SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle): Airborne Induced Experimental Bordetella bronchiseptica Pneumonia in Strain 13 Guinea Pigs	5. TYPE OF REPORT & PERIOD COVERED: Infections Study Sep - Nov 1984
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s): C.J. Trahan, E.H. Stephenson, J.W. Ezzell, W.C. Mitchell	8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS: SGRD-UIR Animal Resources Division, USAMRIID Ft. Detrick, MD 21701-5011	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: TSP-24
11. CONTROLLING OFFICE NAME AND ADDRESS: SGRD-UIZ-D Commander, USAMRIID Ft. Detrick, MD 21701-5011	12. REPORT DATE: Jan 1986
	13. NUMBER OF PAGES
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report): Not Classified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

To be published in Laboratory Animal Science

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Bordetella bronchiseptica, Strain 13 guinea pig, bronchopneumonia aerosol exposure, bordetellosis, Cavia porcellus

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Strain 13 guinea pigs were evaluated as an animal model to study the molecular interactions of Bordetella sp and to evaluate the efficacy of B. bronchiseptica bacterins in inducing a protective immune response. When guinea pigs were exposed to small particle aerosols of varying concentrations of virulent B. bronchiseptica, a spectrum of disease was produced that ranged from inapparent to fulminant. Clinical signs began by day 4 after exposure, and were evidenced by anorexia, weight loss, respiratory distress, and serous to purulent nasal discharge. Pathologic alterations were limited to the respiratory system.

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Moribund animals exhibited a suppurative, necrotizing bronchopneumonia and necrotizing tracheitis. Typically, the bacteria were eliminated from the lungs by day 28, while significant concentrations of bacteria continued to persist in the laryngeal area and in the trachea. The median infectiouns dose (ID_{50}) and the median lethal dose (LD_{50}) were estimated to be 4 CFU and 1,314 CFU, respectively. These data suggest the guinea pig will be a valuable model system in which to study Bordetella sp-host cell interactions, as well as evaluate potential B. bronchiseptica immunogens.

(2)

Airborne Induced Experimental *Bordetella bronchiseptica* Pneumonia in Strain 13 Guinea Pigs

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Bordetella bronchiseptica in Guinea Pigs

The animals described in this report were procured, maintained, and used in accordance with the Animal Welfare Act and the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

Abstract

To evaluate the efficacy of a commercial bacterin in protecting Strain 13 guinea pigs against fatal Bordetella bronchiseptica pneumonia, it was necessary to establish the infectivity and disease pathogenesis induced by virulent organisms. When guinea pigs were exposed to small-particle aerosols of varying concentrations of virulent B. bronchiseptica, a spectrum of disease was produced that ranged from inapparent to fulminant broncho-pneumonia. Clinical signs began by day 4 after exposure, and were evidenced by anorexia, weight loss, respiratory distress, and serous to purulent nasal discharge. Pathologic alterations were limited to the respiratory system. Moribund animals exhibited a suppurative, necrotizing bronchopneumonia and necrotizing tracheitis. In animals that survived the challenge, the bacteria were eliminated from the lungs by day 28, while significant concentrations of bacteria continued to persist in the laryngeal area and in the trachea. The median infectious dose and the median lethal dose were estimated to be 4 colony forming units (CFU) and 1,314 CFU, respectively. These data suggest the guinea pig will be a valuable model system in which to study Bordetella sp - host cell interactions, as well as evaluate potential B. bronchiseptica immunogens.

Bordetella bronchiseptica, a recognized pathogen of the mammalian respiratory tract (1), has been shown to be the etiologic agent of infectious atrophic rhinitis in swine (1,2,3), infectious tracheobronchitis in canines (1,2,4), and epizootic pneumonia in guinea pigs (Cavia porcellus) (5,6,7). Clinical signs of B. bronchiseptica infection in swine include nasal discharge with hemorrhage, impaired breathing, impaired growth, and hypoplasia or atrophy of nasal turbinate bones, with resultant twisting or shortening of the snout (3). In the canine, signs of B. bronchiseptica infection are a moist, hacking cough with excessive accumulation of tracheal mucus, bilateral mucopurulent nasal discharge, vomiting, weight loss, and pulmonary complications (1,4). Typical clinical signs of B. bronchiseptica infections in guinea pigs include inappetence, dyspnea, nasal and ocular discharge, and insensitivity to sound, all of which are most prominent 24 hr prior to death (8, 9). Inbred strains of guinea pigs, i.e. Strain 2 and Strain 13, are more susceptible to B. bronchiseptica pneumonia than are outbred strains (8).

