Protein and Antigenic Heterogeneity among Isolates of *Bacillus piliformis*

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Protein and antigenic heterogeneity among isolates of *Bacillus piliformis*, the etiologic agent of Tyzzer’s disease, were investigated. The seven isolates utilized in this study were originally isolated from naturally infected animals of different animal species and diverse geographical locations. Isolates were propagated in mammalian cell lines, and bacterial extracts were prepared. Protein and antigenic profiles were compared among isolates, using Coomassie blue-stained polyacrylamide gels and Western blot (immunoblot) analyses, respectively. Results showed differences in protein and antigen banding patterns, indicating diversity among isolates. Western blots probed with serum preabsorbed with a heterologous bacterial extract revealed that numerous antigens have different electrophoretic mobilities among isolates but apparently share common epitopes. Immunodominant cross-reactive antigens may be candidate proteins useful for development of improved serologic diagnostic tests, allowing identification of animals infected with a wide range of *B. piliformis* isolates.

*Bacillus piliformis* is a unique pathogen unlike any other bacterium described to date. The organism is an extremely large (10 to 40 μm), rod-shaped, gram-negative, sporeforming, motile, obligate intracellular bacterium. The organism was originally described by Ernest Tyzzer and designated as *B. piliformis* based on morphology alone (20). *B. piliformis* is recognized as the causative agent of Tyzzer’s disease, an often fatal enterohepatic disease which has been described for a wide range of domestic, laboratory, and wild animal species (7, 12, 23). *B. piliformis* infections have also been reported in primates (15), and there is suggestive evidence that it may be a human pathogen (6). The acute form of the disease is characterized by severe necrohemorrhagic enteritis and multifocal necrosis of the liver (7, 12). Weaning and newborn animals are particularly susceptible to acute disease and may experience a high mortality (1, 3, 17). Seemingly healthy adult animals can harbor a latent form of infection which can develop into acute disease when the animal is physiologically stressed (2, 19, 24). Transmission of the disease is believed to occur by oral ingestion of spores shed into the feces by infected animals (17). A key to control and prevention of the disease lies in detection of acutely and latently infected animals.

At present, diagnosis of the disease is difficult. The obligate intracellular nature of the pathogen precludes diagnosis by standard cell-free bacterial culture techniques (1, 4, 20). Histopathologically, the organism is extremely difficult to detect in tissue sections stained with routinely used stains such as hematoxylin-eosin. In most specimens, the organism can only be detected by staining of acutely infected intestinal or hepatic tissues with special stains such as silver (12). Serologic tests have been hampered by the apparent lack of cross-reactivity among *B. piliformis* isolates. Using hyperimmune serum and gel immunodiffusion assays, Fujiwara et al. (8, 9) showed that the lack of cross-reactivity is apparently due to antigenic heterogeneity among isolates.

The present study was undertaken to characterize the proteins and antigens from multiple isolates of *B. piliformis* by polyacrylamide gel electrophoresis (PAGE) and Western blotting (immunoblotting). Identification of common antigens shared among multiple isolates may be useful in development of improved serologic tests capable of identifying animals infected with a wide range of *B. piliformis* isolates.

MATERIALS AND METHODS

**Bacterial isolates and animal infections.** The seven isolates of *B. piliformis* utilized in this study were isolated from naturally infected animals. Isolate designations and sources are given in Table 1. Diagnosis of acute Tyzzer’s disease was confirmed by silver staining of hepatic or intestinal biopsies collected at necropsy. To isolate bacteria from acutely infected animals, portions of the liver or cecum were removed and frozen at −80°C until needed. Tissue homogenates (25%, wt/vol, in phosphate-buffered saline, pH 7.4 [PBS]) were prepared, and 0.5 ml was used to inoculate 21- to 28-day-old Mongolian gerbils (Meriones unguiculatus) per os (21). Gerbils were obtained from a cloned colony with no prior history of Tyzzer’s disease. Animals were euthanized 4 to 6 days postgavage, and the livers were aseptically removed. Minced liver tissue was used to inoculate subconfluent mammalian cell cultures.

To isolate *B. piliformis* from latently infected animals, 28-day-old gerbils were placed on bedding collected from cages housing latently infected animals (13). After 9 days, gerbils were euthanized and necropsied. Tissue samples were removed for histopathologic examination and for storage at −80°C. Since large numbers of *B. piliformis* were observed in the cecal mucosa and not in hepatocytes, cecal homogenates were prepared. Gerbils, 21 days old, were gavaged with 0.5 ml of homogenate. At 4 to 5 days postin-
occlusion, livers were aseptically removed and used to infect mammalian cell cultures.

