Virulence Factors of *Bordetella bronchiseptica* Associated with the Production of Infectious Atrophic Rhinitis and Pneumonia in Experimentally Infected Neonatal Swine

R. MARTIN ROOP II,^{1*} HUGO P. VEIT,¹ RICHARD J. SINSKY,^{1†} SANDRA P. VEIT,¹ ERIK L. HEWLETT,²
AND ERVIN T. KORNEGAY³

Department of Pathobiology,¹ Virginia-Maryland College of Veterinary Medicine, and Animal Science Department,³ Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, and Divisions of Clinical Pharmacology and Geographic Medicine, Department of Internal Medicine, University of Virginia School of Medicine, Charlottesville, Virginia 22908²

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Four strains of *Bordetella bronchiseptica* (CSU-P-1, 64-C-0406, 1120-A-83-013, and B205BT) with defined virulence for neonatal swine were examined, and an attempt was made to correlate the presence of certain in vitro phenotypic characteristics with the ability of a particular *B. bronchiseptica* strain to produce turbinate and lung lesions in piglets. All of the strains except CSU-P-1 colonized the nasal passages of the pigs heavily, and strains 1120-A-83-013 and B205BT produced moderate to severe nasal and lung lesions in experimentally infected piglets. All of the strains attached equally well to porcine tracheal ring explant cultures, and all of the strains except CSU-P-1 produced smooth, hemolytic colonies on Bordet-Gengou medium, agglutinated porcine erythrocytes, and possessed adenylate cyclase activity. Strains 1120-A-83-013 and B205BT produced considerably higher levels of dermonecrotic toxin activity than did strains CSU-P-1 and 64-C-0406. These results indicate that production of nasal and lung lesions in neonatal piglets by the *B. bronchiseptica* strains tested can be directly correlated with their level of dermonecrotic toxin production.

Infectious atrophic rhinitis (IAR) is a common swine respiratory disease which can result in the destruction of the nasal turbinates and associated tissues of affected animals. In addition, swine affected with IAR often have a higher incidence of pneumonia, possibly due to the reduced ability of the damaged turbinates to effectively filter the air going into the lungs or to subsequent pulmonary infection by the nasal pathogens (or both). IAR is considered one of the top five swine disease problems worldwide and results in significant annual economic losses for the swine industry as the result of reduced weight gain efficiency of severely affected animals and the costs of vaccines and antibiotics used for the prophylaxis and treatment of the disease (8). *Bordetella bronchiseptica* is the primary pathogen associated with IAR in young pigs (27), although it appears that the severe turbinate lesions sometimes seen in slaughter-age pigs may be the result of a mixed infection with *B. bronchiseptica* and toxigenic strains of *Pasteurella multocida* (21). *B. bronchiseptica* also causes a bronchopneumonia in swine, which can have a high mortality rate in young piglets (27).

There appears to be considerable variation among *B. bronchiseptica* strains in their ability to colonize and produce turbinate atrophy and pneumonic lesions in young pigs (4, 24). This situation has important implications in the treatment of commercial pig herds colonized by *B. bronchiseptica*. Avirulent strains may provide a form of natural protection against the introduction of a more virulent *B. bronchiseptica* strain into a herd, either by blocking available sites for colonization or by continuously stimulating localized immunity in infected animals. *B. bronchiseptica* strain variation with respect to virulence in pigs also must be

considered when selecting strains for use as bacterins or when formulating subcellular vaccines. Unfortunately, at present the only means of determining the virulence of a particular *B. bronchiseptica* strain for pigs is to infect neonatal swine experimentally. This is costly and time-consuming. The purpose of the study described in this report was to attempt to determine in vitro which phenotypic characteristics of *B. bronchiseptica* strains would be useful for predicting virulence in neonatal pigs.

