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Two Types of Bacteria Adherent to Bovine Respiratory Tract Ciliated Epithelium

A. T. HASTIE, L. P. EVANS, AND A. M. ALLEN

Department of Medicine, Thomas Jefferson University, Philadelphia, PA (ATH, LPE); and Microbiological Associates, Inc., Rockville, MD (AMA)

Abstract. Two hundred sixty tracheas were obtained from a Philadelphia abattoir under permit from the Department of Agriculture; the tracheas were excised from predominantly Holstein calves of both sexes that weighed approximately 250 kg. Tracheas were transported in normal saline to the laboratory at Thomas Jefferson University, Philadelphia, Pennsylvania. Evidence of bacteria adherent to the tracheal epithelium was found in specimens from 20/24 of these tracheas. The epithelium from each of five tracheas was placed in glutaraldehyde fixative for transmission electron microscopic examination. Epithelium from each of 12 other tracheas was placed in formaldehyde fixative for light microscopic examination. Microscopically, 13 of these 17 bovine tracheal epithelia were observed to contain bacteria located longitudinally parallel to and between cilia and microvilli of ciliated cells. Preparations of ciliary axonemes isolated from the epithelium of seven additional bovine tracheas also contained these bacteria in sections viewed by a transmission electron microscope. These bacteria had two different ultrastructural morphologies: filamentous with a trilaminar-structured cell wall and short with a thick, homogeneously stained cell wall beneath a regularly arrayed surface layer. The short bacillus had surface carbohydrates, including mannose, galactose, and N-acetylgalactosamine, identified by lectin binding. The filamentous bacillus was apparently externally deficient in these carbohydrates. Irnrnunogold staining revealed that the filamentous bacillus was antigenically related to cilia-associated respiratory (CAR) bacillus, which has been identified in rabbit and rodent species. Significantly decreased numbers of cilia were obtained from tracheal epithelium heavily colonized by the filamentous bacilli, suggesting a pathologic change in ciliated cells.

Key words: CAR bacillus; cattle; cilia; lectins.

Cilia-associated respiratory (CAR) bacillus has been reported in rats, mice, guinea pigs, and rabbits.[7,13,14,16,24] In infections characterized by bronchitis, bronchiectasis, and mucopurulent pneumonia, this bacillus densely covers ciliated epithelial cells.[7,15,24] It has been transmitted experimentally[1-3] and passaged in chicken embryo allantoic fluid;[1] but has not been cultured in cell-free media.[1] Immunofluorescent and enzyme-linked immunosorbent assays have been developed for the detection of CAR bacillus infection and immune response.[11,12] In the course of other studies concerning lectin binding to respiratory tract cilia[2] and the composition of the ciliary membrane,[4] a bacterium identical to the CAR bacillus was observed for the first time in cattle. In addition, a second type of bacterium, morphologically distinct from the filamentous CAR bacillus, was observed adhering to the bovine tracheal ciliated cells. Epithelia heavily infected with the filamentous bacillus yielded decreased numbers of ciliary axonemes, suggesting a pathologic alteration of the ciliated cells.

Materials and Methods

Two hundred sixty bovine tracheas, usually in groups of four to six on each occasion, were obtained over a 5-year period by permit from the Department of Agriculture from an abattoir in Philadelphia. Twenty-four of these tracheas constituted the source of epithelial tissue on which the present report is based. These 24 trachea have been assigned consecutive case numbers based on the date of acquisition: case No. 1, 9/23/88; 2, 10/5/88; 3, 10/20/88; 4, 12/16/88; 5, 2/15/89; 6, 3/1/89; 7, 3/5/90; 8–9, 3/22/90; 10, 3/28/90; 11, 4/18/90; 12, 6/6/90; 13-16, 6/14/91; 17-20, 6/19/91; and 21-24, 6/26/91. The cattle from which the tracheas were excised were predominantly Holstein of both sexes and approximately 200 kg and were obtained from farms in Pennsylvania, Maryland, and other nearby states. Animals accepted by Meat and Poultry Inspectors were exsanguinated. The tracheas were excised and immediately transported in normal saline (0.9% NaCl) to the laboratory (Thomas Jefferson University, Philadelphia, PA). A second vigorous rinse with fresh saline was performed to remove surface debris and secretions. A small section of epithelium was excised for

