Adherence of *Bordetella bronchiseptica* 276 to Porcine Trachea Maintained in Organ Culture

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Two organ culture models have been adapted for porcine tracheae in order to study colonization by *Bordetella bronchiseptica*. Rings or segments excised from tracheae of newborn piglets were incubated overnight at 37°C in a nutrient medium under 5% CO₂–95% air conditions. Tracheal segments were infected with *B. bronchiseptica* 276, and after different incubation times, bacterial counts were done. *B. bronchiseptica* adhered well to tracheal maintained in culture, and no statistically significant differences between the two models were observed. Noninfected tracheal mucosa maintained a normal appearance for several days, whereas infected mucosae showed typical damage caused by *B. bronchiseptica*, namely, loss of ciliary activity and cilia and sloughing of ciliated cells. Our data indicated that porcine tracheal organ culture could be advantageously used to study in vitro colonization by *B. bronchiseptica*.

Porcine atrophic rhinitis is a multifactorial disease characterized by severe necrosis in the epithelia of the upper respiratory tract (19, 22). *Bordetella bronchiseptica* is considered a primary causative agent of atrophic rhinitis (11, 24). Infection by this microorganism is transmitted by aerosol droplets, and the bacteria can be localized on respiratory epithelial cells; the bacteria are noninvasive and remain on the respiratory surfaces. The bacterium excretes a tracheal cytotoxin which is known to be ciliostatic and which causes extrusion of ciliated cells from tracheal organ cultures (4, 26). *B. bronchiseptica* is known to adhere preferentially to ciliated epithelial cells (24). Although the mechanism is not completely understood, this property of adherence seems to be an important first step for successful colonization and infection of the porcine nasal cavity (19).

Numerous assays have been used to study the adherence of *Bordetella* species to respiratory cells. Host specificity was demonstrated with an in vitro assay of the adherence of *Bordetella* species to tracheal ciliated cells from different species (9, 25). Ciliated epithelial cell outgrowths from canine respiratory tracts were used to test the effect of *B. bronchiseptica* on ciliary activity (5) and to verify the influence of virulence determinants on induced ciliostasis (6). Adherence of *B. bronchiseptica* on hamster lung fibroblasts was also studied (20). Swine nasal epithelial cells were used to investigate in vitro adherence of *B. bronchiseptica* (12, 13, 17, 27) and to compare the adherence of *B. bronchiseptica* and *Pasteurella multocida*, the other causative agent of atrophic rhinitis (16, 19).

Tracheal organ cultures permit the study of the pathogenesis of organisms which damage mucosal cells by attachment and toxin production (18). Two main models have been proposed for maintaining tracheae in organ culture: the tracheal ring method (8) and the matrix-embed/perfusion technique (10). They have both been used almost exclusively to maintain rodent tracheae in culture (3, 8, 10, 24). Aside from the work of Roop et al. (21), who studied attachment of radio labeled *B. bronchiseptica* cells to tracheal rings after an incubation time of 2 h, no studies have looked extensively for the potential use of porcine tracheal organ culture to study the *B. bronchiseptica* colonization process.

The purpose of this study was to adopt the two aforementioned models for porcine tracheae to quantitate the adherence of *B. bronchiseptica* and evaluate the damage this microorganism causes to the upper respiratory tract epithelium.

**MATERIALS AND METHODS**

**Bacterial strain and growth conditions.** Strain 276 of *B. bronchiseptica*, originally isolated from a pig with severe atrophic rhinitis (23), was provided by J. M. Rutter, Institute for Research on Animal Diseases, Compton, United Kingdom. Bacteria, grown overnight on blood agar plates at 37°C, were suspended in Eagle minimal essential medium (MEM) at the desired concentrations (10⁶, 10⁵, or 10⁴ CFU/ml). The suspensions were diluted and 0.1-ml samples were plated on blood agar to obtain exact inoculum counts.