MATERIALS AND METHODS

Bacterial strains and virulence in neonatal swine. The origin of the *B. bronchiseptica* strains used in this study along with results obtained from experimental infection of neonatal pigs with these strains are given in Table 1. Strain CSU-P-1 was obtained from M. Collins, College of Veterinary Medicine, Colorado State University, Fort Collins. Strain 64-C-0406 was obtained from R. F. Ross, College of Veterinary Medicine, Iowa State University, Ames. Strains 1120-A-83-013 and B205BT were obtained from J. Hargis, Animal Health Research Department, Pfizer, Inc., Terre Haute, Ind. The relative virulence of each of the *B. bronchiseptica* strains used in this study for neonatal swine was assessed in experimental infection studies. A complete description of these studies is given elsewhere (H. P. Veit, R. M. Roop II, D. W. Byrd, R. J. Sinsky, and E. T. Kornegay, submitted for publication). Briefly, neonatal swine obtained from sows showing low levels of *B. bronchiseptica* antibodies were exposed to aerosols of one (of the above four) *B. bronchiseptica* strain twice before they reached 10 days of age. Colonization of the nasal cavity was assessed by swabbing before aerosol exposure, at 48 h after each aerosol exposure, and at approximately 18 days of age. At 4 weeks of age, the animals were euthanatized and necropsy was performed. Nasal passages of the animals were swabbed just before

* Corresponding author.

† Present address: School of Public Health, University of Alabama at Birmingham, Birmingham, AL 35233.

TABLE 1. *B. bronchiseptica* strains used in this study and their virulence in neonatal swine^a

Strain	Origin	Colonization	Lesion production	
			Turbinate	Pneumonic
CSU-P-1	Porcine: nasal	—	—	—
64-C-0406	Canine: nasal	+	— ^b	— ^b
1120-A-83-013	Porcine: pneumonic	+	+	+
B205BT	Porcine: nasal	+	+	+

^a Colonization of the nasal cavity and lesion production was determined in experimental infections in neonatal swine. A brief description of these experimental infections and a reference for a more complete description is given in Materials and Methods.

^b Strain 64-C-0406 has previously been reported to be avirulent in pigs by Ross et al. (24).

euthanasia. Lung, tracheal, and (when warranted) other tissues were collected aseptically at necropsy for culture. Sectioned snouts were assessed by gross observation for turbinate atrophy, and a lesion score was assigned based on a modification of a standardized system used for scoring atrophic rhinitis lesions (26). Lungs were examined by gross observation, and pneumonic lesions were noted. Nasal turbinate, lung, and tracheal tissue were fixed in 10% phosphate-buffered Formalin, sectioned, stained with hematoxylin and eosin, and examined by light microscopy.

Colonial morphology and hemolysis on BG agar. *B. bronchiseptica* strains were grown for 48 h at 37°C on Bordet-Gengou (BG) medium supplemented with 15% defibrinated sheep (BGS) or horse (BGH) blood. Colonies were examined grossly and with a stereo dissecting microscope. Colonies were graded for smooth or rough colony texture, domed or umbonate elevation, and production of a zone of hemolysis. The scheme of Peppler and Schrupf (22) was used for describing colonial phenotypes.

Attachment to porcine tracheal rings. Piglets (1 to 4 weeks old), *B. bronchiseptica* negative by culture, were killed, and the tracheas were aseptically removed. Tracheas (3 to 6 cm long) were cut into 3-mm-thick rings with a sterile cutting device, and the rings were placed into six-well, flat-bottom tissue culture plates (Flow Laboratories, McLean, Va.) containing 3 ml of modified Eagle minimal essential medium (Flow Laboratories) supplemented with 2.22 g of NaHCO₃ per liter, 0.1 g of pyruvic acid per liter, 0.2 g of L-glutamine per liter, 4.76 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) per liter, 100,000 IU of penicillin per liter, 100 mg of streptomycin per liter, 2.5 mg of amphotericin B per liter, and 10% bovine fetal serum per well. The tracheal rings were incubated at 37°C under an atmosphere of 20% O₂ and 5% CO₂ and examined daily to ensure that ciliary beating was present. Culture medium was changed after 48 h. Tracheal rings (48 to 72 h old) were washed three times with phosphate-buffered saline (PBS; 0.21 M NaCl, 0.1 M NaH₂PO₄/Na₂HPO₄, pH 7.4) and transferred to modified Eagle minimal essential medium, supplemented as above but without the antibiotics, just before use in the assay.

