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What is This?
Colonization of the Pharyngeal Tonsil and Respiratory Tract of the Gnotobiotic Pig by a Toxigenic Strain of Pasteurella multocida Type D

M. R. Ackermann, N. F. Cheville, and J. E. Gallagher

US Department of Agriculture, Agricultural Research Service, National Animal Disease Center, Ames, IA

Abstract. Seven-day-old gnotobiotic pigs were inoculated intranasally with Pasteurella multocida and euthanatized 2, 5, 9, and 14 days after inoculation. Tissues from the oropharynx and respiratory tract of pigs were cultured quantitatively and analyzed microscopically. Pigs remained afebrile and alert, except one that died of acute fibrinopurulent pneumonia. Pasteurella multocida was isolated in greatest numbers from the pharyngeal tonsils, but only in low numbers from turbinates, trachea, lung, spleen, and liver. Significant histologic changes were limited to the tonsils. Infected pigs developed mild tonsillitis with lymphocytic hyperplasia, and accumulation of cell debris and bacteria in crypts. Capsular antigens of P. multocida, identified on tissue sections with rabbit anti-capsular polysaccharide antibody and immunocytochemical reagents, were confined to the crypt lumen. Ultrastructurally, bacteria were free within crypt material or within phagosomes of macrophages or neutrophils. In a second experiment, 5-day-old pigs were infected with Streptococcus suis type 2, followed by toxigenic Pasteurella multocida at 7 days of age; one pig died of streptococcal septicemia. Pigs developed a mild tonsillitis, and both bacteria were cultured from the tonsillar crypts for up to 14 days after infection. These studies show that a toxigenic strain of Pasteurella multocida, which is a causative agent of atrophic rhinitis, can colonize the tonsil and respiratory tract of gnotobiotic pigs for up to 14 days. In addition, colonization can occur concurrently with Streptococcus suis type 2.

Key words: Atrophic rhinitis; adherence; Streptococcus suis; swine; tonsillitis.

Pasteurella multocida can be isolated from the respiratory tract of swine. Isolates of toxin-producing capsular type D strains are most often associated with atrophic rhinitis, while type A strains commonly occur in lung lesions and pneumonia. Experimentially, nasal infection by toxigenic P. multocida can produce lesions of atrophic rhinitis, and the purified toxin of P. multocida type D clearly produces turbinate atrophy. Turbine atrophy also occurs when the toxin is injected intraperitoneally or in the dermis. Some researchers think, therefore, that Pasteurellae colonizing the respiratory tract or tonsil may release toxin that is taken up systemically by the lymph or blood. Little is known about the extent to which toxigenic strains colonize, and isolation of significant numbers of toxigenic Pasteurella from nasal swabs in the field is often inconsistent and variable.

The precise relationship of P. multocida with other porcine respiratory pathogens is unclear. Intranasal infection with Bordetella bronchiseptica clearly causes severe rhinitis, which is enhanced by superinfection with P. multocida. Although toxins of both are destructive to swine nasal epithelial cells in vitro, when live bacteria are added to such tissue cultures, B. bronchiseptica adheres to epithelial cells at three times the rate of P. multocida. In any event, both bacteria can cause atrophic rhinitis, and they may act alone or be synergistic. It has been suggested that, when resistance of nasal mucosa is weakened by B. bronchiseptica or other non-infectious agents, toxigenic P. multocida becomes established to produce toxin.

This experiment was designed to determine whether a toxigenic strain, P. multocida type D (4533), can colonize the oropharynx and respiratory tract of gnotobiotic pigs and, if so, detect the precise sites of colonization. Streptococcus suis, a common inhabitant of porcine tonsils, was used in a second experiment to determine if an established microbial population would affect colonization by P. multocida.

Materials and Methods

Pigs

Piglets were removed from pregnant sows by caesarian section under halothane anesthesia and placed in a gnotobiotic isolator unit. Piglets (Nos. 1–4, 8, 9; 5–7; 10–15; 16, 17; 18 to 20) were taken from five different sows at different times and received different inocula. All pigs were clinically normal, and no growth occurred when fecal swabs, taken at birth and prior to inoculation, were streaked on aerobic or
Table 1. Number of colony forming units (CFU) × 10^9 Pasteurella multocida/g of tissue recovered from gnotobiotic pigs inoculated at 7 days of age and necropsied at various times post-inoculation.