**Tracheal rings method (method A).** Tracheal ring cultures were prepared by the methods of Collier (8) and Baker and Marcus (3). Tracheae were excised from newborn piglets and then divided into rings between the cartilage with a sterile scalpel. The rings were placed in 35-mm tissue culture dishes and incubated overnight at 37°C in a 5% CO₂–95% air atmosphere with 1.5 ml of MEM containing penicillin (500 U/ml) and streptomycin (500 μg/ml).

**Matrix-embed/perfusion culture technique (method B).** The model described by Gabridge and Hoglund (10) was adapted for porcine tracheae. The modified device that held the trachea was a Teflon block (3.2 by 7.6 by 1.3 cm) (Fig. 1). The perfusion blocks were sterilized in 70% ethanol and air dried in a laminar flow hood just before use. The tracheae were excised from newborn piglets, surplus mucus was removed, and the tracheae were divided into 12- to 15-mm segments and placed in the perfusion blocks. The openings of the tracheae were sealed by two sterile Teflon plugs placed in the wells. MEM containing 1.5% molten agarose was poured slowly over the segments, providing them with nutrients and holding them in place. The plugs, along with any fragments of agarose remaining in the wells or in the

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tracheal lumen, were removed. MEM (3.0 ml) containing antibiotics was then poured into the wells. Each perfusion block was placed in a 100-mm plastic petri dish and held in place by 30 ml of 1.5% agar, which provided moisture to the culture system and facilitated handling. A plastic lid was used to maintain the sterility of the system. The petri dishes were then incubated overnight at 37°C in a 5% CO₂-95% air atmosphere on a rocker platform (Belloco Biotechnology, Vineland, N.J.) set at 3 cycles/min.

Evaluation of ciliary activity. At each step of the experiment, the rings prepared by method A were examined for ciliary activity by observation through an inverted microscope. Ciliary vigor was expressed as normal (+ + +), reduced (+ +), very reduced (+), or absent (−). Before infection, only rings with normal vigor, i.e., those which exhibited ciliary activity over more than 80% of the epithelial border, were used for the experiment. Tracheal segments prepared by method B were cut into rings at the end of the experiment, and therefore, ciliary activity could be evaluated only at that moment.

Infection. Rings and segments were rinsed two to three times with fresh MEM without antibiotics and infected with the bacterial suspension. After a given period, the ciliary activity was evaluated, and some rings were prepared for bacterial counts, histopathology, or electron microscopy. For the matrix-embed/perfusion system, central rings were processed.

Bacterial counts. Rings were washed once in phosphate-buffered saline (0.01 M, pH 7.2) to remove nonadherent bacterial cells. Rings were then placed in 2.0 ml of phosphate-buffered saline containing 1% Triton X-100 (1) and agitated vigorously for 1 min with a mixer (The Vortex Manufacturing Co., Cleveland, Ohio). Dilutions were made and inoculated onto blood agar plates. To permit comparison of adherence among different assays, an adherence index was modified from the work of Arp and Brooks (1) to compensate for slight variations in concentrations of bacterial inocula: antilogarithm_{10} of [log_{10} CFU/ring] − [log_{10} CFU/ml of inoculum] + 4).

Results were compared for statistical significance by using Student’s t test.

Histopathology. Tracheal rings were fixed in buffered Formalin, embedded in paraffin, and cut into 6-μm sections.

Sections were stained with hematoxylin-phloxin-saffron and Gram stained.

Scanning electron microscopy. Tracheal rings were prepared for scanning electron microscopy as previously described (7). Samples were fixed in 5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.0) at room temperature for 2 h. They were dehydrated through graded ethanol from 30 to 100%, critical-point dried in CO₂, mounted on aluminum stubs, and sputter coated with gold-palladium. Samples were then examined with a microscope (S-530; Hitachi) at an accelerating voltage of 20 kV.

