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A Porcine Model for the Evaluation of Virulence of *Bordetella bronchiseptica*

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Summary

Studies of virulence factors of *Bordetella bronchiseptica* require a suitable system. Such a system was devised in colostrum-deprived, caesarean-derived pigs, aged 7 d. In two different experiments, pigs ($n=11$) were inoculated intranasally with 10^6 colony-forming units of the virulent strain 4609. In the same way, further pigs ($n=11$) were inoculated with a strain (B133) of unknown virulence. No significant differences between 4609 and B133 colonization were seen. However, colonization of the turbinates was significantly higher than that of the trachea, lung and tonsil, and a significantly higher degree of colonization was present at 11 d post-inoculation (PI) than at 15 days. Moderate turbinate atrophy was present by 11 d PI, and peribronchiolar fibrosis was present at 15 days. Immunocytochemical methods showed that all pigs had bacterial antigen in the ciliated cells of the turbinates and trachea, and in the lung; some pigs also had antigen in the bronchi. Bacterial antigen was present in some bronchioles and within the cytoplasm of pulmonary macrophages and neutrophils. This model should prove useful for comparing strains of *B. bronchiseptica* and isogenic mutants deficient in putative virulence factors. © 1997 W.B. Saunders Company Limited

Introduction

Bordetella bronchiseptica is associated with pneumonia and atrophic rhinitis in swine (Duncan *et al.*, 1966; Magyar *et al.*, 1988; Chanter *et al.*, 1989; Chung *et al.*, 1990; Ackermann *et al.*, 1991b; Gagne and Martineau-Doize, 1993). This bacterium colonizes ciliated cells of the respiratory tract and can induce mild to severe forms of turbinate atrophy, rhinitis and bronchopneumonia (Duncan *et al.*, 1966; Gagne and Martineau-Doize, 1993). When *B. bronchiseptica* infection occurs concurrently with *Pasteurella multocida*, either spontaneously or experimentally, particularly severe turbinate atrophy often occurs (Miniats and Johnson, 1980; Chanter *et al.*, 1989; Cowart *et al.*, 1989; Chung *et al.*, 1990; Foged, 1992). *B. bronchiseptica* produces a variety of toxins and adhesins that are probably essential for colonization and tissue damage (Magyar *et al.*,

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1988; Magyar and Glavits, 1990; Lendvai *et al.*, 1992; Bemis and Burns, 1993; Elias *et al.*, 1993; Walker and Weiss, 1994).

Assessment of potential *B. bronchiseptica* virulence factors *in vivo* requires a suitable model system. Previous studies have demonstrated the gross and microscopical pathology of *B. bronchiseptica* infection in swine (Duncan *et al.*, 1966; Harris and Switzer, 1968; Gagne and Martineau-Doize, 1993). However, a model system capable of demonstrating a number of microbiological, histopathological and immunocytochemical parameters of both the upper and lower respiratory tract may be necessary to reveal the significance of putative virulence factors. Assessment of such factors would seem increasingly important in view of the recent isolation of strains of *B. bronchiseptica* from cases with moderate turbinate atrophy and nasal conchal inflammation but no toxigenic *P. multocida*, despite extensive testing of concha and tonsil (Magyar and Rimler, 1991). These strains of *B. bronchiseptica* can express a variety of toxins (such as dermonecrotic toxin, adenylate cyclase and tracheal cytotoxin) and adhesins (such as filamentous haemagglutinin and pertactin) (Magyar and Glavits, 1990; Bemis and Burns, 1993), but the importance of these virulence factors cannot be stated with certainty. The present report describes the development of a model in caesarean-derived, colostrum-deprived (CDCD) pigs aged 7 days that may be useful in virulence studies of *B. bronchiseptica*.

Materials and Methods

Organisms

Bordetella bronchiseptica strain 4609 is a virulent strain that is toxigenic in mice (Ackermann, *et al.*, 1991b). *B. bronchiseptica* strain B133 is a strain of unknown virulence isolated by one of us (C.G.-W.). Though not directly demonstrated here, both strains used in this study have been shown to produce dermonecrotic toxin and express *bvg*-regulated virulence factors.