Transmission of infectious B. bronchiseptica to susceptible animals is considered to occur commonly via aerosol droplets. However, studies of the basic pathogenic mechanisms of B. bronchiseptica in guinea pigs and swine have been limited to the exposure of these experimental animals to naturally

infected colonies (5) or by intranasal inoculation of test animals with various concentrations of virulent strains of the organism (3,10,11,12,13, 14,15,16,17). In the canine, aerosol methods of challenge have been used (18, 19, 20, 21), but the challenge dose of virulent organisms has not been quantified routinely.

The conventional production colony of Strain 13 guinea pigs at USAMRIID has experienced periodic epizootics of acute, fatal B. bronchiseptica pneumonia. Until recently, the outbreaks were being controlled through a vaccination program using a bacterin produced at the National Institutes of Health (5). When the laboratory-produced bacterin became unavailable, it became necessary to establish the efficacy of other available immunogens. However, before such efficacy evaluation can be made, an understanding and knowledge of the infectivity and pathogenesis of disease induced by the etiologic agent must first be gained. Consequently, this study was designed to define the infectivity and basic pathogenesis of B. bronchiseptica infection in Strain 13 guinea pigs infected via aerosol exposure.

Materials and Methods

Animals: Inbred Strain 13, female, 6-week-old guinea pigs (390-450 g) were obtained from the Institute's conventional production colony. Selected animals did not exhibit humoral antibodies to B. bronchiseptica, as determined by two separate sera evaluations, conducted 24 and 14 days prior to challenge. Sera for antibody assay were obtained from 2 ml blood samples drawn from the anterior vena cava (22). Blood withdrawal procedures were accomplished after ketamine[1] (30 mg/kg) and xylazine[2] (6 mg/kg) administration. Each animal was ear-tagged for identification and individually housed in polycarbonate cages maintained within ventilated racks.[3] Open formula guinea pig ration[4] and water were provided ad libitum throughout the study.

Experimental Design: Groups of four guinea pigs were given selected doses of aerosolized B. bronchiseptica (Table 1). An additional group of four animals, serving as controls, was exposed to an aerosol of sterile diluent, tryptose phosphate broth (TPB). The animals were clinically evaluated twice daily for 28 days after exposure. Four moribund animals were selected for determination of bacterial concentrations in select tissues and for evaluation of pathologic alterations. The four control animals, as well as four animals

that survived the aerosol challenge exposure, were similarly evaluated on day 28 after exposure.

Bacterium: A frozen (-70°C) stock culture of a guinea pig isolate of B. bronchiseptica, Strain SHGP-1, was revived on 5% sheep blood agar at 37°C. One loop (5 mm diameter) of hemolytic colonies was inoculated into 100 ml TPB in a 500 ml cotton plugged flask. The culture was aerobically incubated for 20 hr, with reciprocal shaking (100 reciprocations per min), at 37°C. Following incubation, the culture was chilled on ice and the absorbance of the culture was measured at 420 nm, 1.0 cm light path. The absorbance value, when multiplied by a factor of 3.46×10^7, was determined to correlate directly to the number of colony forming units (CFU)/ml of culture (J. W. Ezzell, unpublished data). Aliquots of the culture were centrifuged at 10,000 x g for 15 min at 4°C. Cell pellets were then resuspended with appropriate volumes of TPB to obtain the desired concentrations of B. bronchiseptica. Concentration estimations were subsequently verified by colony count assays.

Respiratory Challenge: Animal exposures were performed using whole-body, dynamic aerosol equipment as described previously (23). The Henderson-type aerosol transit tube was modified by incorporation of an animal exposure box. Each animal was exposed for 10 min to a predetermined concentration of

B. bronchiseptica. Respiratory minute volumes were estimated in accordance with Guyton's formula (24). Total inhaled doses were calculated using the minute volumes and the aerosol concentrations of bacteria delivered.