Transmission electron microscopy. Tracheal rings were prepared for transmission electron microscopy as previously described (15). Briefly, rings fixed as mentioned above were postfixed with 2% osmium tetroxide for 2 h and dehydrated in a graded series of acetone washes. Samples were then washed twice in propylene oxide and embedded in Spurr low-viscosity resin. Thin sections were poststained with uranyl acetate and lead citrate and examined with an electron microscope (Philips 2011) at an accelerating voltage of 60 kV.

RESULTS

Two organ culture models, the tracheal rings method and the matrix-embed/perfusion technique, were adapted for porcine tracheae and were used to study colonization by *B. bronchiseptica* 276. Adherence of *B. bronchiseptica* was quantitated, and the results obtained with both organ culture models according to the inoculum size and the incubation time are given in Tables 1 and 2, respectively. The inoculum size varied from 10^7 to 10^9 CFU/ml. The bacterial strain adhered to porcine tracheae in a concentration-dependent manner; a plateau was reached at around 10^8 CFU/ml, and a concentration of 10^9 CFU/ml was chosen for all further experiments. We found with the tracheal rings method that

<p>| TABLE 1. Effect of inoculum size on the adherence of B. bronchiseptica 276 to porcine trachea |
|---------------------------------|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Inoculum size (CFU/ml)</th>
<th>Adherence index ± SD^a</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^7</td>
<td>19 ± 20</td>
<td>26 ± 25</td>
<td>&lt;1</td>
</tr>
<tr>
<td>10^8</td>
<td>181 ± 191</td>
<td>26 ± 25</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>10^9</td>
<td>148 ± 140</td>
<td>11 ± 4</td>
<td>11 ± 4</td>
</tr>
</tbody>
</table>

^a Mean results of two different experiments in which to tracheal rings were quantitated each time. Rings were maintained in culture by the tracheal rings method (method A) and the matrix-embed/perfusion technique (method B).

<p>| TABLE 2. Effect of incubation time on the adherence of B. bronchiseptica 276 to porcine trachea |
|---------------------------------|---------------------------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Adherence index ± SD^a</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>41 ± 21</td>
<td>25 ± 20</td>
<td>25 ± 20</td>
</tr>
<tr>
<td>8</td>
<td>196 ± 231</td>
<td>16 ± 16</td>
<td>16 ± 16</td>
</tr>
<tr>
<td>24</td>
<td>358 ± 285</td>
<td>129 ± 206</td>
<td>129 ± 206</td>
</tr>
<tr>
<td>48</td>
<td>1,057 ± 1,244</td>
<td>92 ± 126</td>
<td>92 ± 126</td>
</tr>
</tbody>
</table>

^a Mean results of three different experiments in which two tracheal rings were quantitated each time. Rings were maintained in culture by the tracheal rings method (method A) and the matrix-embed/perfusion technique (method B).
adherence of *B. bronchiseptica* increased slightly with time; the increase was not observed with the matrix-embed/perfusion technique. Although the mean adherence was greater with the tracheal rings method, the differences observed between the two models were statistically insignificant (*P* > 0.25). However, we should be very careful with the interpretation of the statistical analysis, considering the small number of samples involved. By using the tracheal rings method, we successfully maintained some rings for up to 16 days and were able to detect adherent *B. bronchiseptica* for the complete duration of the experiment (data not shown).

Since identical results were obtained for the two models studied, the following observations on ciliary activity evaluation, histopathology, and electron microscopy applied to...
both models. We compared the ciliary activity of noninfected and infected rings and found that the former retained their normal ciliary activity (+ + +) for several days after being in culture while the latter progressively lost their activity (from +++ to +) within 7 h after infection with *B. bronchiseptica* 276. At various incubation times, some rings were Formalin fixed and prepared for histopathology. Control, noninfected rings showed a normal mucosa covered by a ciliated epithelium (Fig. 2A); rings which were in contact with *B. bronchiseptica* for 4 h had the same appearance, and gram-negative rods were seen at the apices of ciliated cells (Fig. 2B). After 7 h of incubation with *B. bronchiseptica* 276, the mucosa showed some degree of necrosis with some gram-negative rods associated with the remaining cilia (Fig. 2C). After 24 h or more of contact with the bacterium, the epithelium was completely ulcerated and cellular debris was present in the lumen (Fig. 2D).