Experimental Animals and Design

In two different experiments, pigs were derived by caesarean section of pregnant sows and housed in individual isolators. At 5 days of age, the pigs were placed together in a single pen in a room with filtered air and controlled heating and lighting. In all, 11 pigs (five in a first experiment and six in a second) were inoculated at 7 days of age with a virulent strain (no. 4609) of *B. bronchiseptica* suspended in Tris-buffered saline (TBS) (0·5 ml/nostril; 10^6 colony forming units [cfu]/ml). At 3 days and 15 days post-inoculation (PI), the nasal cavities were rinsed with TBS, and the washings were plated on sheep blood agar. Two pigs in the first experiment and three pigs in the second were scheduled to be killed by pentobarbital overdose at 11 days PI and subjected to post-mortem examination; the remaining pigs were dealt with similarly at 15 days PI. However, one pig died before 11 days PI in each experiment. Ventral nasal conchae, tonsil, trachea and lung were taken for bacterial culture; additional samples of these tissues were fixed in 10% neutral buffered formalin for histopathological and immunocytochemical examination. *B. bronchiseptica* was identified from primary isolation plates by a recently developed bordetella-specific hybridization assay (Register *et al.*, 1995). A *B. bronchiseptica* porcine strain (no. B133) of unknown virulence was also tested, exactly as described for strain 4609. Two pigs receiving *B. bronchiseptica* B133 died before 11 days PI in the first experiment; none died in the second.

Table 1
Microbiological observations in pigs inoculated with *Bordetella bronchiseptica* strain 4609 or B133

Bacterial Strain	*Day post-inoculation*	*Number of pigs*	*Bacterial counts (cfu [10^6]/g ± SEM) in*			
			concha	*trachea*	*lung*	*tonsil*
4609	11	4	224·3 ± 156·7	25·4 ± 18·4	6·7 ± 3·0	0·58 ± 0·33
	15	5	38·7 ± 32·3	40·1 ± 14·8	15·6 ± 6·6	0·32 ± 0·15
B133	11	4	304·0 ± 163·4	67·6 ± 51·0	81·4 ± 52·9	0·05 ± 0·05
	15	5	64·8 ± 58·8	57·0 ± 36·1	24·6 ± 15·8	0·144 ± 0·11

* Concha colonized to a significantly higher degree ($P<0.05$) than trachea, lung, tonsil.

Immunochemistry

B. bronchiseptica antigen was detected in tissue sections with an alkaline phosphatase detection system (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA). Briefly, slides were incubated with normal (undiluted) goat serum for 10 min, rinsed, and then incubated with a 1 in 5000 dilution of the primary antibody (polyclonal rabbit anti-*B. bronchiseptica* 4609) at room temperature for 1 h. Control sections either lacked primary antibody or contained an irrelevant rabbit polyclonal antiserum. In addition, this primary antibody gave no trace of labelling when tested on lung and conchal tissues from pigs free of *B. bronchiseptica*. A 1 in 40 dilution of the secondary antibody (biotinylated goat anti-rabbit) was incubated with slides for 30 min. Sections were then incubated with streptavidin-phosphatase and phosphatase substrate and counterstained with Mayer's haematoxylin. Turbinate perimeter ratios were calculated as previously described (Collins *et al.*, 1989).

Statistical Analysis

Aspects of colonization were analysed by the general Linear Models Procedure of the Statistical Analysis System (SAS Institute, Cary, NC, USA). The comparisons were made in respect of strain, tissue, day PI (11 vs 15) as well as of tissue (regardless of strain or day). Turbinate perimeter ratios (TPRs) were regarded as significant when $\leq 1{\cdot}0$ (Collins *et al.*, 1989).

Results

Infection with B. bronchiseptica *4609*

Pigs had a slight cough and reduced appetite by day 2 PI. Death occurred in one of five pigs in the first experiment before day 11 PI and one of six in the second experiment. No pigs died after day 11 PI.

At day 3 PI, nasal washings yielded an average of $3{\cdot}1 \times 10^6$ cfu; by day 15, this number had dropped slightly to $1{\cdot}4 \times 10^6$. The highest numbers of bacteria per gram of tissue were isolated from the nasal concha, trachea and lung (Table 1). Very few bacteria were isolated from the tonsil. The nasal conchae were colonized to a significantly higher degree ($P<0{\cdot}05$) than other tissues 11 days PI. *B. bronchiseptica* was isolated from the two animals that died before day 11 PI, but bacterial counts were not made, because replication of the bacteria after death might have falsely elevated the numbers.