Bacterial Assay: Colony assay procedures were used to quantitate B. bronchiseptica in aerosol samples and tissue swab specimens. Assays for aerosol samples were performed by inoculating 5% sheep blood agar with 0.2 ml amounts of serially diluted test samples. Culture plates were incubated at 37°C for 24 hr, then the concentration (CFU/ml) was determined. Samples from the larynx, trachea, and lungs were obtained by aseptic swabbing of the tissues. Swabs were streaked onto blood agar, MacConkey agar, and urea slants for identification and quantitation of tissue levels of B. bronchiseptica. The identity of isolated colonies as B. bronchiseptica was accomplished using the Gram stain and accepted biochemical tests (25).

Serology: Humoral antibody was quantitated with an enzyme-linked immunosorbent assay (ELISA). Each well of 96-well tissue culture plates[5] was coated with 50 µl of antigen comprised of B. bronchiseptica bacterin[6] diluted 1:4 in phosphate-buffered saline (PBS), pH 7.3. After drying overnight at ambient temperature, the plates were heated to 60°C for a 5 min period, then allowed to cool. Methanol (50 µl) was added to each well and allowed to dry

at ambient temperature. To prevent nonspecific binding of serum antibody, each well was blocked with 300 µl of 5% powdered milk in PBS containing 0.05% Tween-20 (PBS-T), incubated for 30 min at ambient temperature, then washed four times with PBS-T. Serial dilutions of sera, using 0.5% gelatin-PBS-T (100 µl), were made in test wells. Following incubation for 2 hr at 37°C, sera were removed by vacuum, and wells were washed four times with PBS-T. Horseradish peroxidase-protein A conjugate (appropriately diluted in PBS-T containing 5% fetal calf serum) was added (100 µl) to each well. After incubation for 30 min at ambient temperature, the conjugate was removed by vacuum and the wells were washed four times with PBS-T. Chromogenic substrate (1.0 mg/ml of 2,2 azino-di 3-ethylbenzthiazoline sulfonic acid[7] in 0.1 M citrate buffer containing 0.003% H_2O_2) was added (100 µl) to each well. The reaction was stopped after 20 min with 50 µl of 10% sodium dodecyl sulfate. Absorbance was determined at 405 nm using a Biotek EIA reader[8]. Titers, expressed as the reciprocal of serum dilution, were considered positive at 32. This was a twofold dilution higher than the highest positive reaction, due to non-specific binding of titrated antibody, noted in blank control wells (i.e., wells without antigen).

Pathology: Tissues for histopathologic examination were fixed in a

solution of 10% formaldehyde in PBS (pH 7.4), embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin.

Results

Infectivity and Lethality: Guinea pigs that inhaled 43 or less B. bronchiseptica organisms exhibited minimal clinical signs of disease, and only one death was noted (Table 1, Group 1 through 5). A thin, scanty, serous nasal discharge and slight anorexia were seen in affected animals of these groups. Recipients of higher doses of B. bronchiseptica began to show signs of anorexia, weight loss, and respiratory distress by day 4 after exposure (Table 1, Group 6 through 9). Signs in some of these animals progressed to a copious, purulent, bilateral nasal discharge; dehydration; severe tachypnea; extreme lethargy; and death. In several animals, the purulent discharge became sanguineous just prior to death. Using probit analysis, the median infectious dose (ID_{50}) and the median lethal dose (LD_{50}) were estimated to be 4 CFU (95% CL 0.4 to 42) and 1,314 CFU (95% CL 113 to 26,385), respectively. Mean time to death of infected guinea pigs correlated directly with the inhaled dose of infectious bacteria, being 6.2 days for those animals receiving approximately 325 LD_{50} (4.3×10^5CFU).

Gross Pathology: Four moribund guinea pigs from Groups 8 and 9 were necropsied on day 7 after exposure. Severity of the lesions varied among the animals. All animals exhibited congested conjunctivae, dried mucopurulent

crusts in the medial and lateral canthi, and occlusive crusts in the nares. Transparent, straw-colored fluid of varying quantity was consistently observed in the thoracic cavity. Similarly, each animal had white to blood-tinged frothy material in the trachea. Multiple pulmonary lesions were observed throughout the parenchyma of the middle and caudal lobes in each animal. The lesions were irregularly-shaped, well-demarcated, variable in size (0.5-2.0 cm), and reddish-gray. No other gross lesions were observed.

