

Effect of vaccination on experimental infection with *Bordetella bronchiseptica* in dogs

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Objective—To determine comparative efficacy of vaccines administered IM and intranasally, used alone or sequentially, to protect puppies from infection with *Bordetella bronchiseptica* and determine whether systemic or mucosal antibody response correlated with protection.

Design—Randomized controlled trial.

Animals—50 specific-pathogen-free Beagle puppies.

Procedure—In 2 replicates of 25 dogs each, 14-week-old puppies that were vaccinated against canine distemper virus and parvovirus were vaccinated against *B bronchiseptica* via intranasal, IM, intranasal-IM, or IM-intranasal administration or were unvaccinated controls. Puppies were challenge exposed via aerosol administration of *B bronchiseptica* 2 weeks after final vaccination. Clinical variables and systemic and mucosal antibody responses were monitored for 10 days after challenge exposure. Puppies in replicate 1 were necropsied for histologic and immunohistochemical studies.

Results—Control puppies that were seronegative before challenge exposure developed paroxysmal coughing, signs of depression, anorexia, and fever. Vaccinated puppies (either vaccine) that were seronegative before challenge exposure had fewer clinical signs. Puppies that received both vaccines had the least severe clinical signs and fewest lesions in the respiratory tract. Vaccinated dogs had significantly higher concentrations of *B bronchiseptica*-reactive antibodies in serum saliva before and after challenge. Antibody concentrations were negatively correlated with bacterial growth in nasal cavity and pharyngeal samples after challenge exposure.

Conclusions and Clinical Relevance—Parenterally and intranasally administered vaccines containing *B bronchiseptica* may provide substantial protection from clinical signs of respiratory tract disease associated with infection by this bacterium. Administration of both types of vaccines in sequence afforded the greatest degree of protection against disease. (*J Am Vet Med Assoc* 2001;218:367–375)

Bordetella bronchiseptica has been associated with respiratory tract disease in dogs since the early 1900s when it was wrongly implicated as the cause of canine distemper.¹⁻³ After a virus was determined to be the cause of canine distemper,⁴⁻⁶ questions arose con-

cerning the primacy of *B bronchiseptica* as a pathogen in the respiratory tract of dogs.⁷⁻⁹ However, results of several studies, encompassing naturally acquired and experimentally induced infections with *B bronchiseptica*, indicate that the bacteria can cause respiratory tract disease in dogs without the involvement of other agents.¹⁰⁻¹⁵ There are recent conflicting data concerning genetic variation amongst isolates of *B bronchiseptica* that infect dogs and other host species.^{16,17} Presently, data are lacking that indicate that reported genetic differences¹⁷ are directly indicative of differences in virulence; however, it has been proposed that currently available vaccines may be less efficacious in conferring immunologic protection to dogs infected with recent (genetically different) isolates.¹⁷

Infectious tracheobronchitis caused by *B bronchiseptica* acting alone or in concert with viruses such as canine parainfluenza virus and canine adenovirus-2 has a worldwide distribution and occurs commonly when dogs are housed in groups such as in kennels and veterinary hospitals.^{18,19} This prompted the development of single and combination parenterally and intranasally administered vaccines to control *B bronchiseptica* infections.²⁰⁻²⁵ In part, because of variability in experimental models, there has been controversy regarding which vaccine-stimulated immune responses confer protection from *B bronchiseptica* infection; however, it has generally been thought that local immunity is essential for protection, and parenteral vaccination is less effective.^{15,18,19} Nevertheless, it has been proposed that protocols incorporating intranasally and parenterally administered vaccines are prophylactically superior to administering either vaccine alone¹⁸; however, to date, there have been no data to support or refute this concept.

The objectives of the study reported here were to determine comparative efficacy of parenterally (IM) and intranasally administered vaccines, used alone or sequentially, to protect puppies from infection with a recently isolated virulent strain of *B bronchiseptica* and determine whether systemic or mucosal antibody response correlates with protection.

Materials and Methods

Dogs—Specific-pathogen-free Beagles were obtained from a commercial breeder.⁹ Puppies were maintained in isolated facilities beginning at 3 weeks of age and weaned at 6 weeks of age. At this time, they had negative test results for *B bronchiseptica* by use of bacteriologic culture of deep nasal and pharyngeal swab specimens and had agglutination titers of < 1:16, which indicated lack of exposure to *B bronchiseptica* and low or no maternal antibodies. Puppies were vaccinated at 6, 9, and 12 weeks of age with a commercial canine

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vaccine^b against canine distemper virus, parvovirus, adenovirus-2, and parainfluenza virus. Prior to inclusion in the study, puppies were again determined to be seronegative for antibodies against *B bronchiseptica* and had negative bacteriologic culture results for *B bronchiseptica*, determined by use of nasal and pharyngeal swab specimens.

Experimental design—Upon arrival at the experimental site at 7 weeks of age, 50 healthy puppies were randomly assigned to 1 of 5 groups, with 2 replicates of 5 puppies for each group. Individual identification of puppies by ear tattoo and appropriate treatment groups were recorded. All treatment groups were housed separately inside biosecurity level-2 containment rooms to prevent cross contamination. *Bordetella bronchiseptica* vaccines were administered IM^c or intranasally,^d according to label instructions; however, each treatment group had a unique vaccination protocol that assured the last dose of vaccine and the challenge were administered at the same age for each group (18 and 20 weeks, respectively). Administration of the first *B bronchiseptica* vaccine no earlier than 14 weeks of age allowed the completion of the vaccination series with canine distemper virus, parvovirus, adenovirus-2, and parainfluenza virus, which minimized chances of any vaccine-mediated interference with the induction of immune responses. In addition, administration of the last dose and challenge at the same age allowed for equal comparisons among groups (Appendix 1).