electron microscopic observation on trachea Nos, 7- 10 and 12 or for light microscopic observation on trachea Nos. 13-24. The remainder of each of these and the entire length of all the other tracheas were used for isolation of cilia" or ciliary axonemes,? which were extracted from the epithelium and separated from cell debris by differential centrifugation. Over the past 8 years, We have routinely examined and video recorded cilia and ciliary axoneme preparations under oil immersion phase contrast microscopy for axoneme yield, ciliary activity by the addition of ATP and contamination. Preparations were examined for identifiable bacterial cells and epithelial cell debris as causes of contamination." Estimates of yield (cilia or ciliary axoneme numbers per videomonitor field) were scored as excellent (> 300/field), good (l50-300/field), fair (50-I SO/field), or poor « SO/field). A microscopic field on the video monitor measured 86 x 110 μm at a magnification of 2,200x, Criteria were employed for assuring acceptable ciliary axoneme material to be used in various experiments; any preparations that had fewer than 150 axonemes/field, were inactive in ciliary beating, and had unacceptably high concentrations of contamination were not used for further study.

Electron microscopy

Epithelial tissues from trachea Nos. 7- 10 and 12 were placed in 2,5% glutaraldehyde in phosphate buffered saline (PBS, pH 7.4) at 4 C, post-fixed in 1% osmium tetroxide, dehydrated through a series of graded ethanols, and embedded in Spurr's low viscosity medium. Thin sections were stained with uranyl acetate and lead citrate and examined by an Hitachi H7000 transmission electron microscope at 75 kV.

Measurements of bacterial diameter and length were made only on those cells that were sectioned exactly perpendicular or parallel to their long axis. Diameters were measured on bacteria from four different tracheas, Nos. 1, 2, 8, and 9, and lengths were derived from bacteria on trachea No, 9. The limiting thickness of the sections (approximately 60 nm) and the requirement that the entire length of the bacterium appear in the section may have biased the measurements in favor of filamentous bacilli with shorter dimensions. Quantitation of bacteria per length of epithelial tissue was derived from trachea No. 9 with both bacterial types adherent and from trachea No. 10 with only the short bacilli adherent.

Immunostaining of filamentous bacilli in ciliary axoneme preparations from bovine trachea Nos. 9 and 16 was performed on fornvar-carbon-coated copper grids in duplicate. Aliquots of the two axoneme preparations in buffer (20 mM Tris-HCI, pH 8.0, 50 mM KCl, 4 mM $MgSO_4$, I mM dithiothreitol, and 0.5 mM ethylenediaminetetraacetic acid) that contained the bacteria were placed on the grids, and excess fluid was removed by filter paper. The grids were incubated in a 1/50 dilution of rat anti-CAR bacillus antiserum in 0.5% ovalbumin in PBS for I hour. The antiserum was produced in five weanling female seronegative F344/NCr rats by intranasal inoculation of CAR bacillus. The antiserum was serologically tested and found negative for antibody to nine infectious agents *(Mycoplasma pulmonis*, Kilham rat virus, lymphocytic choriomeningitis virus, mouse adenovirus, pneumonia virus of mice, rat coronavirus [RCV/SDAj, reovirus type 3, Sendai virus, and Toolen virus HI) of rats other than CAR bacillus. The grids were rinsed and then incubated in 1/50 dilution of goat anti-rat IgG-coated colloidal gold particles (10 nm size; E·Y Laboratories, San Mateo, CA) in 0.5% ovalbumin in PBS for I hour. After rinsing, each grid was stained with I% aqueous uranyl acetate, Control grids were similarly treated except for a substitution in the first incubation step with rat serum negative for CAR bacillus antibody. This control serum was from a F344/NCr rat that came from a CAR bacillus-negative colony but was not otherwise kept in germ-free conditions.

