Detection of shared antigenic determinants on whole *Moraxella bovis* pili by use of antisera to cyanogen bromide-cleaved *M. bovis* pilus protein

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**Objective**—To determine the ability of antisera against cyanogen bromide-cleaved pili from 4 strains of *M. bovis* to react with whole or non-denatured pili.

**Sample Population**—Antisera to 4 strains of *M. bovis* produced by New Zealand White rabbits.

**Procedure**—Pili from 4 strains of *M. bovis* were collected and purified. Pilus proteins (pilin) were cleaved, using cyanogen bromide. Whole pili and cyanogen bromide-cleaved pili were injected into rabbits. Antiserum were serially diluted, reacted with 4 strains of *M. bovis*, and examined by immunoelectron microscopy and indirect immunofluorescence.

**Results**—Antiserum to whole pili aggregated and distorted pili from homologous strains, but pili from heterologous strains were unaffected. Antiserum to cleaved pili fragments resulted in partial aggregation and thickening of homologous and heterologous pili, suggestive of heterospecific antibodies. Attachment of antibodies to pili was detected by indirect immunofluorescence, indicating a strong reaction of antiserum to whole pili with homologous pili. Weak cross-reactions were evident with certain heterologous strains. In contrast, antiserum to cleaved pili fragments reacted strongly with pili from homologous and heterologous strains.

**Conclusions and Clinical Relevance**—We detected shared antigenic determinants on pili from various strains of *M. bovis* that were not immunogenic after cleavage of pilus protein with cyanogen bromide, and antiserum produced to protein fragments reacted with whole pili from heterologous strains of the organism. Vaccines produced from cyanogen bromide-treated pili may induce broader immunity against infectious bovine keratoconjunctivitis than that provided by currently available vaccines. (Am J Vet Res 2001;62:1279–1284)

*Moraxella bovis* causes a highly contagious and debilitating ocular disease in cattle known as infectious bovine keratoconjunctivitis (IBK). Only piliated strains of the bacterium produce the disease, because pili facilitate attachment of the organism to the corneas of cattle. Nonpiliated strains do not attach to the cornea and are unable to produce disease in experimentally inoculated susceptible calves.

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was an isolate from a calf from Newport, Tenn, and was identified on the basis of its in vitro characteristics. Piloted bacteria were grown on tryptose blood agar base supplemented with 5% bovine blood (BBA). Piloted colony forms of *M. bovis* were maintained by careful selection and cloning of hemolytic flat colonies that had indented the agar. Bacteria were subcultured onto BBA every 24 hours and incubated overnight at 37°C or kept frozen at −70°C in 40% glycerol in trypticase soy broth.

Pilus isolation and purification—Colonies of piloted *M. bovis* from cultures that had been incubated overnight were selected. Sterile cotton-tipped swabs were used to streak cultures onto BBA in 10 stainless-steel pans (20 × 37 cm). Pans were incubated overnight, and bacteria were collected into 60 ml of chilled (4°C) 0.05M Tris-buffered physiologic saline solution (pH 8.0) with 0.01M sodium azide (TBS). Cells were washed once in TBS by use of centrifugation at 13,000 × g, and the pellet was resuspended in 60 ml of chilled 0.01M Tris buffer (pH 9.5) with 0.01M sodium azide (TB). Pili were sheared from cells by agitation with a homogenizer set at 70% speed for 5 minutes at 4°C. Cells then were removed by use of centrifugation (3,000 × g for 30 minutes) and discarded. Supernatant was dialyzed overnight at 4°C in 6,000- to 8,000-molecular weight restriction dialysis tubing, against 2 L of 0.05M TBS. Pili that precipitated were collected by use of centrifugation at 13,000 × g for 1 hour. They then were resuspended in 30 ml of 0.01M TB. Material that did not return to suspension was removed by centrifugation at 23,000 × g for 1 hour at 4°C, and pilus-rich supernatant fluid was dialyzed as described previously. Alternating cycles of differential centrifugation and dialysis were repeated until the pilus preparation was free of cellular contaminants, which was determined by use of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and negative-stain electron microscopy.

