye; 4, severe edema and facial inflammation, abundant nasal and eye secretion, and totally closed eye.

Statistical analysis. For each trial a test of maximum probability was performed. The adjustment was done using a test of maximum probability with mixed effects. Each facial side of each animal was considered as random effects. The polynomials for each trial were found to be different. The mean score of the clinical signs was calculated by dividing the sum of the clinical signs score of each facial side of all the chickens within a group by twice the total number of chickens in the group. The mean score was compared between the groups of each trial for each day of observation using the "Student *t*-test". A value of $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

The clinical-sign scores for treated and untreated birds challenged with the different strains of *A. paragallinarum* are shown in Table 1. In Trial 1, the application of the disinfectant significantly reduced the clinical signs of infectious coryza, starting from the fourth day PI. The highest mean score of the group treated with the disinfectant was 0.40 on the fourth day PI, and the highest mean score in the nontreated group was 1.06 on the eighth day.

On the other hand, the results of Trial 2 showed a lower mean score in groups treated with one or two daily sprayings (groups D and E) in comparison with the group C, which was not treated with the disinfectant, during the first 4 days of observation (second, fourth, sixth, and eighth days PI). In the treated groups, the mean score ranged from 0.20 to 0.56 and from 0.10 to 0.50 for one and two daily sprayings, respectively; the mean score of group C varied between 0.85 and 1.05. There were no statistical differences between the groups treated with either one or two daily sprayings. On the last day of observation (10th day PI), no significant differences were found within any of the experimental groups.

In Trial 3, conducted with unvaccinated birds, the mean scores were higher than in Trial 2. The only significant difference was a lower mean score in group H (two daily sprayings) compared with the other groups at the second day PI. No significant differences were registered in the other days (fourth, sixth, eighth, and 10th days PI). Thus, the disinfectant was not efficient in the prevention of the horizontal transmission of the bacteria among unvaccinated birds. Similarly, Bragg (5) showed that the use of another disinfectant, didecil dimethyl amonium chloride, in challenged unvaccinated laying hens could help to reduce horizontal transmission of *A. paragallinarum*, although it was not enough to prevent clinical disease. Furthermore, Bragg and Plumstead (7) have demonstrated that a continuous disinfection program can contribute to the control of infectious avian diseases.

It is important to note that the mean score in vaccinated birds in Trial 2 (group C) did not exceed a score of 1.05, with a highest individual score of 2 (data not shown). In contrast, the mean score reached 2.2 (group F) in Trial 3, with high scores of 4 observed in several animals (data not shown). There was statistical difference between vaccinated and nonvaccinated birds without treatment with the disinfectant at the peak of infection between the fourth and the eighth days. These results indicate that vaccines remain the main method for the control of infectious coryza and that vaccines can reduce both the severity and the duration of the clinical signs of the disease.

In Trial 4, the application of the disinfectant significantly decreased the clinical signs of infectious coryza, in front of challenge with strain H32 (serovar C), for the entire period of the study. The highest mean score of the group treated with the disinfectant was 0.31 on the eighth day PI, while the highest mean score was 1.10 on the sixth day PI in the nontreated group. These results are similar to the results obtained in Trial 1.

In general, the three serovars showed similar pathogenicity when tested under this experimental model of infection. All groups had a similar peak of infection between the fourth and eighth days PI followed by recuperation, as evidenced by lower scores. Soriano *et al.* (18) studied the virulence of nine serovar reference strains of *A. paragallinarum* and found that virulence differences existed among them. In the same way, Bragg (6) showed that the South African serovar C isolates appeared to be more virulent than the South African serovar A or B isolates. It is known that serovar B strains of *A. paragallinarum* from Argentina are highly pathogenic and different from many other strains examined around the world (22). In this work, we used a horizontal infection model based on seeder birds that challenged vaccinated birds (except in Trial 3). In addition to the commercial vaccine, these birds were given an experimental vaccine, which contained the same strain used for their challenge 6 wk later. Vaccinated birds were less susceptible to *A. paragallinarum* infection and consequently these aforementioned differences of virulence between serovars were not manifested in these trials.

On the basis of our results, we recommend that, in order to reduce the horizontal spread of *A. paragallinarum* in vaccinated birds, the disinfectant should be given in the drinking water and also in at least one environmental spraying per day. Our results demonstrate that the exclusive application of disinfectant program is not effective in unvaccinated birds and clearly supports the need for an efficient vaccination program in association with disinfection and strict biosecurity measures.

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