Bacterial strains to be used in the attachment assay were grown in the following manner. Growth from a BGH plate was used to inoculate 5 ml of Stainer-Scholte broth (25) in a screw-cap tube (16 by 125 mm). This culture was incubated at 120 rpm in a shaker-water bath at 37°C overnight. The growth from this tube was used to inoculate a 250-ml Erlenmeyer flask containing 50 ml of Stainer-Scholte broth supplemented with 100 µCi of L-[2,3,4,5-³H]proline (ICN Radiochemicals, Irvine, Calif.) and incubated for 6 h at 37°C

(120 rpm). The bacterial cultures were harvested by centrifugation at 15,000 × *g* (0 to 4°C) for 10 min and washed once in sterile PBS. The cells were resuspended in sterile PBS to an optical density of 1.0 at 525 nm (1.7 × 10⁹ CFU/ml) for use in the attachment assay.

Immediately before the assay, the tracheal rings were cut aseptically into thirds, and seven pieces were placed into each polypropylene tube (17 by 100 mm). Four tubes were used for each time point per strain tested. Labeled bacterial suspension (1 ml) in PBS was added to each tube. Tubes were incubated with shaking (37°C, 120 rpm) for 2 h. Tubes containing 1 ml of each labeled bacterial suspension alone were also incubated in this manner for each strain tested. At 0, 1, and 2 h, tubes containing rings were removed and the rings were washed twice with sterile PBS. Rings were placed into scintillation vials, 5 ml of NCS Tissue Solubilizer (Amersham Corp., Arlington Heights, Ill.) was added to each vial, and the vials were incubated in a water bath at 50°C overnight. After 2 h of incubation, tubes containing labeled cells only were removed and 0.1- and 1.0-ml samples from these tubes were placed into scintillation vials and solubilized as just described. Scintillation fluid (Tritonol; 18 ml) (7) was then added to each vial, and the samples were counted on a liquid scintillation counter (model LS 8100; Beckman Instruments, Inc., Fullerton, Calif.). The counts obtained from the solubilized samples taken from the tubes which contained labeled bacteria only were used to calculate the radioactivity per milliliter of cell suspension added to the rings for each strain tested. The amount of nonspecific adherence, calculated from the tracheal rings that were removed and washed without incubation, was subtracted from the radioactivity present in the incubated and washed tracheal rings. This value was divided by the total amount of radioactivity added to each tube to determine the percent adherence for each strain tested.

Hemagglutination. Agglutination of porcine erythrocytes was tested by use of a modification of the procedure described by Bemis and Plotkin (3). Citrated porcine erythrocytes were washed twice in PBS and resuspended in PBS to a final concentration of 0.5% (vol/vol). Bacterial cells were grown for 48 h at 37°C on BGS. Growth from these plates was suspended in PBS to obtain an optical density of 1.0 at 525 nm (1.7 × 10⁹ CFU/ml). Cell suspension (50 µl) was placed into each of two wells in the first row of a 96-well microtiter plate for each bacterial cell suspension to be tested, and serial twofold dilutions through 1:1,024 were made with PBS. PBS alone served as a negative control. The porcine erythrocyte suspension (50 µl) was added to each well, and the contents of the wells were mixed by rotating the plates gently. Plates were incubated at room temperature for 4.5 h. Hemagglutination titers were expressed as the reciprocal of the highest dilution (before the addition of erythrocytes) of bacterial cell suspension showing agglutination of the erythrocytes.

Adenylate cyclase activity. Whole-cell suspensions were assayed for adenylate cyclase activity by use of a previously described procedure (11). Bacterial suspensions were prepared by suspending 24-h growth from a BGS plate in PBS to an optical density of 0.2 at 600 nm (10⁸ CFU/ml).