<table>
<thead>
<tr>
<th>Pig Group</th>
<th>Pig No.</th>
<th>Days After Inoculation</th>
<th>Inoculum*</th>
<th>Number of CFU × 10^9 P. multocida/g of Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>P. multocida</td>
<td>Turbinate</td>
</tr>
<tr>
<td>Experimental</td>
<td>1</td>
<td>2</td>
<td>4,760</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>4,760</td>
<td>13</td>
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<tr>
<td></td>
<td>3†</td>
<td>7</td>
<td>4,760</td>
<td>248,220</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9</td>
<td>4,760</td>
<td>783</td>
</tr>
<tr>
<td></td>
<td>5</td>
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<td>6,350</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9</td>
<td>6,350</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>14</td>
<td>6,350</td>
<td>95</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Intranasally inoculated, 0.5 ml/nostril. Numbers represent CFU/ml.
† Died with severe fibrinopurulent pneumonia and pericarditis.

anaerobic cultures for bacteria. Pigs were fed a sterile diet of SPF Lac (Pet Ag, Hampshire, IL) and monitored daily.

Experimental design

Three regimens of inoculation were followed: 1) P. multocida alone; 2) S. suis followed by P. multocida; and 3) P. multocida followed by S. suis. Pig Nos. 1–4 and 5–7 were infected intranasally by placing 0.5 ml culture of P. multocida (4,760 and 6,350 colony-forming units [CFU/ml, respectively] into each nostril (0.5 ml/nostril) at 7 days of age. During inoculations, all pigs were held in an upright position. Pig Nos. 8 and 9 were given 0.5 ml of sterile tryptose broth. Pig Nos. 10 to 13 were infected intranasally with S. suis (240,000 CFU/ml, 0.5 ml/nostril) at 5 days of age, followed by P. multocida at 7 days of age. Pig Nos. 14 and 15 were given 0.5 ml of sterile tryptose broth. To reverse the order of exposure, pig Nos. 16, 17 and 18, 19 were infected intranasally with P. multocida (1,200 and 4,300 CFU/ml, respectively, 0.5 ml/nostril) at 7 days of age and S. suis (150 and 6,150 CFU/ml, respectively) at 14 days of age. All infected pigs received P. multocida at 7 days of age and were necropsied at 2, 5, 9, and 14 days post-inoculation with P. multocida or when they became moribund; control pigs were necropsied on post-inoculation days 2 and 14. Fecal swabs were taken immediately before removal from the isolator. Control pigs were free of organisms, whereas pure colonies of P. multocida or S. suis/P. multocida combinations (only from those infected with both organisms) were grown from infected pigs.

Bacteriology

Pigs were euthanized by an intravenous pentobarbital overdose, and weighed specimens of turbinate, tonsil, trachea, lung, liver, spleen, and ileum were ground in saline with a Ten Broeck grinder. Colony-forming units/g were determined by serial dilutions of homogenates on blood agar plates. Pasteurella multocida was identified by colony morphology, iridescence after growth on dextrose starch agar, and biochemical fermentation of selected colonies. Streptococcus suis was identified by colony morphology, slide agglutination of selected colonies, and gram stains of selected colonies. Number of organisms/g of tissue was tabulated (Tables 1, 2, and 3).

Table 2. Number of colony forming units (CFU) × 10^9 Pasteurella multocida and Streptococcus suis/g of tissue recovered at necropsy from gnotobiotic pigs inoculated at 5 days of age with S. suis and at 7 days of age with P. multocida. The pigs were necropsied at various times after the second inoculation.

<table>
<thead>
<tr>
<th>Pig Group</th>
<th>Pig No.</th>
<th>Days After Inoculation with P. multocida</th>
<th>Inoculum*</th>
<th>Number of CFU × 10^9 P. multocida and S. suis/g Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P. multocida</td>
<td>S. suis</td>
<td>P. multocida</td>
</tr>
<tr>
<td>Experimental</td>
<td>10</td>
<td>2</td>
<td>15,600</td>
<td>240,000</td>
</tr>
<tr>
<td></td>
<td>11†</td>
<td>2</td>
<td>15,600</td>
<td>240,000</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>5</td>
<td>15,600</td>
<td>240,000</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>14</td>
<td>15,600</td>
<td>240,000</td>
</tr>
<tr>
<td>Control</td>
<td>14</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Intranasally inoculated, 0.5 ml/nostril. Numbers represent CFU/ml.
† Died of S. suis septicemia.
Pathology

Tissue specimens from the same organs taken for bacteriology were fixed in 10% neutral buffered formalin for 24 hours. Turbinates were decalcified in EDTA (ethylenediamine-tetraacetic acid, tetrasodium salt dihydrate) Decalcifying Solution (Baxter Scientific Products, McGaw Park, IL) for an additional 24 hours after fixation. Tissues were routinely processed, embedded in paraffin, and cut at 5 to 7 μm. Sections were stained with hematoxylin and eosin, Giemsa, and Brown-Brenn Gram Stain. Replicate unstained sections of tonsil and lung were used for immunocytochemistry using an immunoperoxidase technique. Briefly, a 1:5,000 dilution of rabbit anti-\( P.\) \( multocida \) capsular polysaccharide (Dr. R. Rimler, USDA Agricultural Research Service, National Animal Disease Center, Ames, IA) was used as primary antibody, followed by identification of the antibody using an immunohistochemical kit (Vector Laboratories, Burlingame, CA). Normal rabbit serum was used as a control.