Any adverse clinical signs that developed after vaccinations were recorded. At 20 weeks of age, 2 weeks after the final vaccination of all treatment groups, all puppies, including unvaccinated controls, were challenge exposed with a total dose of approximately 10¹⁰ bacteria.

Each puppy's overall condition and respiratory tract signs were recorded twice daily for 10 days following challenge by a single blinded investigator during a 20-minute observation period. Physical examination included determination of rectal temperature, respiratory rate, thoracic auscultation, and observations that assessed the presence of spontaneous coughing and retching, nasal discharge, pharyngeal erythema, signs of depression, and anorexia. Clinical scores were assigned, according to predetermined criteria (Appendix 2).

Serum for determination of IgG concentration and samples of saliva for determination of IgG and IgA concentrations were collected from all dogs prior to vaccination on days 0, 14, and 28, prior to challenge on day 42, and 10 days following challenge. All samples were frozen at -70 C. The number of bacteria cultured from nasal and pharyngeal swab specimens and lung lavage fluids obtained at necropsy were determined by use of plate counting methods. *Bordetella bronchiseptica*-specific IgG (serum and saliva) and IgA (saliva) concentrations were determined by use of ELISA and agglutination (serum) analysis of specimens obtained at the end of replicate 1 and at the termination of the study (by use of combined samples from both replicates); all samples were analyzed during a single set of assay procedures. Blood was collected at day 42 prior to challenge and 3, 5, and 10 days following challenge for determination of CBC.

Ten days after challenge, all puppies in the first replicate were humanely sacrificed by administration of an overdose of barbiturate. Complete gross necropsies were performed, and detailed histologic and immunohistochemical examinations of the respiratory tract were performed.

At necropsy, nasal and pharyngeal swab specimens were taken, and the right cranial lung lobe was lavaged with 25 ml of saline (0.9% NaCl) solution. Lung lavage fluid was clarified by use of centrifugation. Persons who conducted various testing procedures, including histopathologic and immunohistochemical evaluations, were blinded as to treatment group.

***Bordetella bronchiseptica* inoculum and experimental infection**—The Regina-1 isolate of *B bronchiseptica* was obtained from the lungs of a puppy that died with severe suppurative pneumonia in 1995. Tissues from this puppy yielded negative results for canine distemper virus on the basis of immunohistochemical staining. Following positive identification²⁶ as *B bronchiseptica*, typical phase-I (virulent morphotype) colonies²⁷ were harvested with a bacterial loop and stored in 5% inositol in horse serum at -70 C. Third passage was used for experimental infections. For inoculation, a confluent log phase (24 hours) colony was scraped off each of 4 **Bordet Gengou (BG)** agar^c plates and suspended in 100 ml of sterile saline solution. Puppies were sedated via IV administration of diazepam^f (0.5 mg/kg [0.23 mg/lb] of body weight) and ketamine^g (10 mg/kg [4.5 mg/lb]) and administered approximately 3 ml of aerosolized inoculum that was delivered during a 10-minute period by use of an individual mask connected to a nebulizer.^h The inoculum was back-titered by making 10-fold dilutions, streaking BG plates, and determining growth at 24 and 48 hours.

Pathologic and immunohistochemical analyses—Lungs were removed in toto and photographed from the dorsal view. Any areas of gross pulmonary lesions were measured by use of a computerized digitizer.ⁱ Standard blocks of tissue were collected from the nasal turbinates (midsagittal section of the nose), trachea (immediately distal to the larynx and cranial to the bifurcation), and all lung lobes, fixed in neutral-buffered 10% formalin, and processed routinely for histology. Inflammatory changes were scored 0 to 4+. Scoring was based on the relative assessment of the following variables: amount of intraluminal exudate (mucus and inflammatory cells), amount of hyperplasia in mucosal epithelium, amount of transepithelial migration of inflammatory cells, and amount of infiltration in the lamina propria by inflammatory cells. Immunohistochemical identification of *B bronchiseptica* in tissues was performed by use of described techniques.²⁸ Briefly, sections cut from blocks of formalin-fixed paraffin-embedded tissue were reacted with either diluted rabbit anti-*B bronchiseptica* antiserum^j or normal rabbit serum (negative control). Following reaction with the primary antibody, tissues were incubated with rabbit IgG antisera before viewing the reaction product by use of an avidin-biotin complex technique, as described.²⁸ A positive control tissue from a dog with naturally acquired *B bronchiseptica* was also stained. Amounts of *B bronchiseptica* viewed in situ were scored 0 to 4+.

Quantitation of *B bronchiseptica*-reactive antibodies—An agglutination test for antibodies that reacted with *B bronchiseptica* was performed, as described.²⁹ Briefly, formalin-fixed *B bronchiseptica* and doubling dilutions of canine sera were incubated in V-bottom wells for 24 hours at 20 C, and wells were visually assessed for agglutination (failure to form a distinct button of antigen at the bottom of the well). Controls included serum obtained from an unvaccinated dog (negative control) and immune serum from a dog vaccinated with *B bronchiseptica* (positive control). The ELISA for IgG and IgA antibodies that were reactive with *B bronchiseptica* were performed, as described,³⁰ with minor modifications. Briefly, 96-well flat-bottomed microtiter plates^k were coated overnight at 20 C with washed sonicated *B bronchiseptica* in carbonate coating buffer (7.5 µg/well). The bacterial antigen was prepared from confluent 24-hour cultures of the Regina-1 isolate, as for the challenge inoculum, except that the growth from 5 BG plates was pooled and resuspended in 20 ml of saline solution prior to dividing into aliquots and freezing at -70 C until used. The optimal dilution of antigen was determined in a standard checkerboard design, using immune canine serum. Plates were washed by immersion in phosphate-buffered saline solution that contained 0.05%