Dermonecrotic toxin activity. *B. bronchiseptica* strains were tested for dermonecrotic toxin (DNT) activity in guinea pigs by use of a modification of the technique described by Nakai et al. (17). Cells grown overnight in 5 ml of Stainer-Scholte medium at 37°C with shaking were used to inoculate a 1-liter flask containing 200 ml of Stainer-Scholte medium, and the culture was incubated at 37°C with shaking at 120

TABLE 2. Biological properties of *B. bronchiseptica* strains

Strain	Colonial phenotype ^a	Hemagglutination titer (range) ^b	DNT activity (range) ^c	Adenylate cyclase activity ^d	
				- Calmodulin	+ Calmodulin
CSU-P-1	Dom ⁻ Scs ⁻ Hly ⁻	0 (0)	18 (0-50)	0.05	0.08
64-C-0406	Dom ⁺ Scs ⁺ Hly ⁺	64 (32-128)	83 (0-100)	2.36	9.13
1120-A-83-013	Dom ⁺ Scs ⁺ Hly ⁺	37 (32-64)	667 (400-800)	2.29	12.98
B205BT	Dom ⁺ Scs ⁺ Hly ⁺	32 (32)	533 (400-800)	3.87	11.49

^a Nomenclature of Peppler and Schrupf (22) is used for describing colonial morphology of *B. bronchiseptica* strains of BG agar. Dom designates domed colony type, Scs designates smooth colony surface, and Hly indicates production of a zone of hemolysis around the colony.

^b Hemagglutination titers are expressed as the reciprocal of the highest dilution of a bacterial cell suspension that agglutinated porcine erythrocytes. Values given are the average of six determinations. Numbers in parentheses represent the range of values obtained for each strain.

^c DNT activity is expressed as the reciprocal of the highest dilution of cell lysate which produced a positive reaction (necrotic area of >5 mm in diameter) in guinea pig assay. Each strain was tested at least five times, and the numbers in parentheses are the range of values obtained.

^d Specific activity expressed in nanomoles of [³²P]ATP converted to [³²P]cAMP per minute per milligram of protein, with and without 1 μ M calmodulin added to the reaction mixture. Each value is the average of at least two determinations.

rpm on a rotary shaker for 6 to 8 h. The cells were harvested by centrifugation at $15,000 \times g$ (0 to 4°C) for 10 min and resuspended in 0.01 M Tris, pH 7.8, to an optical density of 1.0 at 650 nm (2×10^9 CFU/ml). The cell suspension was centrifuged again as just described, and the cell pellet was resuspended in a 0.5 volume of Tris. The resulting cell suspension was lysed by sonicating at 60% full power (Sonic Dismembrator, model 300, 3/4-in. [1.9-cm] titanium tip; Artek Systems Corp., Farmingdale, N.Y.) in 30-s bursts with 30 s of cooling between bursts. The cell suspension was sonicated in a beaker that remained immersed in ice throughout the lysing procedure. Lysis of the cells was indicated by a change from a turbid suspension to translucence. Samples of the suspension were also checked periodically by phase microscopy. Cell lysates were stored in 2-ml portions at -40°C until use.

DNT activity of cell lysates was assayed by the following procedure. A sample of cell lysate was thawed at room

temperature. The sample was immediately placed at 4°C upon thawing and used within 8 h. Cell lysates from strains 64-C-0406, 1120A-83-013, and B205BT were diluted 1:100, 1:200, 1:400, and 1:800 in sterile 0.01 M Tris, pH 7.8. Strain CSU-P-1 was diluted 1:10, 1:20, 1:50, and 1:100 in the same diluent. Each dilution (100 μ l) was injected into a site on the back of a depilated guinea pig. Three strains were tested in each animal, and sterile Tris was injected into a site as a negative control (total of 13 sites per animal). Necrotic lesions were measured at 48 h, and lesions of >5 mm in diameter were considered a positive reaction. DNT titers were expressed as the highest dilution of cell lysate giving a positive reaction. At least five determinations were made for each strain tested.