Six 1-mm cubes of tonsil were fixed in 2.5% glutaraldehyde with 0.05 M cacodylate buffer (pH 7.4) for electron microscopy. Tissues were dehydrated in alcohols, post-fixed in osmium tetroxide, and embedded in epoxy resin. Semi-thin sections, 1 μm in thickness, were stained with toluidine blue and examined by light microscopy. Ultra-thin sections of selected areas were stained with lead-citrate and uranyl acetate and examined on a Philips electron microscope. One 0.5 × 0.5 cm section of tonsil was fixed in 2.5% glutaraldehyde with 0.05 M cacodylate buffer for scanning electron microscopy. These tissues were then dehydrated in alcohols, post-fixed in osmium, sputter-coated with gold, and examined on a Cambridge scanning electron microscope at the Veterinary Pathology Department of Iowa State University (Ames, IA).

Results

Bacteriologic findings

All pigs were free of anaerobic and aerobic bacteria at the start of the experiment. \( Pasteurella\) \( multocida \) was isolated in highest numbers from the tonsil from all but pig No. 3 (Table 1).

\( Pasteurella\) \( multocida \) was also isolated, with some consistency, from the trachea, turbine, and lung. Large numbers of \( P.\) \( multocida \) were isolated from all six tissues of pig No. 3 that died of fibrinopurulent pneumonia. Both \( P.\) \( multocida \) and \( S.\) \( suis \) were isolated from the tonsil of pig Nos. 10, 12, 13, 18 and 19 (Tables 2, 3). \( Streptococcus\) \( suis \) was not isolated from tissues of pig Nos. 16 and 17.

Pathologic findings

Significant histologic changes were limited to the tonsil in all infected pigs, and no histologic lesions were seen in turbinates, tracheas, livers, and spleens of infected pigs. Pig Nos. 3 and 11 died of acute fibrinopurulent pneumonia and septicemia; \( P.\) \( multocida \) and \( S.\) \( suis \) were isolated from tissues of respective pigs. These two pigs are excluded from the following data.

\textit{Tonsil}. The mucosa of tonsils from control pigs were thin grossly. Histologically, the crypts contained small amounts of material consisting of sloughed keratinocytes and mucus (Fig. 1). The tonsillar lymphoid tissue contained small follicles of lymphocytes with few lymphoblasts.

In infected pigs, tonsillar lesions were similar regardless of inoculum and the degree of involvement progressively increased with time. Two days after inoculation, crypt epithelium was thickened and contained marked increases in numbers of intraepithelial leukocytes including neutrophils and macrophages (Fig. 2). Dilated lymphatic vessels containing moderate numbers of small lymphocytes were within the subepidermal connective tissue and also subadjacent to the tonsillar lymphoid tissue. Eosinophils, which were evenly distributed in low numbers throughout the tonsillar lymphoid tissue of control pigs, were decreased in number.

Samples at 5 to 14 days after infection contained mucus, squamous cells, degranulate neutrophils, and debris in lumens of distended crypts. Capsular antigen

Table 2. Continued.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|c|c|}
\hline
 & \multicolumn{2}{c|}{\text{Number of CFU} \times 10^7 \text{ P. multocida} \text{ and S. suis/g Tissue}} \\
 & \text{Tonsil} & \text{Spleen} & \text{Liver} \\
\hline
\text{P. multocida} & \text{P. multocida} & \text{P. multocida} & \text{P. multocida} & \text{P. multocida} & \text{S. suis} & \text{S. suis} & \text{S. suis} \\
\hline
9 & 1,564 & 250 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 2,027 & 0 & 33 & 0 & 12 & 0 & 37 & 0 \\
93 & 437 & 0 & 26 & 0 & 0 & 0 & 0 & 0 \\
68 & 794 & 714 & 0 & 2,120 & 0 & <1 & 0 & <1 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\
\hline
\end{tabular}
\end{table}
Table 3. Number of colony forming units (CFU) × 10³ of Pasteurella multocida and Streptococcus suis/g of tissue recovered at necropsy from gnotobiotic pigs inoculated at 7 days of age with *P. multocida* and at 14 days of age with *S. suis*. The pigs were necropsied at various times after the first inoculation.