Protein cleavage by cyanogen bromide—Purified pili were cleaved with cyanogen bromide, using the method of Gross and Witkop. Approximately 3 mg of pilin in 0.01M TB was mixed with 5 ml of 0.1M HCl (final concentration) to which 50 mg of cyanogen bromide was added. This mixture was incubated overnight at room temperature (20°C), diluted to 20 ml with distilled water, and lyophilized.

Polyacrylamide gel electrophoresis—Pilus preparations were diluted at least 4-fold with sample buffer (0.25M Tris-HCl, 0.5% SDS, 2.5% glycerol, and 1 µg bromophenol blue/ml), incubated for 4 minutes at 100°C, and electrophoresed in a vertical slab of polyacrylamide, using the discontinuous buffer system. A 3-cm stacking gel (4.0% acrylamide in 0.125M Tris, pH 6.8) layered onto an 11.5-cm 12% polyacrylamide gel. Lanes for each panel were as follows: 1, molecular weight standards; 2, untreated pilin; and 3, cyanogen bromide-treated pilin. Molecular weight standards were ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD). Notice that 3 bands were detected in lane 3, corresponding to the bands detected in lane 2. The second band from the top in lane B, lane 3 was probably a partially cleaved pilin (PCP) fragment. Stain = Coomassie blue.

Indirect immunofluorescence—Suspensions of bacteria from cultures grown for 24 hours on BBA were prepared with deionized water. Ten drops of the suspensions were placed on ethanol-cleaned glass microscope slides, air-dried, and fixed for 1 minute in absolute methanol. Serial 2-fold dilutions of antisera (1:10 to 1:2,560) were made with PBS solution (PBSS), and a drop of each dilution was placed on a spot of bacteria and incubated for 10 minutes in a humid chamber at 37°C. Slides were washed 3 times (5 minutes/wash) in PBSS-Tween 20 and air-dried. Goat antirabbit IgG fluorescein iso-thiocyanate conjugate (used at the highest dilution in PBSS that still resulted in maximal fluorescence) was placed on the spots, incubated, and washed, as previously described. Slides then were examined by use of epifluorescence microscope (400X magnification). Intensity of fluorescence was rated subjectively from minimal (+) to maximal (++) fluorescence. Antibody titers were expressed as the inverse of the highest dilution of antisera that resulted in fluorescence of ≥ 1+.

Figure 1—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of EPP-63 (A) and NPTn (B) pilus protein (pilin) in 12% polyacrylamide gels. Lanes for each panel were as follows: 1, molecular weight standards; 2, untreated pilin; and 3, cyanogen bromide-treated pilin. Molecular weight standards were ovalbumin (45 kD), carbonic anhydrase (31 kD), soybean trypsin inhibitor (21.5 kD), and lysozyme (14.4 kD). Notice that 3 bands were detected in EPP-63 treated pilin (panel A, lane 3). The top band represented uncleaved pilin (UCP), and the 2 bottom bands represented cleaved pilin fragments (PF-1 and PF-2). Four bands were detected in NPTn cyanogen bromide-treated pilin (panel B, lane 3). The top and bottom 2 bands corresponded to the bands detected in lane 2. The second band from the top in panel B, lane 3 was probably a partially cleaved pilin (PCP) fragment. Stain = Coomassie blue.
Results

Cyanogen bromide cleavage of pilin—Three to 4 bands were seen in SDS-PAGE gels following use of silver stain (Fig 1). Cyanogen bromide-treated EPP-63 pilus preparations produced 3 peptide bands. One was residue of uncleaved pilin (UCP), which was identified by comparison with samples of UCP. Two bands of pilin fragments (PF; PF-1 and -2, respectively) were fragments produced as the result of cleavage of the protein at 2 methionine residues. An additional band (partially cleaved pilin) was seen occasionally in cyanogen bromide-treated NPTn pilus preparations. This band likely represented incomplete cleavage of pilin at 1 of the methionine residues.