RESULTS

Strain virulence in neonatal swine. Strain CSU-P-1 did not colonize the nasal cavity or lungs of aerosol-exposed neonatal pigs, and no nasal turbinate or pneumonic lesions were seen in these animals at necropsy. Strain 64-C-0406 did colonize both the nasal passages and lungs of experimentally infected pigs but did not produce gross nasal turbinate or lung lesions. Strains 1120-A-83-013 and B205BT colonized infected animals and produced both gross nasal turbinate and lung lesions.

Colonial morphology and hemolysis. In the system described by Peppler and Schrupf (22), *B. bronchiseptica* colonies on BG agar are scored for domed elevation (Dom⁺), smooth colony surface (Scs⁺), and hemolysis (Hly⁺). Strain CSU-P-1 formed colonies with a Dom⁻ Scs⁻ Hly⁻ phenotype on both BGS and BGH plates. The other three *B. bronchiseptica* strains used in this study, 64-C-0406, 1120A-83-013, and B205BT, demonstrated a Dom⁺ Scs⁺ Hly⁺ colonial phenotype on both BGS and BGH plates at 48 h (Table 2). In addition, colonies of CSU-P-1 were larger at 48 h on both BGS and BGH plates than those formed by the other three strains (1.4 versus 0.8 mm average diameter).

Attachment to porcine tracheal rings. After 1 h of incubation of labeled cells with porcine tracheal rings, CSU-P-1 demonstrated a higher level of attachment than did the other three strains (Fig. 1). However, after 2 h of incubation, all four strains showed comparable levels of attachment to porcine tracheal rings.

Hemagglutination of porcine erythrocytes. Whole-cell suspensions of *B. bronchiseptica* strains 64-C-0406, 1120-A-83-013, and B205BT agglutinated porcine erythrocytes at dilutions of 1:32 and greater, whereas CSU-P-1 demonstrated only slight hemagglutination in an undiluted bacterial cell suspension (Table 2).

FIG. 1. Attachment of *B. bronchiseptica* strains CSU-P-1, 64-C-0406, 1120-A-83-013, and B205BT to porcine tracheal ring explants.

Dermonecrotic toxin activity. Early studies showed that the four strains of *B. bronchiseptica* produced considerably more DNT when grown in Stainer-Scholte medium than when grown in brucella medium (data not shown). Lysates of strains 1120-A-83-013 and B205BT consistently produced necrosis in the backs of guinea pigs at dilutions of 1:400 and 1:800. Strain 64-C-0406 produced weak necrosis consistently at dilutions of 1:100 or lower but never at higher dilutions. CSU-P-1 never produced lesions at dilutions greater than 1:50 (Table 2).

Adenylate cyclase activity. Strains 64-C-0406, 1120-A-83-013 and B205BT demonstrated adenylate cyclase activity which was stimulated by the addition of calmodulin (30), but strain CSU-P-1 did not (Table 2). Preliminary studies of reisolates from piglets indicate that passage of strains 1120-A-83-013 and B205BT led to an increase in adenylate cyclase activity, whereas the activity of 64-C-0406 appeared to be relatively unaffected by pig passage. However, whole-cell suspensions from BG plates, urea extracts of Stainer-Scholte broth-grown cells, and assays designed to measure adenylate cyclase toxin activity in S49 lymphoma cells consistently indicated that strain 64-C-0406 produced equal or greater amounts of adenylate cyclase activity when compared with strains 1120-A-83-013 and B205BT (data not shown), even after reisolation of these strains from piglets. These observations are currently under investigation.