Other rings were fixed and prepared for scanning electron microscopy. A control ring, in culture for 72 h, showed a normal mucosa entirely covered by ciliated cells (Fig. 3A). Rings infected with the bacteria from 1 to 72 h looked almost the same, but they were less ciliated than noninfected rings, and many rod-shaped bacteria were associated with the cilia, occasionally forming microcolonies (Fig. 3B and C). A 24-h-infected ring had a totally different appearance; some cells were sloughing from the surface, and there was a scarcity of cilia and some bacterial association with the remaining cilia (Fig. 3D). Transmission electron microscopy was also performed on infected tracheal samples. Four to seven hours after infection, gram-negative rod-shaped bacteria were seen in the interciliary space (Fig. 3E). Damage to the rings infected for 24 h was extensive (Fig. 3F). Bacteria were seen in contact with the remaining cilia, apparently bound by thin filaments radiating from the bacterial cells. These filaments may represent fimbriae, since *B. bronchiseptica* 276 is fimbriated (Fig. 4). Consistent microscopic lesions of the tracheal mucosa colonized by *B. bronchiseptica* include intimate attachment of the bacteria to the cilia, leading to formation of microcolonies and eventual loss of ciliated epithelium.

**DISCUSSION**

We adapted two organ culture models, the tracheal rings and the matrix-embed/perfusion chamber, to study the ad
herence of *B. bronchiseptica* 276 and to evaluate its effect on the epithelium of the porcine upper respiratory tract. Both systems appeared to be adequate models for such a study. However, the matrix-embed/perfusion system seemed to reproduce in vivo conditions more closely than did the tracheal rings method (2). Use of tracheal segments instead of rings eliminates some factors that could provide nonspecific attachment; bacteria can attach to the outside of the trachea, and the scalpel creates some tracts at the end of the tracheal ring which may entrap bacteria so that they cannot be removed from these areas, even after many rinses (10). Also, since the rings are kept in the medium without any movement, a resultant mucus accumulation could affect adherence and prevent proper contact with oxygen, which could affect the metabolism of the cells. By keeping the perfusion blocks on a rocker platform, these inconveniences can be eliminated.

Adherence of *B. bronchiseptica* 276 was studied with the two organ culture models. No statistically significant differences were observed between the two models, although adherence to the tracheal rings appeared greater. The factors mentioned above that may lead to attachment of the bacteria to sites other than the tracheal epithelium may explain the difference observed between the two models.

Another purpose of this work was to evaluate the effect of the adherence of *B. bronchiseptica* 276 to the epithelium. Inverted microscopy showed that good ciliary activity could be preserved in control, noninfected rings, while infected rings lost this activity almost completely after a few hours; histopathological observations confirmed these results by showing marked differences in the appearance of the epithelium fixed at different times after infection with this strain. Scanning electron microscopy confirmed the association of the bacteria with ciliated cells, particularly with the cilia. Bovine erythrocyte agglutinin, a nonfimbrial surface molecule, has recently been identified as a possible adhesin responsible for binding to porcine nasal epithelium (14). The role of *B. bronchiseptica* fimbriae is not clear; the present work represents the first observation of the involvement of thin filaments in the colonization process. Damage typically associated with *B. bronchiseptica* infection was reproduced in both models of organ culture. In conclusion, we consider that both models may be advantageously used to evaluate the adherence of *B. bronchiseptica* as well as other porcine respiratory pathogens to porcine epithelium of the upper respiratory tract.

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**LITERATURE CITED**

Adherence of *B. bronchiseptica* to porcine trachea.