Aliquots of ciliary axoneme preparations from three tracheas, Nos. I, 2, and 4, were incubated with colloidal gold particles coated with lectins from *Canavalia ensiformis, Bauhinia purpurea*, and *Dolichos biflorus* (E·Y Laboratories, San Mateo, CA), pelleted by centrifugation, washed, and repelleted. Both nontreated and lectin-treated axonemal pellets were processed for embedding, sectioning, and staining as described above. The axoneme preparation from trachea 4 had some short bacilli, but there were too few bacteria per section for quantitation. The other two axoneme preparations, from trachea Nos. I and 2, had sufficient numbers of short bacilli for scoring. Only the axoneme preparation from trachea No, 2 had very limited numbers of the filamentous bacilli for quantitation (proportionately < 1% of the number of ciliary axonemes). No distinction was made in the number of apparent lectin binding sites per bacterial cell because this number would be influenced by the extent of bacterial surface area in the section. Approximately 50 cells of the short bacillus were scored (+ or - lectin) for each of the three lectin types in each axoneme preparation from trachea Nos. I and 2 (presented as mean ± standard deviation). Approximately 30 cells of the filamentous bacillus were scored (+ or - lectin) for the three lectin types in the axoneme preparation from trachea NO.2.

Light microscopy

From 12 tracheas, Nos. 13-24, obtained on three separate days (four each day), small sections of epithelium, 0.5- x l-cm piece per trachea, were excised and placed in 10% formalin in PBS (pH 7.4). The remainder of each trachea was treated for isolation of ciliary axonemes.? Axoneme yield in equal aliquots from each tracheal preparation was scored as excellent, good, fair, or poor on the basis of approximate axoneme number/videomonitor field (> 300, 150-300, 50-ISO, or < 50, respectively) from oil-immersion phase-contrast light microscopic videorecordings. These estimates were generated without prior knowledge by the investigators (A. T. Hastie or L. P. Evans) of bacterial infection of the epithelium. The excised pieces of epithelium were embedded in paraffin and sectioned and stained by the Warthin-Starry technique and with hematoxylin and eosin. These pieces were scored for the level of filamentous bacterial infection (+++ = heavily infected; ++ = moderately infected; + = lightly infected; or 0 = none) without knowledge by the investigator (A. M. Allen) concerning yields of ciliary axonemes from each trachea. Statistical analysis of the data was performed by determination of the rank correlation coefficient.[18]