Immunoelectron microscopy—Three reactions were observed. Nonimmune rabbit antiserum and whole-pilus antisera incubated with heterologous strains of *M. bovis* did not produce detectable changes in pilus morphology (Fig 2). Whole-pilus antisera reacted with homologous bacteria produced noticeable aggregation, thickening, and distortion of the pili. Antisera to cleaved pilin produced noticeable aggregation and thickening of the pilus for homologous and heterologous strains, except that antiserum to cleaved NPTn pilin incubated with strain IBH-64 produced a thickening of the pilus without causing aggregation.

Indirect immunofluorescence—Attachment of rabbit anti-pilus and anti-PF antibodies to pilated homologous and heterologous strains of *M. bovis* was documented by results of indirect immunofluorescence (Fig 3, Table 1). A total lack of fluorescence was
Antiserum to NPTn whole pili did not react with pili from heterologous strains. Although antiserum to NPTn whole pili yielded antibody titers of 10,2560 for the homologous strain, it did not cross-react with any heterologous pili. Antiserum to NPTn whole pili had a titer of 2,560 for homologous pili and for pili on strains FLA-64 and EPP-63, but it did not react with bacterial cell surfaces. Titer of this antiserum to IBH-64 pili was 320.

Antiserum to IBH-64 whole pili had a titer of 1,280 with homologous pili and cross-reacting titers of 160 to pili and cell surfaces of strains NPTn and EPP-63. This serum yielded a titer of 320 to the cell-surface antigens of strain FLA-64 but did not react with pili. Titer of antiserum to cleaved IBH-64 pilin was >2,560 for IBH-64 pili and yielded titers of 1,280 to NPTn pili and 640 to strains FLA-64 and EPP-63 pili.

Antiserum to FLA-64 whole pili reacted strongly with homologous pili, but it reacted only with cells of heterologous strains. Antiserum to cleaved FLA-64 pilin had a titer of >2,560 for homologous pilus and cross-reacted with pili on IBH-64, NPTn, and EPP-63, yielding titers of 1,280, 640, and 320, respectively.

### Discussion

The 4 strains of *M. bovis* used in this study were capable of producing IBK after inoculation into the eyes of cattle. When incorporated into vaccines, they are capable of inducing protective immunity against a homologous strain of the organism. However, cattle vaccinated with any of these strains were not protected against infection with heterologous strains of *M. bovis*.

Use of cyanogen bromide for treatment of pilus preparations from these 4 strains produced 3 to 5 bands in silver-stained SDS-PAGE gels. Cyanogen bromide cleaves proteins at the carboxyl side of methionine residues, and *M. bovis* pilin contains 2 such amino acids at positions 7 and 86 of the polypeptide. These amino acids and the resulting cleavage with cyanogen bromide are analogous to that reported for gonococcal pilin, which has methionine residues at positions 7 and 92.

The band closest to the top of the gel (Fig 1) in lanes containing cleaved pilin corresponded to that of untreated pilus and likely represented UCP. The band immediately below the UCP band in cleaved pilin preparations from strain NPTn may have represented a peptide composed of the small N-terminal fragment and the central fragment. In another study, cleavage of the peptide bond in gonococcal pilin at this location was less efficient (20% complete) than cleavage of the peptide bond between the central and carboxy-termi

### Table 1—Titers determined by use of indirect immunofluorescence for rabbit anti-*Moraxella bovis* pilus sera against pilus preparations from strains of *M. bovis*