Tween 20 and incubated in blocking buffer after a 1:50 dilution of canine serum was added to each replicate well. Following incubation for 1 hour, peroxidase-conjugated goat anti-canine IgG or IgA¹ diluted in blocking buffer was added. Plates were again incubated for 1 hour prior to washing and addition of a substrate (2,2N-azino-di-[3-ethyl-benzthiazole-6-sulfonate]), according to the manufacturer's instructions.^m Controls included serum and saliva obtained from unvaccinated dogs (negative control) and immune sera from dogs vaccinated with *B bronchiseptica* (positive controls) as well as blanks (wells that contained washing buffer only). Saliva was collected on cotton-tipped swabs, as described.³¹ **Optical density (OD)** values were converted by use of a computerized programⁿ to ELISA units (percentage of positive control) by use of the following formulas, using a modification of described methods³²:

- 1) net OD = mean OD of controls or test samples – mean OD blank
- 2) $\frac{\text{net OD sample} - \text{net OD negative control}}{\text{net OD positive control} - \text{net OD negative control}} \times 100 = \text{ELISA units}$

Bacteriologic culture—To quantitate growth of *B bronchiseptica* in various organs, selective culture methods²⁶ and standard semiquantitative techniques were used.³³ Briefly, swab specimens from the nasal cavity, pharynx, and lung (first replicate only) were inoculated onto 1 quadrant of MacConkey and peptone agar plates. A sterile bacteriologic loop was then used to sequentially streak the 4 quadrants of each plate without flaming the loop until all quadrants were streaked. Plates were incubated at 37 C and checked at 24 and 48 hours for growth of microorganisms. Suspicious colonies (ie, organisms that grew as nonlactose fermenters on MacConkey agar and as blue colonies on peptone agar) were quantitated as follows: no growth, 0; growth on the first quadrant, 1+; growth in first and second quadrants, 2+; growth in first 3 quadrants, 3+; growth in all quadrants, 4+. Data derived from peptone agar (plate counts) were analyzed statistically. To confirm that quantitated colonies were *B bronchiseptica*, typical colonies were subcultured on blood agar and tested by use of conventional methods of identification. Tests on identification strips^o used for gram-negative organisms as positive confirmation of *B bronchiseptica* were TSI (alkaline), urease (+), citrate (+), and arginine (–).

Statistical analyses—The study was a randomized complete-block (replication) design with room of dogs as the experimental unit; individual puppies were subsampled.³⁴ Data were analyzed by use of a computerized statistical program.^{34p} All continuous response variables were analyzed by use of a general linear mixed model or a general linear repeated-measures mixed model, and all subjective (categorical) variables were analyzed by use of the Cochran-Mantel-Haenszel statistic. For the response variables (percentage of days that puppies coughed, percentage of days with signs of depression), a general linear mixed model was used, as follows:

$$Y_{ijk} = \mu + \tau_i + \beta_j + \alpha_{ij} + \varepsilon_{ijk}$$

where Y_{ijk} is the response variable, μ is the overall constant, τ_i is the random effect of the i th block (replication), β_j is the fixed effect of the j th treatment, α_{ij} is the random error used to test treatment, and ε_{ijk} is the random residual effect.

Percentage values of these variables were transformed to the arc sine before analysis, and the treatment least squares means were back transformed to percentages after analysis. If treatment effects were significant ($P \leq 0.05$), 1 *df* contrast among treatment means were made. For the response variables (temperature, respiratory rate, WBC count, subsets of WBC, ELISA units of IgG in serum, ELISA units of IgA in saliva, ELISA units of IgG in saliva, agglutination titers), a

general linear repeated-measures mixed model was used, as follows:

$$Y_{ijkl} = \mu + \tau_i + \beta_j + \alpha_{ij} + \delta_k + \beta\delta_{jk} + \varepsilon_{ijkl}$$

where Y_{ijkl} is the response variable, μ is the overall constant, τ_i is the random effect of the i th block (replication), β_j is the fixed effect of the j th treatment, α_{ij} is the random error used to test treatment, δ_k is the fixed effect of the k th day of study, $\beta\delta_{jk}$ is the fixed effect of treatment by day of study interaction, and ε_{ijkl} is the random residual effect.

Total WBC and differential cell counts and ELISA titers (units) were transformed to the natural log and the agglutination titers were transformed to the log base 2 before analysis. The treatment least squares means were back transformed after analysis, and 1 *df* contrast at each day of study among treatments was made. These transformations change data in such a way that the assumptions necessary for an ANOVA procedure are more nearly met. The categorical response variables, bacterial counts (0 to 4+) on plates inoculated with swab specimens from the nose and pharynx, were analyzed by use of the Cochran-Mantel-Haenszel statistic, which is a χ^2 test that adjusts for blocking. If the treatment effects were significant ($P < 0.05$), pairwise treatment differences were made by use of the Cochran-Mantel-Haenszel statistic. Pearson correlation coefficients among concentrations of serum IgG, salivary IgA, and salivary IgG and recovery of *B bronchiseptica* from the nose and pharynx (bacterial plate counts) were calculated. A probability value of $P \leq 0.05$ was considered significant in all tests.

Results

Clinical signs—No adverse effects were detected following administration of either of the vaccines to any puppies in either replicate.

Replicate 1—Coughing was observed in unvaccinated control puppies beginning approximately 36 hours after aerosol infection with *B bronchiseptica*. This coughing continued in control puppies until the end of the observation period on day 10 after infection. Coughing in these puppies was often paroxysmal and accompanied by retching. In contrast, substantially less coughing was observed in puppies that received either vaccine alone or in sequence. Coughing was not observed in several puppies that received both vaccines. In addition to coughing, all unvaccinated puppies had pyrexia, anorexia, and signs of depression at 1 or more observation times on days 2 to 4 after infection.