DISCUSSION

Colonial morphology and hemolysis on BG agar is the basis for assigning strains of *B. bronchiseptica* to the phase designations that are often described for these organisms (18, 19). Phase I colonies are small, smooth, domed, and hemolytic. Phase IV colonies are large, rough, and umbonate, often with an irregular edge. Phases II and III are intermediates between phases I and IV. Phase I colony-producing strains have generally been regarded as virulent, and the production of colonies of phases II through IV has been associated with a reduction or loss of virulence (8). Some confusion has resulted from the use of the phase I to IV scheme for describing *Bordetella* colonies, especially with respect to the phase II and III colonial phenotypes (8). In addition, *Bordetella* sp. strains can undergo phase variation after repeated transfer on laboratory media, and hemolytic reactions are often dependent upon the species origin of the blood used. For these reasons, some investigators have suggested that alternative schemes be used for characterizing *B. bronchiseptica* colonies (1, 22). Peppler and Schrupf (22) presented a system for describing *B. bronchiseptica* colonial phenotypes based on colonial elevation, colonial surface texture, and hemolysis on BG agar that is very useful in this regard. Strains 64-C-0406, 1120-A-83-013, and B205BT used in this study consistently produced small, smooth, domed, hemolytic colonies (Dom⁺ Scs⁺ Hly⁺) at 48 h on BGS. CSU-P-1 formed large, rough, umbonate colonies (Dom⁻ Scs⁻ Hly⁻) at 48 h on this medium. Phase variation did not present a problem with the strains tested in this study. All four strains retained their colonial characteristics when the plates were refrigerated after 48 h of growth at 37°C. Inoculation of subcultures from these plates onto fresh BGS yielded colonies identical to those of the parental culture, even after the parental culture had been refrigerated for up to 2 months. Colonial variation on BG medium was, however, noted for these strains on two occasions. Some of the cultures grown on BG from tracheal swabs taken from

experimentally infected pigs at necropsy showed a combination of Dom⁺ Scs⁺ Hly⁺ and Dom⁺ Scs⁺ Hly⁻ colonial phenotypes on the same plate. It was also noted that streaking a Stainer-Scholte broth culture of strains 64-C-0406, 1120A-83-013, or B205BT onto BGH yielded a very few Dom⁻ Scs⁺ Hly⁺ colonies mixed in with a vast majority of Dom⁺ Scs⁺ Hly⁺ colonies. Interestingly, BG plates inoculated from brucella broth cultures of these same three strains yielded only Dom⁺ Scs⁺ Hly⁺ colonies. These phenomena were not investigated further. However, from the results of our studies, it can be presumed that Dom⁺ Scs⁺ Hly⁺ strains of *B. bronchiseptica* are not always virulent for neonatal pigs. This is supported by the work of Collings and Rutter (4), who showed that phase I strains of *B. bronchiseptica*, which presumably showed the Dom⁺ Scs⁺ Hly⁺ phenotype, from a human, a monkey, and a dog did not produce turbinate lesions or pneumonia in experimentally infected piglets.

Bordetella species preferentially attach to the ciliated epithelium of the upper respiratory tract of susceptible animals, and for this reason the ability to attach to cilia has been hypothesized to be an important virulence factor for these organisms (2, 16, 28, 31). In this regard, the results of our attachment assay in porcine tracheal ring explants seemed paradoxical when first examined. Strain CSU-P-1 attached to the rings twice as well as the other three strains at 1 h of incubation. At 2 h, all four strains showed comparable levels of attachment (Fig. 1). This is in direct contrast to what was expected, since CSU-P-1 was unable to colonize experimentally exposed animals. One possible explanation is that there appear to be two different mechanisms by which *B. bronchiseptica* can attach to eucaryotic cells. Plotkin and Bemis (23) showed that *B. bronchiseptica* attaches to hamster lung fibroblasts (HLF) by two distinct mechanisms. One is nonspecific and mediated by cations; both smooth and rough strains of *B. bronchiseptica* can attach to HLF cells by this mechanism. The other mechanism of attachment relies on a specific protein ligand which recognizes specific sites on the HLF cells; only smooth *B. bronchiseptica* strains attach to HLF by this mechanism. It is possible that the results of our attachment assay were caused by the CSU-P-1 strain attaching to the tracheal rings by a nonspecific type of attachment which quickly reached saturating levels and the other three strains attaching via the specific means of attachment which involved specific binding sites. This would explain the apparent difference in the kinetics of the binding of the strains to the tracheal rings (Fig. 1).