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Strain of <em>Moraxella bovis</em></th>
<th>NPTn</th>
<th>IBH-64</th>
<th>FLA-64</th>
<th>EPP-63</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole pilin</td>
<td>NPTn</td>
<td>&gt;2,560</td>
<td>160†</td>
<td>1,280 &gt;10‡</td>
<td>&lt;10‡</td>
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<tr>
<td></td>
<td>IBH-64</td>
<td>160†</td>
<td>1,280 &gt;10‡</td>
<td>&lt;10‡</td>
<td></td>
</tr>
<tr>
<td></td>
<td>FLA-64</td>
<td>80‡</td>
<td>80‡</td>
<td>&gt;2,560</td>
<td>80‡</td>
</tr>
<tr>
<td>Cleaved pilin</td>
<td>NPTn</td>
<td>1,280</td>
<td>320</td>
<td>1,280</td>
<td>1,280</td>
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<tr>
<td></td>
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<td>&gt;2,560</td>
<td>640</td>
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<tr>
<td></td>
<td>FLA-64</td>
<td>640</td>
<td>1,280</td>
<td>&gt;2,560</td>
<td>320</td>
</tr>
<tr>
<td>Nonimmune rabbit serum</td>
<td></td>
<td>&lt;10§</td>
<td>&lt;10§</td>
<td>&lt;10§</td>
<td>&lt;10§</td>
</tr>
</tbody>
</table>

*Titers of 1,280 to *M. bovis* cell-wall associated antigens. †Titer of 160 to *M. bovis* cell-wall associated antigens. ‡Titer of 320 to *M. bovis* cell-wall associated antigens. §Did not fluoresce to any part of the cell with nonimmune rabbit serum. The NPTn whole-pilus antiserum had an antibody titer of 2,560 to cell-wall-associated antigens of a nonpiliated variant of NPTn.
immunofluorescence supported those data generated by use of immunoelectron microscopy. Data generated in our laboratory indicated that immunofluorescence is more sensitive than immunoelectron microscopy for detection of pilin antibodies (data not shown). Antibodies to whole pilin reacted noticeably with pili from homologous strains. In some combinations, cross-reactions with these antisera were detected; however, antibody titers of whole-pilus antisera for heterologous pili always were much lower than titers of homologous reactions. In contrast, all antisera to cleaved pilin had high antibody titers to heterologous and homologous pili.

Whole-pilus antisera reacted, as determined by use of indirect immunofluorescence, with varying intensities to antigens associated with bacterial cell walls of homologous and heterologous strains. This reaction was observed even in a nonpiliated variant of NPTn that reacted with anti-NPTn whole-pilus antisera. At least 2 possibilities exist to explain this observation. It may have been attributable to conserved pilin fragments accumulating in the periplasmic space prior to their transport outside the cell. Protein precursors to pilin produced within the bacterial cell have a small conserved leader sequence that is cleaved from the protein prior to its incorporation into the pilus structure.22 Also, it is possible that cell wall components may have been in the pilus preparations at concentrations undetectable by SDS-PAGE or negative-stain electron microscopy but still capable of eliciting an antibody response. However, all the cleaved-pilin antisera reacted primarily with the pilus and not with the cell walls. It appears that the cleavage process resulted in preparations that did not contain immunogenic concentrations of the protein leader sequence or cell wall components.

Analysis of our data revealed conserved antigenic determinants on intact pili of at least these 4 strains and perhaps numerous additional strains of M bovis. Although specific antibodies readily react with these epitopes in the whole pilus, they are not immunogenic. Cleavage of pilin with cyanogen bromide uncovers these sites and renders them immunogenic. If these sites are conserved and render them immunogenic, monoclonal antibodies raised against pilin may have the potential to detect shared antigenic determinants in pili from divergent strains of Moraxella bovis.24 Further, monoclonal antibodies developed against conserved epitopes in pili of other bacteria also may be useful for the detection of pilin antibodies (data not shown). Antisera to pilin are more sensitive and specific than antisera to cell walls of M bovis.

The conserved leader sequence of M bovis pilus is cleaved in the cell,24 perhaps during pilus synthesis, and is joined to the pilin subunit to form the pilus.25,26 The pilus is excreted from the cell,25,27 and it associates in vivo with the cell surface28 and is able to adhere to and infect host cells.29 These studies indicate that these conserved epitopes are important for the adherence of M bovis to the host. The conserved leader sequence that is cleaved from the protein of pili was suggested to have a conserved structure, and this is supported by the presence of conserved cysteines in the leader sequence of M bovis pilus.46

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