<table>
<thead>
<tr>
<th>Pig Group</th>
<th>Pig No.</th>
<th>Days After Inoculation with</th>
<th>Inoculum*</th>
<th>Number of CFU × 10³ of <em>P. multocida</em> and <em>S. suis</em>/g Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>P. multocida</em></td>
<td><em>S. suis</em></td>
<td>Turbinate</td>
</tr>
<tr>
<td>Experimental</td>
<td>16</td>
<td>9</td>
<td>1,200</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>17</td>
<td>14</td>
<td>1,200</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>9</td>
<td>4,300</td>
<td>6,150</td>
</tr>
<tr>
<td></td>
<td>19</td>
<td>14</td>
<td>4,300</td>
<td>6,150</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>14</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Intranasally inoculated, 0.5 ml/nostril. Each number represents colony forming units/ml.

of *P. multocida* and Gram-positive cocci seen in Brown-Brenn-stained sections were restricted to the lumen of tonsillar crypts (Fig. 3). Five days after inoculation, foci of large blastic lymphocytes surrounded epithelium deep in the crypts (Fig. 4). Germinal centers were not obvious, but the blast cell foci expanded progressively with time (days 9 and 14).

Ultrastructurally, tonsillar crypts from pigs 2 days after inoculation had neutrophils between squamous cells and within the crypt lumen. Also, within the crypt lumen were increased numbers of sloughed crypt lining cells that had lost their plasma membrane interdigitation with adjacent cells; the membranes were often linear with decreased numbers of desmosomes. Large oval lipid droplets, within crypt lining cells of control pigs, were reduced in number (Fig. 5). At 5 to 14 days after inoculation, crypt lumens contained large amounts of necrotic cell debris, degenerate and necrotic crypt lining cells, neutrophils, and few macrophages and lymphocytes (Fig. 6). Bacteria with Gram-negative structure were rarely seen but were identified within phagosomes of neutrophils and macrophages. Bacteria with Gram-positive structure were present between desquamated crypt lining cells, within the crypt debris, and in phagosomes and phagolysosomes of neutrophils and macrophages (Fig. 7). Moderate numbers of lymphocytes were between squamous cells lining crypts, as were macrophages and neutrophils. Most crypt squamous cells lacked the large lipid vacuoles, had decreased amounts of intermediate filaments, and had swollen endoplasmic reticulum and mitochondria (Fig. 6).

![Fig. 1](image1.png)  
**Fig. 1.** Tonsil; control pig. One crypt lumen contains flat, concentrically arranged keratinocytes. Sparse amounts of lymphoid tissue surround the crypts. HE.

![Fig. 2](image2.png)  
**Fig. 2.** Tonsil; pig 5 days post-inoculation with *Pasteurella multocida*. The crypt epithelium is infiltrated by moderate numbers of neutrophils. Crypt lining cells are swollen and hypertrophied in areas of intense inflammation. The crypt lumen contains desquamated crypt lining cells and neutrophils. Pericryptic lymphoid tissue is densely cellular due to numerous small lymphocytes. HE.
Table 3. Continued.

<table>
<thead>
<tr>
<th></th>
<th>Tonsil</th>
<th>Trachea</th>
<th>Lung</th>
<th>Spleen</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>P. multocida</td>
<td>S. suis</td>
<td>P. multocida</td>
<td>S. suis</td>
<td>P. multocida</td>
<td>S. suis</td>
</tr>
<tr>
<td>1,315</td>
<td>0</td>
<td>483</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3,676</td>
<td>0</td>
<td>6</td>
<td>0</td>
<td>&lt;1</td>
<td>0</td>
</tr>
<tr>
<td>7,353</td>
<td>2,705</td>
<td>16</td>
<td>10</td>
<td>&lt;1</td>
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</tr>
<tr>
<td>2,862</td>
<td>3,103</td>
<td>12</td>
<td>16</td>
<td>4</td>
<td>18</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

By scanning electron microscopy, crypts of control pigs had narrow lumens and contained small numbers of flat, rhomboidal to polygonal cells (desquamated keratinocytes, Fig. 8). In infected pigs, cellular material within crypt lumens increased with time and extended deep into the branching diverticula of crypts (Fig. 9). The openings of crypts and the diverticula were enlarged due to distension. The pericrypt lymphoid tissue was expanded and densely cellular. Bacteria were not seen with scanning electron microscopy.