TPRs indicated a moderate degree of atrophy in pigs examined on days 11

Table 2
Histopathological, immunocytochemical and other observations in pigs inoculated with *Bordetella bronchiseptica* strain 4609 or B133

Bacterial strain	*Expt. no.*	*Number of pigs*	*Day post-inoculation*	*Conchal lesion**	*Conchal Bb-IR*†	*Lung lesion*	*Bronchial Bb-IR*‡	*Bronchiolar Bb-IR*‡	*Tracheal Bb-IR*†
4609	1	1	5	S;+;1·1	<30%	S	84%	8	<30%
4609	1	2	11	S;++;0·9	<30%	S	55%	6	30–60%
4609	1	2	15	S;++;0·8	<30%	F;N;H	21%	<1	30%
4609	2	1	5	ND	ND	None	0%	0	<30%
4609	2	2	11	S;0;0·8	<30%	S	18%	<1	<30%
4609	2	3	15	S;+;0·8	<30%	F	0%	0	<30%
B133	1	2	4&6	S;+;1·2	30–60%	S	84%	12	30–60%
B133	1	1	11	S;0;0·6	<30%	S;F;H	58%	17	<30%
B133	1	2	15	S;+;0·9	<30%	F,H	0%	0	<30%
B133	2	3	11	S;+;0·9	30–60%	S	17%	7	<30%
B133	2	3	15	S;+;0·7	<30%	F	3%	0	<30%

Scores and numbers are averages from the number of pigs indicated.
Bb-IR, *B. bronchiseptica* immunoreactivity; S, suppurative inflammation; F, fibrosis; N, necrosis; H, hyperplasia; ND, not done.
* Conchal lesion: S, suppurative inflammation; 0, + and ++ represent scores for lymphoplasmacytic inflammation of submucosa (0 = no infiltrates, + = mild, multifocal infiltrates circumscribing submucosal glands, ++ = diffuse infiltrates surrounding submucosal glands and throughout mucosa); the final number (0·6 to 1·2) denotes turbinate perimeter ratio (scores of 1·0 indicate moderate turbinate atrophy and scores of >1·0 indicate no turbinate atrophy).
† Conchal and tracheal Bb-IR: expressed as <30%, 30–60%, or >60% of epithelial surface area showing Bb-IR within section.
‡ In bronchi, Bb-IR in the apical portion of the epithelial cells was assessed as the number of bronchi showing Bb-IR/total number of bronchi in two tissue sections. In bronchioles, Bb-IR was sparse and the total number of bronchioles showing labelling was simply counted.

and 15 PI (Table 2). All animals developed moderate to marked infiltrates of neutrophils within the submucosa and also in the peri-conchal meatus. In addition, minimal to moderate infiltrates of lymphocytes and plasma cells were present in the lamina propria. There was moderate to marked loss of cilia with multifocal erosions of epithelium, similar to that previously described (Duncan *et al.*, 1966; Harris and Switzer, 1968; Gagne and Martineau-Doize, 1993), by days 11 and 15 PI. Significant inflammatory infiltrates were not present in the trachea. Sections of lung showed moderate to dense infiltrates of neutrophils in the lumina of the bronchi, bronchioles and alveoli and varying degrees of cilia loss in the bronchi at 11 days PI. There were multifocal areas of epithelial hypertrophy and hyperplasia of the bronchial and bronchiolar epithelium by day 15. Most cells lining the bronchial and bronchiolar airways had very few cilia, and these airways were surrounded by variable amounts of adventitial fibrosis and occasionally by necrosis with mineralization.

B. bronchiseptica antigen was present to varying degrees in the apical portion of ciliated cells in all sections of the turbinates and trachea, and in most sections of lung (Table 2). In areas denuded of cilia, *B. bronchiseptica* immunoreactivity (Bb-IR) was not present. Bb-IR was present in the cytoplasm of many macrophages and neutrophils, and occasionally in the type II cells that lined the alveolar septa. Occasionally, *B. bronchiseptica* antigen was present

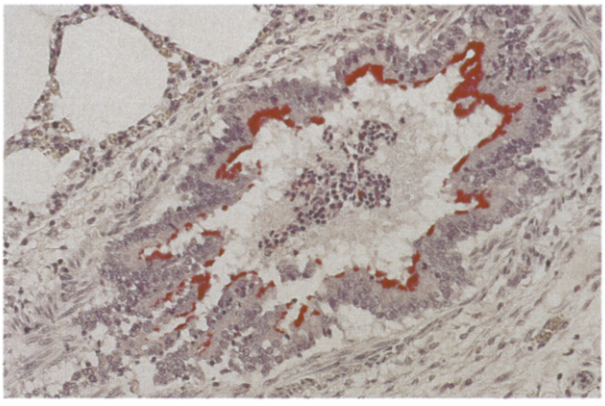

Fig. 1. *Bordetella bronchiseptica*-immunoreactivity (red) in ciliary area of epithelial cells of a bronchus. × 400.

free in the alveolar lumen, associated with small tufts of cilia; such antigen was thought to represent deposits of apical cytoplasm from bronchial or bronchiolar cells.