Replicate 2—Clinical signs were similar in the groups of puppies in replicate 2, with uniform coughing in control puppies from days 2 to 10 after infection and reduced or no coughing in vaccinated puppies. However, retrospective serologic analyses conducted after the termination of the study indicated that 9 puppies (1 puppy in group 2, 5 puppies in group 3, 2 puppies in group 4, and 1 puppy in group 5) had serum antibodies that reacted with *B bronchiseptica* antigens in the ELISA and agglutination tests after arrival but before vaccination. Low numbers of nonpathogenic *Pseudomonas* spp had been cultured from the nasal cavity, pharynx, or both of 7 puppies in treatment groups 1 (2 puppies), 2 (1), and 3 (4) approximately 1 week after arrival at the site. However, there was no apparent relationship between presence of *Pseudomonas* spp and development of antibodies to *B bronchiseptica* prior to

vaccination; some puppies that were seropositive had *Pseudomonas* spp, and others did not. None of these puppies had clinical signs of disease before challenge. Seropositive puppies in replicate 2 were excluded, and data from replicates 1 and 2 were pooled and analyzed, as described.

Analysis of pooled data from all puppies in replicates 1 and seronegative (prior to vaccination) puppies in replicate 2 revealed significantly less coughing in all vaccinated groups on days 4 to 10, compared with the unvaccinated control group (Fig 1). In addition, there was significantly less coughing in treatment groups 4 and 5, which received both vaccines in sequence, compared with group 3, which received intranasal vaccine alone. Unvaccinated control puppies had signs of depression for a significantly greater number of days and had significantly higher temperatures on days 2 to 4 than any of the vaccinated puppies; differences among vaccinated groups for signs of depression were not detected (Table 1, Fig 2).

Pathologic and immunohistochemical findings—

Gross pathologic changes were not detected in the nasal cavity, trachea, or lungs of any control or vaccinated puppy that was examined (replicate-1 puppies). However, microscopic examination of tissues revealed inflammatory lesions of moderate severity in the nasal cavity, trachea, and pulmonary airways of unvaccinat-

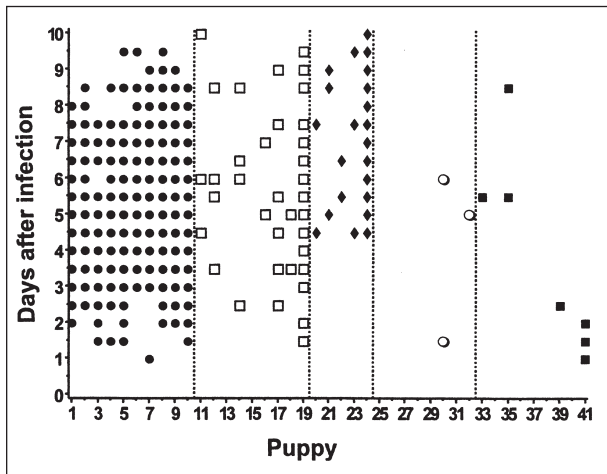


Figure 1—Scatter plot of coughing in puppies after infection with *Bordetella bronchiseptica*. Each symbol represents an observed incident of coughing in an individual puppy. Puppies were unvaccinated controls (solid circles) or were vaccinated via IM (open squares), intranasal (diamonds), IM-intranasal (open circles) or intranasal-IM (solid squares) administration.

Table 1—Mean percentage of days 4 to 10 after challenge exposure with *Bordetella bronchiseptica* in which puppies coughed and had signs of depression

Group	Cough	Signs of depression
1 (Control)	77.7 ^a	23.9 ^a
2	20.9 ^{b,c}	2.8 ^b
3	37.0 ^b	2.4 ^a
4	3.3 ^c	2.8 ^b
5	3.4 ^c	2.8 ^b

^{a-c}Within a column, mean values with different superscripts are significantly ($P \leq 0.05$) different.

See Appendix 1 for description of groups and vaccination schedule.

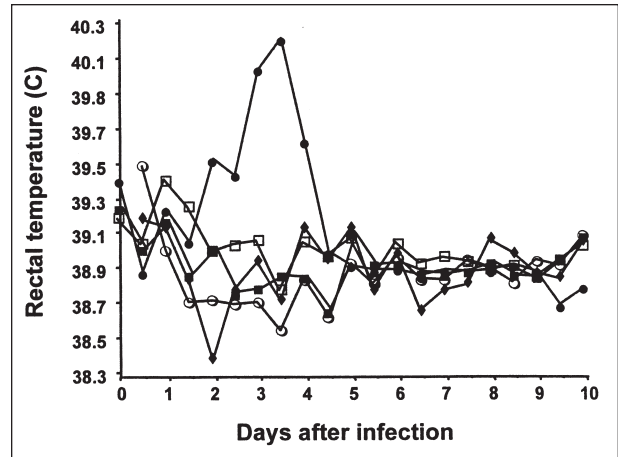


Figure 2—Rectal temperatures (least square group means) in puppies after infection with *B bronchiseptica*. See Figure 1 for key.

ed puppies, and the lowest group mean overall lesion scores were in puppies that received both vaccines sequentially; however, ANOVA could not be performed on data derived from a single replicate. Changes in the airways of upper and lower portions of the respiratory tract of control puppies included hyperplasia of luminal epithelium, transepithelial migration of neutrophils, and a mixed inflammatory cell infiltrate with a predominance of neutrophils in the lamina propria of affected airways. Some inflamed airways had increased amounts of mucus adhered to lining epithelial cells. In lungs of affected control puppies, inflammation was almost exclusively limited to bronchi and large- and medium-sized bronchioles, with minimal to no involvement of small bronchioles and pulmonary parenchyma. Immunohistochemical staining revealed that inflamed airways were colonized by *B bronchiseptica* that were seen lining the luminal surface of epithelium, adhered to cilia, and entrapped in mucus (Fig 3 and 4). In contrast, no or minimal histologic changes and no or minimal bacterial growth were detected in the upper and lower portions of the respiratory tract of puppies that received either vaccine alone or in sequence (Fig 5 and 6).