It also is interesting that the CSU-P-1 strain was able to attach to porcine tracheal rings in vitro but did not colonize the animals in vivo. This strain may not have been able to penetrate the mucus blanket that covers the ciliated epithelium of the upper respiratory tract of pigs. Even if this strain was able to penetrate the mucus and attach to the underlying cilia, it may not have been able to survive in the host long enough after inoculation (48 h) to have been detected by nasal swabbing, as we were never able to reisolate this strain from the pigs after inoculation. CSU-P-1 is equivalent to the phase IV strain of *B. bronchiseptica*. Phase IV strains of *B. pertussis* have been shown to be more susceptible to uptake and killing by macrophages than phase I strains (16), and a similar situation may exist for phase IV *B. bronchiseptica* strains in pigs. Indeed, studies in our laboratory indicate that strain CSU-P-1 is extremely sensitive to killing by normal pig serum (D. W. Byrd, unpublished data). However, another group has reported that phase IV *B. bronchiseptica* can be

recovered after experimental infections in young pigs, although the numbers are greatly reduced (4).

Another property of *B. bronchiseptica* strains that has been presumed to be correlated with virulence is the ability to agglutinate erythrocytes (6). Sheep cells have been used most widely, but *B. bronchiseptica* also agglutinates erythrocytes from dogs, pigs, horses, and guinea pigs (3). We used porcine erythrocytes in our assay and found that strain 64-C-0406 showed the highest hemagglutination titer of the strains tested. This indicates, at least for the strains used in this study, that hemagglutination alone is not always a useful characteristic for predicting the virulence of a *B. bronchiseptica* strain for pigs.

B. bronchiseptica produces a heat-labile, intracellular toxin which produces dermonecrosis when injected intradermally into guinea pigs and mice and is lethal for mice when injected intraperitoneally (15, 17). This material has been called both the heat-labile toxin (15) and the DNT (17), and a detailed characterization of this compound was recently presented by Kume et al. (13). The results of the study reported here (Table 2) indicate that the production of DNT by *B. bronchiseptica* strains may play an important role in their ability to produce turbinate lesions and pneumonia in young pigs. Virulent strains B205BT and 1120-A-83-013 both exhibited high average titers of DNT activity (>500) when cell lysates were assayed in guinea pigs. In contrast, strain 64-C-0406, which colonized the pigs but did not produce lesions, showed an average DNT titer of only 83, and CSU-P-1, which did not colonize aerosol-exposed animals, produced an average DNT titer of 18 in the guinea pig assay. Thus, it appears that a threshold level of DNT production may be involved, as both of the avirulent strains, CSU-P-1 and 64-C-0406, possessed some DNT activity.

Other investigators have also noted a positive correlation between DNT activity or mouse lethality of *B. bronchiseptica* strains and the ability of those strains to produce respiratory disease in pigs (4, 6). Collings and Rutter (4) noted that strains of *B. bronchiseptica* which produced turbinate atrophy in experimentally infected piglets had a much lower 50% lethal dose in mice than did avirulent strains. Elías et al. (6) demonstrated that *B. bronchiseptica* strains isolated from herds of pigs suffering from IAR had a considerably higher average level of mouse toxicity than did strains isolated from nonaffected herds. In addition, Hanada et al. (10) were able to produce turbinate lesions very similar to those seen in naturally occurring IAR by applying a filtered cell lysate from a *B. bronchiseptica* strain with a high level of DNT activity intranasally to piglets for 64 days, and Kume et al. (13) have observed damage to extracted porcine nasal turbinate tissue when it was incubated with *B. bronchiseptica* cell lysates in vitro. Our results support these findings. It has also been suggested that the DNT of *B. bronchiseptica* may play a role in the production of respiratory disease in dogs (9).