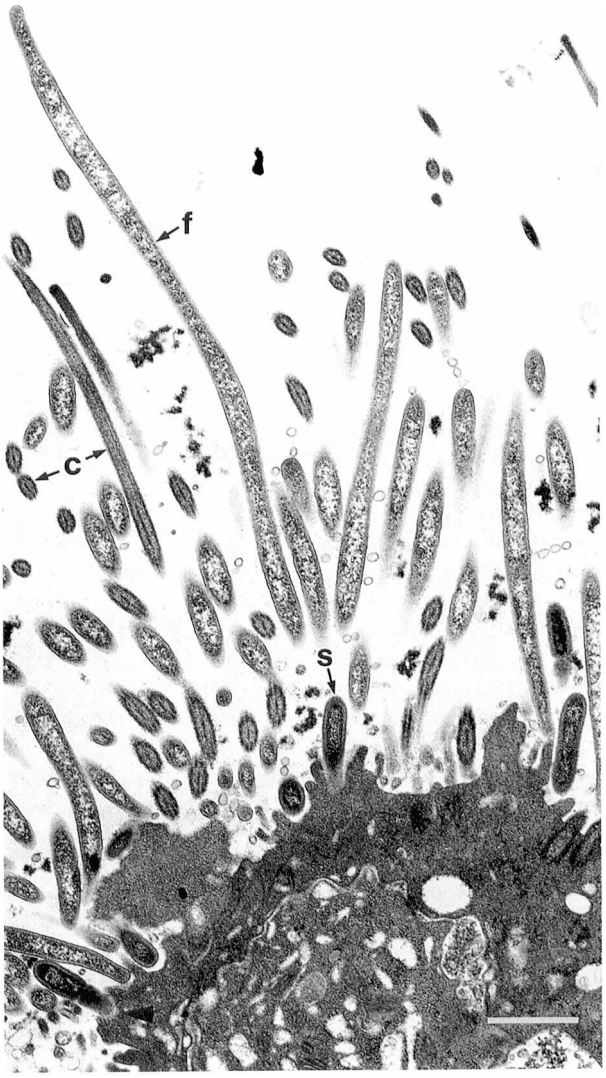

Fig. 1. Transmission electron micrograph. Tracheal epithelium; cow. Two types of bacteria are adherent to the epithelial surface. Many filamentous bacilli (l) and, less frequently, short bacilli (s) are located among the cilia (c) at the epithelial cell's luminal surface. Fewer cilia are present than in normal tracheal epithelium. At the lower left is a short bacillus possibly undergoing cell division (arrowhead). Bar = 1 urn.

Results

On bovine tracheal epithelia, two morphologically distinct types of bacteria were observed, one short and the other elongated or filamentous (Fig. I). These two bacterial types were present as determined by electron microscopic observation on epithelia of five tracheas, Nos. 7-10 and 12, obtained on four separate occasions. Two tracheas, Nos. 9 and 12, contained both bacilli; two, Nos. 7 and 8, contained only filamentous bacilli; and one, No. 10, contained only the short bacillus. Either one or both bacterial forms have also been found by electron microscopic observation in seven different preparations of cilia or ciliary axonemes extracted from bovine trachea Nos. 1-6 and lion seven other dates, suggesting that the microorganisms were present on these tracheal epithelia as well. Three axonemal preparations, from trachea Nos. 2, 5, and 6, contained both bacterial types; three preparations, from trachea Nos. 1,4, and 11, contained only the short bacillus; and one preparation, from trachea No.3, contained only the filamentous type. The II dates on which these 12 trachea were obtained were scattered throughout a 3-year period, without any apparent seasonal occurrence.

Both bacterial types were found interspersed among and lying longitudinally parallel to microvilli and cilia of ciliated cells. The diameters (mean ± standard deviation) of the two types of bacteria, 214 ± 27 nm for the short bacilli (n = 13) and 253 ± 35 nm for filamentous bacilli (n = 88), were similar to that of cilia, approximately 250 nm. Although both were bacilli, the length of the shorter one was 0.85 ± 0.12 urn (n = 10), whereas the length of the other was 4.3 ± 0.7 urn (n = 17) for those bacterial cells contained entirely within a section and apparently extending as far as the cilia from the epithelial surface for others. The short bacillus was often observed with a constriction in diameter suggesting cell division, but no constrictions were observed in the filamentous bacilli (see Fig. I).

There were substantially greater numbers of filamentous bacteria per length of epithelial surface in two tracheas, Nos. 9 and 12, colonized by both types of bacteria. Quantitation of bacteria in trachea No. 9 revealed a total of 376 filamentous bacilli and 29 short bacilli along five different lengths of epithelium with a total length of 174 urn. The epithelial surface in trachea No. 10 was colonized only by the short bacilli, 70 of which were counted along six different epithelial lengths with a total length of 218 urn.

Far fewer cilia, either in longitudinal or cross section, were observed on epithelium colonized by both bacilli (227 cilia per 174 μm) compared with epithelium colonized only by the short bacilli (1,567 cilia per 218 um).

Besides bacterial cell length and density on the epithelial surface, the two bacterial types also differed in other characteristics. The cell wall structure of the filamentous bacillus was trilaminar, resembling that of gram-negative bacteria (Fig. 2). In contrast, the cell wall structure of the shorter bacillus was thick and homogeneously stained beneath a regularly arranged surface array, resembling the cell wall of gram-positive bacteria. The surface array was best observed in oblique sections as peripheral beading, regularly spaced at an average of 13.1 nm (Fig. 3).