Clinical pathologic analyses—Control puppies in replicate 1 had relative and absolute leukocytosis, neutrophilia, and left shift on days 5 and 10 after infection. Similarly, analyses of pooled data revealed significantly higher total WBC count in control puppies on days 5 (except for puppies vaccinated by use of parenteral administration alone) and 10 after challenge, compared with all vaccine treatment groups (Table 2). Control puppies also had significantly higher numbers of circulating segmented neutrophils and monocytes on day 10 after challenge, compared with vaccinated puppies, and significantly higher numbers of band neutrophils on days 3 and 5 after infection.

Antibodies against *B bronchiseptica*—Prior to inclusion in the study, all puppies had agglutination titers against *B bronchiseptica* that were considered negative ($< 1:16$). In replicate 1, after the first dose of the IM or intranasally administered vaccine, there was an



Figure 3—Photomicrograph of a section of trachea from an unvaccinated control puppy that was euthanized 10 days after challenge exposure with *B bronchiseptica*. Notice numerous brown-stained bacterial colonies on the epithelium. On a scale of 0 to 4, inflammation in this specimen was graded 3+, and bacterial growth in culture medium was graded 4+. Immunohistochemical stain; bar = 100 μ m.

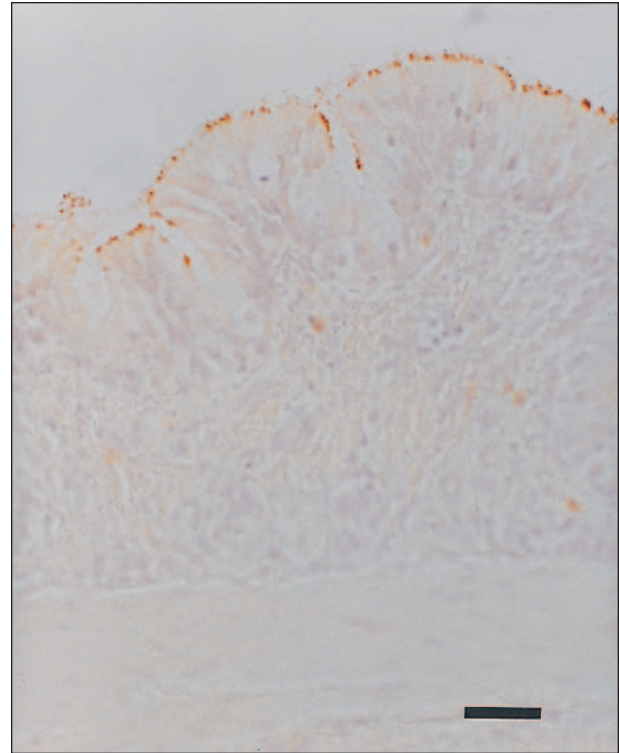


Figure 4—Photomicrograph of a section of bronchiole from an unvaccinated control puppy that was euthanized 10 days after challenge exposure with *B bronchiseptica*. Notice numerous brown-stained bacterial colonies on the epithelium, which is hyperplastic and has multifocal infiltration by inflammatory cells. On a scale of 0 to 4, inflammation in this specimen was graded 4+, and bacterial growth in culture medium was graded 4+. Immunohistochemical stain; bar = 100 μ m.

Table 2—Geometric group mean values for total WBC counts before and after challenge exposure with *B bronchiseptica* in puppies

Group	WBC count ($\times 10^3/\mu$ l)			
	Day 0 (challenge)	Day 3	Day 5	Day 10
1 (Control)	11.4 ^{a,c}	9.4 ^a	17.9 ^a	21.1 ^a
2	8.9 ^b	13.8 ^b	15.2 ^a	15.1 ^c
3	13.3 ^c	11.5 ^{a,b}	11.5 ^b	11.7 ^b
4	11.0 ^{a,c}	12.9 ^b	11.1 ^b	12.1 ^b
5	9.6 ^{a,b}	10.6 ^a	10.0 ^b	11.4 ^b

^{a-c}Within a column, mean values with different superscripts are significantly ($P \leq 0.05$) different.
See Appendix 1 for description of vaccination schedule.

increase in serum IgG antibodies against *B bronchiseptica* within 2 weeks after administration. Serum antibody concentrations were increased upon subsequent IM or intranasal administration of vaccine and after challenge. Salivary IgG and IgA antibodies that were reactive with *B bronchiseptica* were low prior to vaccination and increased after vaccination; however, differences among vaccinated groups were small. After infection, all dogs had substantially increased salivary IgG and IgA antibodies against *B bronchiseptica*, and differences were not apparent among groups. There were no apparent differences in pulmonary IgG and IgA concentrations among the treatment groups.

Analyses of pooled data revealed significantly higher concentrations of serum antibodies against *B bronchiseptica* in all vaccinated groups on the day of challenge, compared with control puppies (Table 3

and 4). Agglutination and serum IgG titers against *B bronchiseptica* increased within 2 weeks after the first vaccination in each of the vaccinated groups. Moreover, serum antibody concentrations increased after subsequent IM or intranasal administration of vaccine and after challenge. Titers of serum antibodies against *B bronchiseptica* in treatment group 5 increased sooner than did titers in treatment group 4; however, treatment group 4 maintained higher antibody titers through day 10 after infection than did treatment group 5. At the time of the second and third vaccinations (days 14 and 28, respectively), treatment group 5 had significantly higher titers than did treatment group 4. Furthermore, significantly higher agglutination titers were detected in puppies that received IM administration of vaccine either alone (treatment group 2) or in sequence with intranasal administration (treatment groups 4 and 5), compared with puppies that received intranasal administration alone (treatment group 3). In addition, these differences persisted on days 3 and 10 after challenge.