The results of this study and those of others indicate that DNT activity is a potential test for predicting the virulence of field strains of *B. bronchiseptica* for pigs; however, more strains must be tested before this can be conclusively shown. A means of testing for DNT activity that does not rely on the use of animals would make the test less expensive, less time-consuming, and more practical for testing a large number of strains. An enzyme-linked immunosorbent assay that makes use of bound antibodies to the purified or partially purified toxin would provide an excellent quantitative measure that would be useful for examining large numbers of samples and would alleviate many of the problems associ-

ated with bioassays, such as animal variation in response to the toxin. A cytotoxic assay with bovine embryonic lung cells has been used to measure the level of heat-labile toxin as an alternative to testing in mice (4); however, with such an assay there is always the question of whether the results are directly comparable to the results of the bioassay.

The finding that DNT activity appears to be directly related to the ability of *B. bronchiseptica* strains to produce turbinate and lung lesions in young pigs has importance in the production of vaccines designed to prevent or reduce the severity of IAR. *B. pertussis* DNT in its toxic form is reported to be a poor antigen. However, it can easily be converted to a toxoid by treatment with Formalin (15). This toxoid is very antigenic and will protect animals against the action of the toxin (15). Hence, if specific antibodies against *B. bronchiseptica* DNT are found to be protective in pigs, then IAR bacterins should be prepared from *B. bronchiseptica* strains which produce high levels of DNT and any acellular vaccines against IAR should include *B. bronchiseptica* DNT. In both circumstances, the vaccine should be prepared in such a way as to reduce or eliminate toxicity while maintaining or enhancing antigenicity, i.e., possibly by Formalin treatment.

Although adenylate cyclase activity appears to be important as a virulence factor in *B. pertussis* infections in mice (29) and has been proposed as a virulence factor in IAR (4, 14, 20), no correlation was observed between adenylate cyclase activity and lesion production for any of the four *B. bronchiseptica* strains used in this study. Adenylate cyclase may, however, play a role in other aspects of IAR or *B. bronchiseptica*-associated pneumonia, such as evasion of the host immune response. The mechanism of action of the *Bordetella* adenylate cyclase in a host is not known with certainty, but it appears that the enzyme can affect a number of eucaryotic cells in vitro by entering the target cell and catalyzing the production of supraphysiologic levels of cAMP (12). Confer and Eaton (5) found that urea extracts of whole-cell-associated *B. pertussis* adenylate cyclase inhibited chemotaxis, phagocytosis, superoxide generation, and bacterial killing in polymorphonuclear leukocytes and inhibited the oxidative burst in alveolar macrophages in vitro. Adenylate cyclase from *B. pertussis* has also been shown to inhibit superoxide generation and the oxidative burst in peripheral blood monocytes and to inhibit target cell lysis by human natural killer cells (12). Indeed, it has been reported that the adenylate cyclase of *B. bronchiseptica* may be an important protective antigen (14, 20).

In summary, work in our laboratory and by others (4, 24) has shown that there is considerable variation among strains of *B. bronchiseptica* in their ability to infect and produce lesions in young pigs. In addition, some phenotypic characteristics of *B. bronchiseptica* that have previously been associated with virulence, namely Dom⁺ Scs⁺ Hly⁺ colonial phenotype on BG agar, hemagglutination, in vitro attachment to ciliated epithelial cells, and adenylate cyclase activity, did not always correlate with in vivo virulence in pigs for the four *B. bronchiseptica* strains that we examined. However, DNT production by *B. bronchiseptica* did show a positive correlation with virulence in pigs.

The information presented in this report should improve the understanding of the host-parasite interaction involved in the production of IAR and pneumonia in young pigs by *B. bronchiseptica* and serve as a basis for future research oriented toward determining the nature of the host-parasite interactions involved in *Bordetella*-associated upper respiratory diseases in other animals, including humans. It may also

prove useful in the development of improved vaccines against IAR. Finally, the knowledge that certain phenotypic characteristics of *B. bronchiseptica* are associated with the production of turbinate lesions and pneumonia in young pigs should stimulate the development of laboratory tests useful for predicting the potential virulence of *B. bronchiseptica* field isolates for swine.

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