Four guinea pigs that survived an infectious challenge were necropsied on day 28 after exposure. Gross pathologic alterations were limited to the lungs. Each of the guinea pigs exhibited multiple, irregularly-shaped, well-demarcated, reddish-gray lesions (1-2 cm diameter) in the middle lung lobe and, to a lesser extent, in the caudal lobe. The lesions often coalesced, and, in one animal, the right middle lobe was completely consolidated. No gross pathologic alterations were observed in the four sham-challenged, control guinea pigs.

Histopathology: Each moribund guinea pig had a suppurative, necrotizing bronchopneumonia and a necrotizing tracheitis. The lumina of the bronchi, bronchioles, termial bronchioles, and alveoli were filled with cellular debris, necrotic epithelial cells, and inflammatory cells entrapped in a

fibrinous exudate. The predominating inflammatory cells were neutrophils, although some macrophages and lymphocytes were present. An eosinophilic homogenous fluid (edema within alveoli and smaller airways) and capillary congestion were present at the periphery of the lesion.

Animals surviving the infectious challenge exhibited smaller areas of granulomatous bronchopneumonia. The lesions were characterized by a fibrocellular debris containing various combinations and numbers of epithelial cells, macrophages, plasma cells, lymphocytes, neutrophils, and cellular debris within the lumina of the bronchi, bronchioles, terminal bronchioles, and alveoli. A demonstrable increase in the thickness of the alveolar interstitium was caused by the presence of fibroblasts, macrophages, plasma cells, and lymphocytes. No histopathologic alterations were noted in the sham-challenged control group animals.

Bacteriology. Guinea pigs that died of acute bronchopneumonia yielded high concentrations of B. bronchiseptica from the larynx, trachea, and lung swabs (Table 2). Gradient effects of bacterially induced disease within the respiratory system could not be demonstrated, since only moribund animals were examined. The animals that survived the infectious challenge, however, did exhibit a gradient-like range in the bacterial concentrations detected in the

organs of the respiratory tract. Typically, there was a significant concentration of bacteria in the laryngeal area and in the trachea that persisted to 28 days after exposure. Bacterial concentrations within the lung parenchyma were at low, almost undetectable, levels by day 28.

Discussion

The whole-body aerosol exposure system yields a preponderance of aerosol particles that are < 1 μm in diameter. Such particles readily penetrate the air passages to the alveoli, which results in an even dispersion throughout the respiratory system. By varying the inhaled dose of infectious B. bronchiseptica, the spectrum of disease that developed in the Strain 13 guinea pigs ranged from an inapparent infection to an acute, fulminant bronchopneumonia. Thus, it is now possible to determine the effect of various levels of exposure on both pathogenesis and the immunogenesis of B. bronchiseptica infection in guinea pigs.

Previous studies of airborne transmission of B. bronchiseptica in animals have been performed principally by intranasal instillation of bacterial suspensions, which yields a large aerosol particle ($\geq$ 5 μm), or by exposure to naturally infected colonies. Aerosol droplets $\geq$ 2 μm will rarely reach the alveoli. Instead, they impinge in the upper respiratory tract. The placement of initially uninfected experimental animals in infected colonies does not allow for control of exposure parameters. Aerosol methodology has been used to induce experimental infection in dogs. However, quantitative techniques were not incorporated in such studies to obtain definitive challenge doses of

organisms. Our approach simulated the natural exposure that occurs when animals are maintained in infected colonies, and can provide the ability to predetermine the level of inhaled dose the animals will receive.

Clinical signs of bordetellosis in the canine, as well as the gross and microscopic pathologic alterations, (1,4,18,26) are quite similar to those we observed in guinea pigs. In the dog, the more prominent clinical signs are a bilateral, mucopurulent, nasal discharge, and a moist, hacking cough. Canines that die exhibit a scant to copious, mucopurulent exudate throughout the respiratory tract, with areas of hemorrhage and consolidation in the lungs. The degree of severity depends on the stage of the disease. The Strain 13 guinea pig model complimented and extended the use of animals in the study of B. bronchiseptica infection, and may prove valuable in elucidating the molecular, pathogenic, and immunogenic mechanisms of B. bronchiseptica infection, especially when the economic difference betwen the two animal models is considered.