At the time of challenge, there were also significantly higher concentrations of salivary IgG against *B bronchiseptica* in all groups that had received the intranasally administered vaccine alone or in sequence with parentally administered vaccine. Significantly higher concentrations of salivary IgA against *B bronchiseptica* were observed in puppies that received IM and intranasally administered vaccines in sequence. On day 10, after

Table 3—Geometric group mean values for reciprocal of agglutination titers against *B bronchiseptica* before and after challenge exposure in puppies

Group	Day -28	Day -14	Day 0 (challenge)	Day 3	Day 10
1	4.3 ^a	4.0 ^a	4.0 ^a	4.2 ^a	207.9 ^a
2	4.0 ^a	94.6 ^b	3,270.2 ^c	2,849.7 ^c	5,607.1 ^c
3	4.2 ^a	4.2 ^a	227.0 ^b	539.9 ^b	2,159.7 ^b
4	22.9 ^b	617.0 ^c	6,980.7 ^c	5,699.4 ^{c,d}	11,740.0 ^c
5	119.2 ^c	2,060.1 ^d	5,191.2 ^c	13,093.9 ^d	8,900.2 ^c

^{a-c}Within a column, mean values with different superscripts are significantly ($P \leq 0.05$) different. See Appendix 1 for description of vaccination schedule.

Table 4—Geometric group mean values for serum IgG titers (ELISA units) against *B bronchiseptica* before and after challenge exposure with *B bronchiseptica* in puppies

Group	Day -42	Day -28	Day -14	Day 0 (challenge)	Day 3	Day 10
1 (Control)	23.5 ^{a,c}	25.5 ^{a,c}	24.6 ^a	29.7 ^a	41.8 ^a	120.7 ^a
2	18.2 ^a	22.2 ^{a,b}	56.1 ^b	103.4 ^b	124.9 ^b	173.1 ^a
3	8.8 ^b	11.9 ^b	16.8 ^a	76.3 ^b	102.7 ^b	192.3 ^a
4	18.1 ^a	43.6 ^{c,d}	67.2 ^{b,c}	111.6 ^b	133.0 ^b	154.3 ^a
5	36.1 ^c	63.6 ^d	106.6 ^c	140.1 ^b	159.2 ^b	188.6 ^a

^{a-c}Within a column, mean values with different superscripts are significantly ($P \leq 0.05$) different. See Appendix 1 for description of vaccination schedule.

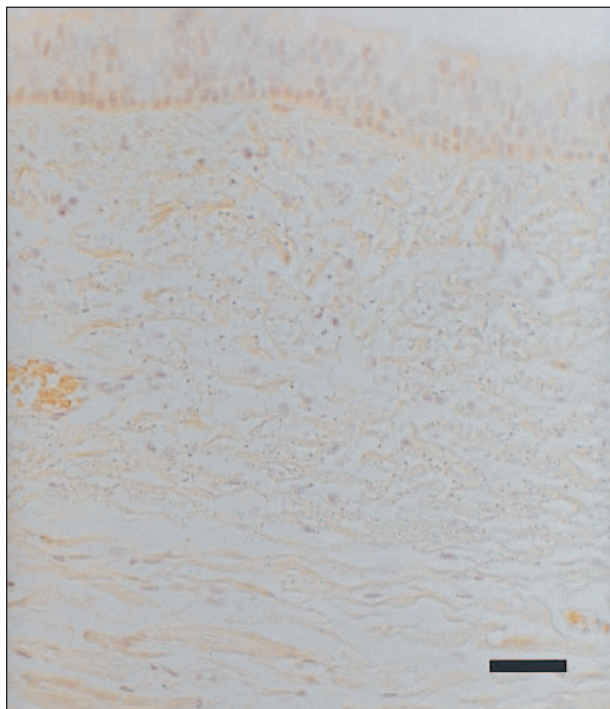


Figure 5—Photomicrograph of a section of trachea from a puppy that was vaccinated via intranasal administration of modified-live *B bronchiseptica* and IM administration of inactivated *B bronchiseptica* and was euthanized 10 days after challenge exposure with *B bronchiseptica*. Notice absence of bacterial colonies on the epithelium. On a scale of 0 to 4, inflammation in this specimen was graded 0, and bacterial growth in culture medium was graded 0. Immunohistochemical stain; bar = 100 μ m.

infection, all vaccinated groups had significantly higher concentrations of *B bronchiseptica*-reactive salivary IgA.

Because many vaccinated puppies had no coughing after challenge inoculation, it was not possible to statistically correlate a specific concentration of IgG or IgA antibodies with the amount of coughing observed; however, the unvaccinated control puppies with low or no serum IgG against *B bronchiseptica* at the time of

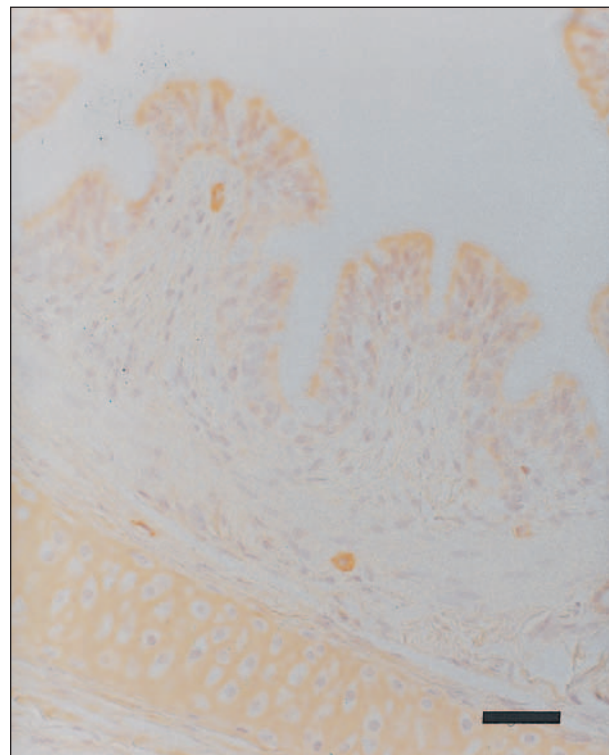


Figure 6—Photomicrograph of a section of bronchiole from a puppy that was vaccinated via intranasal administration of modified live *Bordetella bronchiseptica* and IM administration of inactivated *B bronchiseptica* and was euthanized 10 days after challenge exposure with *B bronchiseptica*. Notice absence of bacterial colonies on the epithelium. On a scale of 0 to 4, inflammation in this specimen was graded 0, and bacterial growth in culture medium was graded 0. Immunohistochemical stain; bar = 100 μ m.

challenge consistently coughed for up to 10 days after challenge (Fig 1).