The filamentous bacterium retained its trilaminar cell wall structure unaltered during the extraction process for preparation of ciliary axonemes that removes

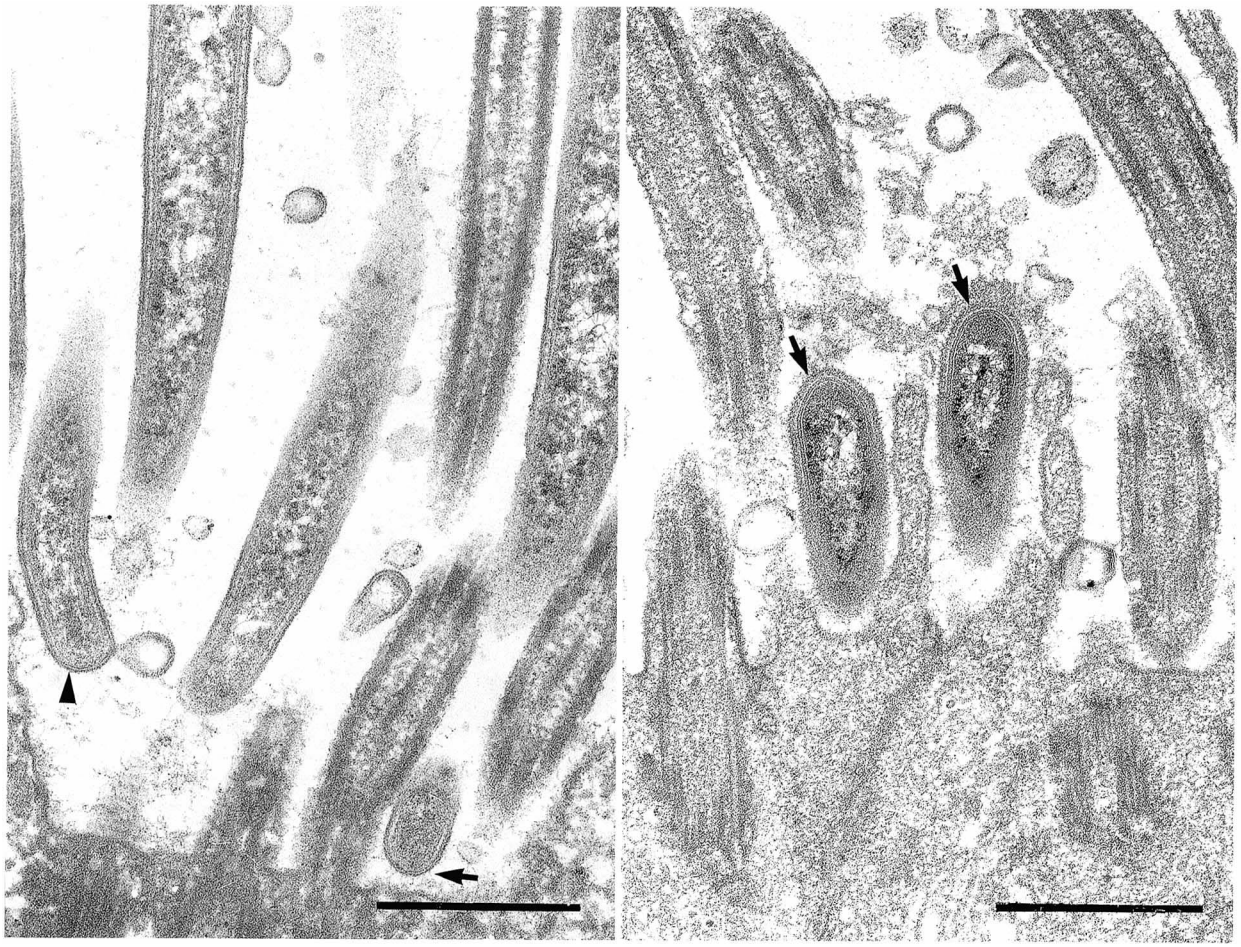

Fig. 2. Transmission electron micrograph. Tracheal epithelium; cow. Filamentous bacilli are adhered to a ciliated cell. The cell wall of the filamentous bacillus is trilaminar (outermost layer dark, light, dark; arrowhead). Thin fibers (arrow) extend between the bacterial cell wall at the lower right edge and the ciliated cell membrane. Bar = 0.5 μm.