Bordetella-induced respiratory infections in humans are caused by B. pertussis. The bacteria cause a superficial infection of the distal airways which produces whooping cough or pertussis pneumonia (27-29). Human disease is characterized by endobronchitis and endobronchiolitis that progresses to

interstitial pneumonia with peribronchitis. The smaller bronchi and bronchioles fill with mucopurulent exudate. In severe infections, necrosis and erosion of the respiratory epithelium often leave shallow ulcers. Bronchial and bronchiolar walls become heavily infiltrated with lymphocytes and plasma cells. Alveolar consolidation is rare, and usually is associated with secondary bacterial infections.

The Strain 13 guinea pig - B. bronchiseptica model may prove to be of value in exploring host cell - Bordetella sp interactions. In humans, disease processes induced by B. pertussis are quite similar to those alterations observed after airborne transmission of infectious bacteria in Strain 13 guinea pigs. Serologically, B. pertussis and B. bronchiseptica share similar antigens. Interactions of these related antigens with host-cell molecular components may direct the onset of the respiratory lesions observed in man and in guinea pigs.

The data obtained in this investigation demonstrated induction, via aerosol transmission, of a spectrum of B. bronchiseptica disease in Strain 13 guinea pigs. Based upon this study, we are now able to evaluate the ability of various immunogens to induce protection against airborne transmitted B. bronchiseptica.

Acknowledgments

The authors thank Ms. T. Abshire, Ms. T. Smith, SGT D. Reasinger, Mr. J. Dominik, and Mr. H. Young for technical assistance, and Dr. D. Bemis, University of Tennessee, Knoxville, TN, for providing the Bordetella bronchiseptica challenge strain.

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Table 1 Mortality and time-to-death responses in guinea pigs after aerosol challenge with graded doses of *Bordetella bronchiseptica*

Group	Inhaled Dose (CFU)	Dead/Total	Significant ELISA Titers[a] (positive/examined)	MDTD[b] (range)
1	0.004	0/4	0/4	------
2	0.04	0/4	0/4	------
3	0.4	0/4	1/4	------
4	4.3	1/4	1/3	12
5	4.3×10^1	0/4	3/4	------
6	4.3×10^2	1/4	3/3	13
7	4.3×10^3	3/4	1/1	9.3 (7-11)
8	4.3×10^4	3/4	1/1	8.0 (7-10)
9	4.3×10^5	4/4	---	6.2 (6-7)
Control	------	0/4	---	------

[a]Sera for titers drawn on day 28. Titers $\geq$ 32 considered significant

[b]Geometric mean days to death

Table 2 *Bordetella bronchiseptica* levels in selected tissues

Group	Number	Status	Day After Exposure	Culture Results[a]		
				Larnyx	Trachea	Lungs
5	1	Survive	28	4+	4+	0
6	1	Survive	28	4+	3+	1+
7	1	Survive	28	2+	0	1+
8	1	Survive	28	3+	3+	1+
8	1	Moribund	7	4+	4+	4+
9	3	Moribund	7	4+	4+	4+

[a]Grading system for *B. bronchiseptica* isolates:

4+ = Urea slant positive at $\leq$ 24 hr; > 100 colonies on blood or MacConkey agar.

3+ = Urea slant positive at $\leq$ 48 hr; 25-100 colonies on blood or MacConkey agar.

2+ = Urea slant negative; 5-25 colonies on blood or MacConkey agar.

1+ = Urea slant negative; < 5 colonies on blood or MacConkey agar.

Footnotes

[1]Vetalar®, Parke Davis, Morris Plains, NJ

[2]Rompun®, Haver-Lockhart, Shawnee, KS

[3]Ventilated Animal Rack®, Lab Products, Inc., Rochelle Park, NJ

[4]NIH Production Guinea Pig Chow, Agway, Inc., St. Marys, OH

[5]96-well Cluster Plates (0.32cm^2/well), Costar, Cambridge, MA

[6]Bronchicine®, Pellen Labs, Inc., Omaha, NE

[7]Sigma Chemical Corp, St. Louis, MO

[8]Biotek Inst., Inc., Burlington, VT