Bacteriologic findings—Clinical signs and pathologic changes were generally associated with colonization of *B bronchiseptica* in the infected puppies in replicate 1. Moderate to large numbers of *B bronchiseptica*

Table 5—Correlation coefficients for recovery of *B bronchiseptica* from nasal and pharyngeal swab specimens and serum IgG, salivary IgA, or salivary IgG concentrations after challenge exposure of 31 vaccinated puppies

Immunoglobulin	Nasal	Pharyngeal
Serum IgG	−0.51 ($P = 0.004$)	−0.42 ($P = 0.018$)
Salivary IgA	−0.35 ($P = 0.054$)	−0.47 ($P = 0.007$)
Salivary IgG	−0.40 ($P = 0.026$)	−0.34 ($P = 0.062$)

were cultured from the nasal cavity, pharynx, and lungs of all (5/5) unvaccinated control puppies as well as from all 10 puppies that received either vaccine alone. In contrast, there was substantially less or no detectable growth of *B bronchiseptica* (6/10) in specimens obtained from the upper and lower portions of the respiratory tract of puppies that received both vaccines in sequence. Results of bacteriologic cultures were comparable to results of visual assessment of bacteria in immunohistochemically stained sections; however, culture was apparently more sensitive in detecting small numbers of bacteria, although these observations could not be tested statistically. Analyses of pooled data revealed that puppies that had received vaccines administered intranasally and IM in sequence had significantly fewer *B bronchiseptica* cultured from nasal and pharyngeal swab specimens on day 10 after infection than did control puppies and puppies that received either vaccine alone. Significant negative correlations existed between *B bronchiseptica*-reactive serum IgG and growth on peptone agar of *B bronchiseptica* recovered from pharyngeal and nasal swab specimens. In addition, there was a significant negative correlation between *B bronchiseptica*-reactive salivary IgG and growth of *B bronchiseptica* from nasal swab specimens and a significant negative correlation between *B bronchiseptica*-reactive salivary IgA and growth of *B bronchiseptica* recovered from pharyngeal swab specimens (Table 5).

Discussion

Results of the study reported here confirm and extend those of previous investigations that have generally documented the efficacy of *B bronchiseptica* vaccines in dogs.²⁰⁻²⁵ Intranasal or IM administration of vaccines resulted in substantially less coughing (the cardinal clinical sign of *B bronchiseptica* infection) in vaccinated puppies, compared with control puppies. Moreover, there was no paroxysmal coughing in vaccinated puppies, and several vaccinated puppies were not observed to cough at any time during any observation period. In addition, vaccinated puppies were generally spared of pyrexia, anorexia, and signs of depression, which developed in unvaccinated puppies. These clinical findings were associated with less bacterial growth in the upper and lower portions of the respiratory tract as well as fewer inflammatory changes associated with bacterial colonization of the mucosa of the respiratory tract.

In studies such as this one, ideally, animals from all experimental groups should be mixed to avoid a “pen effect.” However, because it is postulated that intranasally administered vaccines that contain modified-live *B bronchiseptica* may potentially result in bac-

terial shedding from the nose of vaccinated dogs for a variable period, mixing of puppies in different treatment groups was precluded, because all commingled puppies may have become exposed and immunized by bacteria of vaccine origin. To avoid this possibility and have sufficient *df* to conduct ANOVA, this study was designed to be conducted in 2 replicates, with 5 puppies/treatment group in each replicate.

Laboratory and clinical evidence of infection with *B bronchiseptica* was lacking prior to challenge of dogs in the second replicate; however, there was evidence of antibodies reactive with *B bronchiseptica* in some of the test puppies before vaccination, confounding the analyses. Therefore, descriptive statistical analyses were performed on the first replicate separately, and data from puppies in replicate 2 that remained seronegative until after vaccination were pooled with data from replicate 1 and analyzed as originally intended. We could not completely exclude the possibility of a pen effect in the first replicate; however, no evidence of adaptive immunity (antibodies against *B bronchiseptica*) was detected prior to vaccination, and unvaccinated control puppies remained seronegative until after challenge. Similarly, there was no evidence that contact with seropositive puppies in replicate 2 resulted in adaptive immunity in commingled pen mates prior to vaccination. Therefore, we feel that clinical observations and laboratory findings, which indicated the efficacy of the vaccines, were valid.

Clinical signs other than coughing have not been reported in experimental models of *B bronchiseptica* infection in dogs,^{18,19} although other clinical signs, including pyrexia, anorexia, and signs of depression have been reported in outbreaks of *B bronchiseptica* in kennels.¹⁵ At least 2 factors may have accounted for the apparently more severe disease observed in our experiment: dose of inoculated bacteria and virulence of the infecting *B bronchiseptica* isolate. There has been considerable variability in reported experimental *B bronchiseptica* infections.^{12,20-25} Although it is difficult to quantitate the total dose of bacteria that is delivered to the respiratory tract when using nebulization as a delivery method, we administered a high dose in an attempt to increase the stringency of the efficacy test in order to increase the chances of observing differences between treatment groups. This high dose of bacteria may have accounted for the severity of disease we observed. There has been controversy over differences in virulence among *B bronchiseptica* isolates and the effect of prolonged culture on virulence of the organism in vivo.¹⁹ We used a low-passage inoculum of an isolate obtained from a case of fatal suppurative pneumonia in an attempt to minimize any attenuation of virulence that may develop with prolonged culture and passage.¹² Although we observed more severe clinical disease than has been reported elsewhere and although these results support the concept that low-passage bacteria are more virulent in vivo, the bacterial factors that relate to virulence in vivo have yet to be identified.