Fig. 3. Transmission electron micrograph. Tracheal epithelium; cow. Short bacilli are adhered to a ciliated cell. The cell walls of the short bacilli are thick and homogeneous beneath a regularly arrayed surface layer apparent in oblique sections of the cells (arrows). Bar = 0.5 μm.

the ciliary membrane by detergent solubilization (0.1% Triton X-100, Fig. 4a). The cell wall structure of the short bacillus also remained intact, including the surface array not seen in cross section (Fig. 4b) but observed in oblique sections (Fig. 3).

Ciliary axoneme preparations that contained the filamentous bacilli were examined for immunoreactivity of rat anti-cilia-associated respiratory (CAR) bacillus antiserum to the filamentous microorganism from cattle. The antiserum showed positive recognition of all of the filamentous bacteria, whereas rat serum negative for CAR bacillus antibody did not react with any above background concentrations ($n > 30$ bacterial cells in each category, Fig. 5).

Lectins from *Canavalia ensiformis, Bauhinia purpurea,* and *Dolichos bijlorus* adhered to the surface of the short bacillus, indicating surface carbohydrates that had not been removed by the treatment with detergent. The *Canavalia* lectin, which has affinity for mannose, was attached most frequently: 58 ± 12 (mean ± standard deviation) short bacilli with lectin, 8 ± 8 without. The *Bauhinia* lectin, which has affinity for N-acetylgalactosamine and galactose, was present on 34 ± 5 short bacilli and absent on 18 ± 6. *Dolichos* lectin, which also recognizes N-acetylgalactosamine, was less frequent; it bound to 18 ± 1 short bacilli and was absent on 36 ± 0. Thus, the nearly double amount of *Bauhinia* lectin binding was presumably due to galactose. In contrast, neither *Canavalia* nor *Bauhinia* leetins were attached to the surface of the filamentous bacterium (*0/30* and *0128* cells, respectively). Only three filamentous cells bound *Dolichos* lectin compared with 28 that did not.

Discovery of the presence of a CAR bacillus-type bacterium in cattle and the observation of decreased numbers of cilia on epithelium with adherent filarnen-

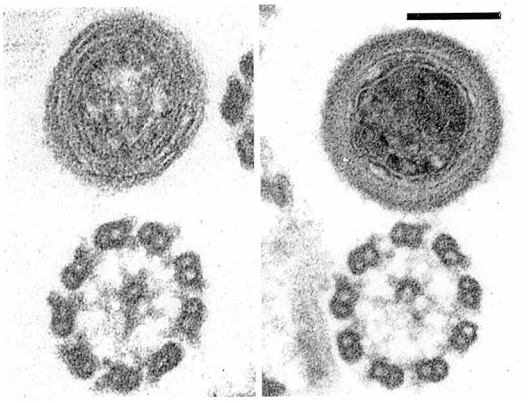

Fig. 4. Transmission electron micrographs, Isolated ciliary axoneme preparations; cow. Filamentous bacillus (Fig. 4a) and short bacillus (Fig. 4b) are present in the upper half of micrograph and cross sections of isolated ciliary axonemes are present in the lower half. The axonemes are cross sections at midshaft between the basal plate and the cap at the distal tip. One of the central singlet microtubules of the axoneme is lost and the ciliary membrane is removed during the process of extraction from the epithelium. The morphology of the bacterium appears unaltered. The regularly arrayed surface layer of the short bacillus (see Fig. 3) is not readily observed in cross section (Fig. 4b), Bar = 0.1 μm in both.