Infection with B. bronchiseptica *B133*

These pigs also developed a cough and ate less feed. In the first experiment two of five pigs died; none died in the second experiment. At 3 days PI, nasal washings yielded an average of $5{\cdot}7 \times 10^6$ cfu; by day 15 this number had dropped slightly to $0{\cdot}8 \times 10^6$. The clinical signs, cultural findings, Bb-IR and histopathology showed trends similar to those produced by *B. bronchiseptica* 4609 (Tables 1 and 2). Neither at day 11 nor day 15 PI were the bacterial counts in conchae, trachea, lung or tonsil from pigs inoculated with strain B133 significantly different from those of pigs inoculated with strain 4609.

Discussion

This work demonstrated that both *B. bronchiseptica* 4609 and B133 colonized and induced lesions in CDCD pigs. The parameters described in this animal model may be useful for comparing different strains of bacteria and isogenic mutants deficient in specific virulence factors (Magyar *et al.*, 1988; Bemis and Burns, 1993). The data obtained referred to both the upper and lower respiratory tract, and the brief time period (15 days) needed to assess the results seemed likely to decrease the chance of contamination by other pathogens, as well as reducing costs.

No obvious differences in the parameters studied were seen between our known virulent strain 4609 and strain B133. However, both strains induced turbinate atrophy and colonized the nasal conchae at a significantly higher level than other tissues. Bb-IR in the nasal conchae was consistently present. Lesser degrees of colonization were present in trachea, lung and tonsil. The significant decrease in colonization from day 11 to day 15 PI may have been

due to inflammatory response and ciliary damage, both of which increased with time.

Several trends were noted, some of them similar to those in previous studies (Duncan *et al.*, 1966; Magyar *et al.*, 1988; Gagne and Martineau-Doize, 1993). Conchal atrophy was not seen in pigs that died before day 11 PI; however, a moderate degree of atrophy was present at days 11 and 15 PI. Other strains of *B. bronchiseptica* may induce a greater degree of turbinate atrophy and lymphoplasmacytic inflammation (Magyar *et al.*, 1988). The high degree of immunoreactivity in the nasal conchae, trachea, bronchi and bronchioles at ≤ 11 days PI was present when there was little time for ciliary damage and loss to occur. By day 15 PI, the loss of cilia on cells lining the conchae and bronchi was associated with decreased amounts of *B. bronchiseptica* antigen. There was no obvious correlation between the degree of Bb-IR and the bacterial count; however, such a correlation was sometimes evident when these two parameters were compared on an individual pig basis (data not shown). Pulmonary fibrosis, a feature of chronic porcine pulmonary bordetellosis, was present in pigs at day 15 PI. The virulence factors responsible for pulmonary fibrosis also have not yet been identified. It is likely that the presence of *B. bronchiseptica* antigen in the cytoplasm of macrophages and neutrophils of the alveoli induced a release of cytokines, such as transforming growth factor-β (TGFβ), that play a role in the development of fibroplasia. The presence of antigen in these cells has not been previously reported.

B. bronchiseptica colonizes tonsil with difficulty, unlike *P. multocida*, which is also associated with atrophic rhinitis and replicates within the lumen of tonsillar crypts, as well as being present within the necrotic cell debris of the crypt lumen (Chanter and Rutter, 1990; Ackermann *et al.*, 1991a). In addition to the low numbers of viable *B. bronchiseptica* present in tonsil, no Bb-IR was present in tonsil sections (data not shown). Apparently, ciliated cells are required for *B. bronchiseptica* colonization and the micro-environment of the tonsillar crypt is not conducive to replication. Although *B. bronchiseptica* antigen was present in macrophages and neutrophils within the lung alveoli, a location that also lacks ciliated cells, this antigen was probably deposited by gravitational force or by air movement.

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