It has been proposed that the immunologic pressure exerted by routine vaccination has promoted genetic diversity in *B bronchiseptica*.¹⁷ Moreover, it has been suggested that the use of current *B bronchiseptica*

vaccines has resulted in the selection of vaccine-resistant isolates within the canine population during the period since the 1970s.¹⁷ Although genetic analysis of the Regina-1 isolate was not performed, our study revealed substantial sparing of clinical disease and bacterial colonization as well as fewer inflammatory lesions in the respiratory tract in vaccinated puppies that were infected with a high dose of a recently isolated field strain; these findings do not support the hypothesis that vaccine-driven bacterial evolution has resulted in the emergence of “escape mutants” that are no longer susceptible to immune responses stimulated by older genetically different low-virulence isolates that are used in current *B bronchiseptica* vaccines.

There has been considerable controversy concerning which host defense mechanisms protect dogs from clinical disease associated with *B bronchiseptica* infection.^{18,19} Early studies did not find a relationship between serum antibodies that react with *B bronchiseptica* and protection from coughing.¹⁴ In contrast, the results of some subsequent studies have supported the concept that systemic antibodies stimulated by parenteral administration of vaccines (or natural infection) substantially decrease signs of disease in dogs that have been exposed to *B bronchiseptica*.^{18,19} Results of our study support the belief that serum antibodies that react with *B bronchiseptica* may protect dogs from clinical disease. However, because there was often no coughing at all in vaccinated dogs, it was not possible to formally address the relationship between serum or nasal antibodies and protection by use of regression analyses. Nevertheless, the fact that vaccinated puppies that had high concentrations of serum IgG or agglutinating antibodies had little or no coughing after challenge suggests that a systemic antibody response to the bacteria may be a reasonable indicator of vaccine efficacy. Although serum and salivary antibody concentrations could not be correlated with coughing because of the discrete nature of the cough data, the significant negative correlations between antibody concentrations and bacterial growth in the nose and pharynx were indicative of the protective effect of the vaccine-induced antibody responses. Similarly, the presence of vaccine-induced serum antibody is a good correlate of immunity against colonization of the respiratory tract by *B pertussis* in children.³⁵

It has been suggested that protection associated with intranasally administered vaccines results from competition between modified-live vaccinal strains of *B bronchiseptica* that colonize the nasal cavity after vaccine administration and naturally acquired or experimentally inoculated field isolates of the bacteria.¹⁸ The fact that no *B bronchiseptica* were cultured from swab specimens or detected immunohistochemically in tissue sections at the time of euthanasia of most dogs that received intranasally administered vaccine alone or in combination with parenterally administered vaccine suggests that substantial colonization by vaccine-derived *B bronchiseptica* and resulting competition with virulent *B bronchiseptica* are not responsible for the efficacy of intranasally administered vaccines.

^aMarshall Farms, North Rose, NY.

^bVanguard puppy 5, Pfizer Animal Health, Exton, Pa.

^cCoughGuard B, Pfizer Animal Health, Exton, Pa.

^dNasaGuard-B, Pfizer Animal Health, Exton, Pa.

^eDifco Laboratories, Detroit, Mich.

^fDiazepam, Sabex Inc, Boucherville, QC, Canada.

^gVetalar, Vetrepharm Canada Inc, London, ON, Canada.

^hUltra-Neb 99, Devilbiss, Somerset, Pa.

ⁱNorthern Eclipse, Epics Imaging Inc, Mississauga, ON, Canada.

^jCourtesy of Register K, USDA, Ames, Iowa.

^kImmunlon-4, Bio Merieux, St Laurent, QC, Canada.

^lBethyl Laboratories Inc, Montgomery, Tex.

^mKirkegaard & Perry Laboratories Inc, Gaithersburg, Md.

ⁿMicroplate Manager, Bio-Rad Laboratories, Hercules, Calif.

^oAPI, Bio Merieux, St Laurent, QC, Canada.

^pPROC GLM, SAS Institute Inc, Cary, NC.

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Appendix 1

Schedule for intranasal and IM administration of *Bordetella bronchiseptica* vaccines and challenge exposure with virulent *B bronchiseptica* in puppies (n = 10/group)

Group	Puppy age (wk)			
	14	16	18	20
1 (Control)	—	—	Challenge	—
2	—	IM	IM	Challenge
3	—	Intranasal	Challenge	—
4	IM	IM	Intranasal	Challenge
5	Intranasal	IM	IM	Challenge

— = Not vaccinated or challenge exposed.

Appendix 2

Assignment of clinical scores in puppies that were challenge exposed by aerosol administration of *B bronchiseptica*

Variable	Finding	Score
Anorexia	Puppy eats food and drinks normally	0
	Puppy does not eat or drink	1
Signs of depression	Puppy bright, alert, and responsive to surroundings	0
	Puppy dull, listless, and nonresponsive to surroundings	1
Coughing or retching	No coughing or retching	0
	Coughing or retching	1
Auscultation	Normal lung sounds in all lung fields	0
	Abnormal lung sounds in 1 or more lung fields	1
Nasal discharge	No nasal discharge	0
	Nasal discharge	1
Fever	Rectal temperature ≤ 99.5 or ≥ 103.4 F* considered abnormal	Temperature to the nearest degree
Respiratory rate	Considered normal if ≤ 25 breaths/min	No. of breaths/15 s
Pharyngeal erythema	Not present	0
	Present	1

*To convert degrees F to C, subtract 32 and multiply by 5/9.