tous bacilli prompted assessment of a possible correlation. An additional 12 bovine tracheas, Nos. 13-24, obtained on three separate occasions were examined by Warthin-Starry staining for the filamentous bacilli (Fig. 6). Preparations of ciliary axonemes obtained from these tracheas were also scored for yield from equivalent lengths of trachea. The results (Table 1) reveal that the filamentous CAR bacillus-type bacteria were present as heavy infections in three of the 12 tracheal samples and as light infections in another five of the 12. Statistical analysis of only those trachea with filamentous bacilli present (n = 8) shows a significant inverse correlation (r = - 0.881, P < 0.005) between increased extent of infection and lower yields of ciliary axonemes. Analysis that included those tracheas without filamentous bacilli confirmed the inverse correlation (r = - 0.405) but was not sufficient to achieve significance for n = 12.

Discussion

Bacillus species, not further identified, have been reported as the most frequent type of bacteria isolated in a study of microflora in the lungs of apparently healthy cattle;" however, this early study examined isolates from swabs or tissue homogenates only and did not include histologic observations. Furthermore, only cell-free culture of isolates was used, which excluded isolation and identification of a cilia-associated respiratory (CAR) bacillus-type microorganism, if present." The present report of two distinct types of bacilli

Fig. 5. Transmission electron micrographs. Ciliary axoneme preparations; cow. Fig. 5a. Filamentous bacilli in control preparations immunostained with rat antiserum negative for cilia-associated respiratory (CAR) bacillus, followed by colloidal gold coated with anti-rat antibody and then uranyl acetate stain. Fig. 5b. The bacilli in duplicate samples are similarly treated, although a substitution with rat anti-CAR bacillus serum is made in the first incubation step. Note the dense covering of the bacterium with round, densely black, gold particles revealing the presence of the rat anti-CAR bacillus antibodies in this figure compared with only background level staining in Fig. 5a. Bar = 0.2 μm in both.

adhering to bovine tracheal epithelium is a consequence of other studies regarding the surface components of ciliated cells.[10,12]

The morphologic characteristics of the short bacillus-a thick homogeneous cell wall and regularly spaced surface array-distinguish it from the filamentous CAR bacterium[7,24] and other microorganisms, including *Bordetella pertussis* and *B. bronchiseptica.i-" Mycoplasma pneumoniae* and *M. pulmonis.i-" Pseudomonas aeruginosa," and Klebsiella pneumoniae,"* which are capable of adhering to ciliated epithelium. The surface array repeat is similarly spaced (13 nm), as observed in *Bacillus sphaericus* NTCC 9602,[8] and may

Fig. 6. Tracheal epithelium; cow. The epithelium is sectioned perpendicularly to the epithelial surface. Fig. 6a. Trachea No. IS. Filamentous bacilli are present in densely packed numbers on the luminal surface. Fig. 6b. Trachea No. 16. Single filamentous bacilli are present on ciliated epithelial cells in a selected section of lighter infection adjacent to more heavily infected regions. Warthin-Starry method. Bar = 10 μm in both.

also be a glycoprotein because mannose, galactose, and N-acetylgalactosamine were identified by lectin binding to the bacterial surface. Glycoprotein surface arrays potentially mediate adherence.[1,21] Carbohydrate ligand and receptor positions would be the reverse of those found in *Bordetella pertussis* and *M. pneumoniae* adherence, which apparently involve specific bacterial adhesion or lectin recognition of epithelial cell surface galactose and sialic acid, respectively.[t-"]

The morphological characteristics of the filamentous bacterium and its colonization of ciliated epithelial cells corresponds exactly with the description of the CAR bacillus.[l-":"] Moreover, the filamentous bacilli in cattle were shown to be antigenically related to the CAR bacillus that infects rats. The lack of lectin binding to the filamentous bacilli indicates negligible surface polysaccharides consisting of mannose, galactose, and N-acetylgalactosamine. A negative periodic acid-Schiff reaction was observed with CAR bacillus from rodents.[''] The detergent treatment in extraction of the ciliary axonemes may have removed noncovalently bound surface carbohydrate from the filamentous bacilli; however, there was no obvious disarrangement of the outer membrane cell wall structure, and recognition of antigenic components was still possible.