Discussion

Studies of colonization of the respiratory tract of pigs by *P. multocida* have traditionally focused on the nasal turbinates. In contrast, this study demonstrates that a toxigenic strain of *P. multocida* can colonize the tonsils of gnotobiotic pigs up to post-inoculation day 14. We feel that tonsillar colonization may play a significant role in the pathogenesis of atrophic rhinitis by serving as a readily available source for toxigenic strains to colonize turbinates following mucosal weakening, or as a location for secretion of pathogenic concentrations of toxin that spread systemically. Toxigenic *P. multocida* may not need to colonize the turbinates to
Fig. 5. Electron micrograph. Tonsil; control pig. Plasma membranes between crypt lining cells have small interdigitating convolutions and densely osmiophilic desmosomes. Most cells in this area contain large, oval, lipid inclusions. One cell lining the crypt lumen is degenerate and has an angular nucleus with peripheralized chromatin, and a large cytoplasmic space. The cell is partially separated from the plasma membrane of the underlying cell. Lead citrate and uranyl acetate. Bar = 1 μm.

Fig. 6. Electron micrograph. Tonsil; pig 9 days post-inoculation with Pasteurella multocida. Tonsillar crypts contain neutrophils, degranulate macrophages and necrotic cell debris. Crypt lining cells are enlarged and contain swollen mitochondria, small cytoplasmic clear spaces, and lack lipid droplets. Between the crypt lining cells is a macrophage (M), and cytoplasm of a neutrophil (N). Lead citrate and uranyl acetate. Bar = 1 μm.

cause atrophy. Studies have shown that turbinate damage can occur following intraperitoneal injection of both crude and purified forms of the P. multocida toxin. We were able to cause mild turbinate atrophy in one pig by applying a total of 150 μg of dermonecrotic toxin directly onto the tonsil (data not shown). Williams et al. caused turbinate atrophy with three weekly intradermal injections of 0.02 μg/kg.

Mechanisms by which P. multocida colonizes the tonsil are unknown. Fimbrial expression by type D strains has been correlated with toxigenicity, and it may be that fimbriae mediate adherence to degenerate crypt lining cells or crypt debris. Fimbriae may bridge across the thick capsule layer of P. multocida. The capsular polysaccharides of this layer mask the hydrophobic components of the bacterial cell wall and inhibit adherence to hydrophobic molecules of the host. The cell debris within the crypts may also support colonization, by protecting bacteria from inflammatory cells, or act as a physical barrier against removal during deglutition. Although type A strains are more adherent to rabbit tracheal and pharyngeal cells than D strains, few type A strains are toxigenic.

The relatively low numbers of P. multocida isolated from turbinates in this study are similar to bacterial numbers of healthy pigs in the field and in another study using gnotobiotic pigs. In field studies, toxigenic
strains of *P. multocida* are isolated in greater frequency, but generally lower numbers, from turbinates of swine from herds with atrophic rhinitis than from swine of healthy herds. In a study using germ-free pigs, toxigenic strains of *P. multocida* caused only mild turbinate changes and low numbers were isolated from the turbinate. Turbinate colonization by *P. multocida* can be enhanced by the presence of toxigenic *B. bronchiseptica*, and it has been suggested that the cytotoxin of *B. bronchiseptica* enhances turbinate colonization by toxigenic *P. multocida*.

Turbinate lesions were not seen in this study and were not expected. Other studies that have produced turbinate atrophy with toxigenic *P. multocida* used a more heavily concentrated inoculum and isolated larger numbers from the turbinate.

Colonization of the respiratory tract by *P. multocida*, in spite of the presence of an established population of *S. suis*, shows that co-colonization is possible. Previous studies have shown that *S. suis* is commonly isolated from the tonsils of pigs. Inability of *S. suis* to grow in the tonsil of pig Nos. 16 and 17 was probably because of the low numbers of organisms used in the inoculum (150 total) and natural immune mechanisms such as phagocytic clearance, IgA opsonization, and complement activation. Although *P. multocida* was isolated from the tonsil in each inoculation regimen, fewer colony forming units (CFU)/gm isolated from pigs with an established *S. suis* infection (pig Nos. 10, 12, 13) may be due to the relatively large inoculum of *S. suis* (240,000 CFU/ml). This higher concentration of *S. suis* was used to maximize colonization by this organism. In contrast, reversed order of exposure (pig Nos. 16–19) did not markedly alter the numbers of *P. multocida*.

**References**

7. Duncan JR, Ross RF, Switzer WP, Ramsey FK: Pathology of experimental *Bordetella bronchiseptica* infec-

Request reprints from Dr. M. R. Ackermann, US Department of Agriculture, Agricultural Research Service, National Animal Disease Center, PO Box 70, Ames, IA 50010 (USA).