Table 1. Estimated yield of cilia and cilia-associated respiratory (CAR) bacillus infection* in bovine tracheae.

Trachea No.	Estimated Ciliary Yield†	Estimated CAR Bacillus Infection‡
Collected 6/14/91		
13	Fair (138)	0
14	Good (206)	+
15	Fair (109)	+++
16	Poor (36)	+++
Collected 6/19/91		
17	Poor (47)	+++
18	Excellent (330)	+
19	Good (186)	0, ME
20	Good (213)	+
Collected 6/26/91		
21	Good (174)	0
22	Excellent (404)	+
23	Fair (135)	+
24	Poor (49)	0

* Yield of ciliary axonemcs and concentration of CAR bacillus infection were ranked from highest to lowest for those eight tracheas. Nos. 14-18, 20, and 22-23, observed to contain the filamentous bacilli. The rank correlation coefficient was $r = -0.881$ ($P < 0.005$).

† Excellent = >300 axonemes/field; good = 150-300; fair 50-150; poor = <50. Mean estimated number of axonemes taken from two different videorecorded light microscopic fields per preparation is given in parenthesis.

‡ +++ = heavy infection; ++ = moderate infection; + = light infection; 0 = no infection. ME = missing epithelium.

In addition to morphological and biochemical differences between the short and filamentous bacilli, the numbers of each type adhering to the epithelial surface was disparate; the filamentous bacilli greatly outnumbered the short bacilli. Even in the absence of competition with the filamentous bacilli, the number of adherent short bacilli increased only twofold per epithelial surface length. This observation may reflect unique binding sites for each bacterial type.

The identification of cattle as hosts for CAR bacillus indicates a broader host range than previously thought. This observation, together with the reported inability to culture the bacillus in cell-free medium,'' poor staining with conventional histologic dyes such as Gram's and hematoxylin and eosin stains, and exceptionally similar appearance to cilia at its epithelial attachment site.' raises the concern that this microorganism may be undetected in many cases and thus may be much more widespread than presently recognized.

A substantial infection of the filamentous bacilli in bovine tracheal epithelium might modify epithelial cells or their responses in many ways and thereby influence any studies using this tissue. For example, binding of

various mediators or cytokines might be altered because of possible changes in apical membrane components. Attempts to establish primary epithelial cultures from bovine tracheal tissue may also be jeopardized by the presence of this bacterium, unless appropriate antibiotics are used. Examination of sections from each trachea by either electron microscopic techniques or Warthin-Starry stain to ascertain presence or absence of the filamentous bacilli takes days and is therefore impractical for immediate use of fresh tissue in experimental study. Ciliary axoneme preparations using ½–¾ of the trachea can be obtained within hours, assessing a larger area than usually examined by a light or electron microscope, and can indicate whether the remainder of the trachea is acceptable for further study. Alternatively, immunohistochemical techniques using anti-CAR bacillus antiserum with epithelial tissue samples could provide equally rapid detection of those trachea with substantial infections of the filamentous bacilli.

The CAR bacillus infections in rodents elicit inflammatory responses leading to pathologic changes in airway tissue.[7,14,24] Although some leukocytic infiltration was observed in the submucosa of the bovine epithelium containing the filamentous bacilli, this was not a consistent observation. There was, however, an inverse correlation of decreased yield of cilia from bovine tracheal epithelia that had high concentrations of adherent filamentous bacilli (Table 1). The small piece of tissue examined may not accurately reflect the condition of the remaining tracheal epithelium, or there may be other reasons, such as viral infections, for loss of ciliary differentiation. Nevertheless, the results suggest that this bacterium may adversely affect the ciliated cell. A loss of cilia could reduce effective mucociliary clearance and lead to more extensive infection of the airway epithelium by the filamentous bacillus.

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