**Cultivation.** *B. piliformis* isolates were propagated in established mammalian cell lines, including embryonic normal mouse liver (BNL CL/2, ATCC TIB73), buffalo rat liver (BRL 3A, ATCC CRL 1442), mouse fibroblast (3T3; MA Bioproducts, Walkersville, Md.), and embryonic mouse fibroblast (17 clone 1; Lawrence Sturman, Albany, N.Y.). Cell lines were grown at 37°C in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum (Hazelton, Lenexa, Kans.) and 2 mM L-glutamine (Sigma Chemical Co., St. Louis, Mo.) in the presence of 10% CO2. Mammalian cell cultures were infected by placing minced liver on subconfluent monolayers and incubating at 37°C, as described elsewhere (T. H. Spencer, J. R. Ganaway, and K. S. Waggie, Vet. Microbiol., in press). After 4 h, medium and minced tissue were decanted and monolayers were given fresh Dulbecco modified Eagle medium with 10% Serum-Plus (Hazelton). Infected monolayers were incubated at 37°C for 2 to 7 days to allow propagation of the bacteria. Bacteria were either subcultured to fresh monolayers when mammalian cells degenerated or harvested for use in PAGE experiments.

**Preparation of bacterial extracts.** Bacterial extracts for use in PAGE experiments were grown in either BRL 3A or 17 clone 1 cell lines. When *B. piliformis* concentrations of 105 to 107 per ml were achieved, monolayers were scraped, and medium containing bacteria and mammalian cells were harvested. Intracellular bacteria were released by sonication on ice for three 30-s bursts. Mammalian cells were removed by centrifugation at 200 × g for 5 min at 4°C. The supernatant was decanted, and bacteria were pelleted by centrifugation at 12,000 × g for 30 min at 4°C. Pellets were washed twice in PBS and suspended in distilled deionized water. Bacterial lysates were prepared by sonication, six 30-s bursts at 4°C, and clarified by centrifugation at 12,000 × g for 5 min. Protein concentrations were determined with the BCA protein assay reagent, following the manufacturer's recommendations (Pierce Chemical Co., Rockford, Ill.).

**Preparation of mammalian cell extracts.** To prepare control reagents, uninfected mammalian cells were grown to confluence and monolayers were harvested by scraping. Suspensions were centrifuged at 200 × g for 5 min at 4°C to pellet eucaryotic cells. Pellets were washed in PBS, disrupted by sonication and clarified as described for bacterial extracts.

**PAGE and Western blotting.** Protein analysis of bacterial lysates was performed by sodium dodecyl sulfate (SDS)-PAGE as described by Laemmli (14). Electrophoresis was carried out at 40 mA constant current, and gels were stained in Coomassie blue. Molecular weight markers were applied to each gel to allow approximate size determinations.

For Western blots, 5 μg of bacterial or mammalian cell extract was loaded in each lane, electrophoretically separated by SDS-PAGE, and transferred to nitrocellulose as described by Towbin et al. (18). Membranes were blocked with 5% nonfat dry milk in PBS and probed with antisera. Bound antibodies were detected by sequential incubation in affinity-purified, biotinylated, anti-species secondary antibody (Vector Laboratories, Burlingame, Calif.), 1% horse serum (Hazelton) diluted in PBS, and avidin-biotin-horse-radish peroxidase conjugate (Vector Laboratories). Blots were developed in a solution of 77 mM CoCl2, 4.3 mM 3,3′-diaminobenzidine, and 0.03% hydrogen peroxide. Colorimetric reactions were terminated by exhaustive rinsing in deionized water.

**Hyperimmune antiserum.** Hyperimmune sera were prepared in female New Zealand White rabbits previously identified as free of antibody to *B. piliformis* by Western blot and enzyme-linked immunosorbent assay (ELISA) analyses of preimmune serum samples. Suspensions of infected liver (10%, wt/vol, in PBS) were prepared and treated with 0.4% Formalin for 30 min. Preparations were emulsified in an equal volume of complete Freund adjuvant, and 1.0 ml of the emulsion was administered subcutaneously and intramuscularly according to National Institutes of Health guidelines for use of complete Freund adjuvant. On day 28, animals were reimmunized with liver suspensions prepared as described above and preparations were emulsified in incomplete Freund adjuvant. Animals were bled and serum samples were harvested 7 days after booster immunizations. Antisera were titrated in Western blots against the homologous antigen preparations and used in all subsequent experiments at serum dilutions which gave strong reactivity against the homologous antigen. Dilutions used ranged from 1:400 to 1:1,000.

**ELISAs.** ELISAs were performed essentially as described by Waggie et al. (22), except that bacteria were propagated in mammalian cell cultures rather than embryonated eggs. Extracts of isolate R or isolate B served as the antigen source.

**FIG. 1.** Silver-stained tissue section of liver from experimentally infected gerbil. *B. piliformis* appeared as intensely stained rods in characteristic "pickup stick" arrays within infected hepatocytes.

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**TABLE 1.** Isolate designations, sources, and cell lines used for propagation of *B. piliformis* isolates

<table>
<thead>
<tr>
<th>Isolate designation</th>
<th>Host species from which isolate was derived</th>
<th>Source</th>
<th>Cell line(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Rabbit, acute infection</td>
<td>Maryland</td>
<td>17 clone 1</td>
</tr>
<tr>
<td>E</td>
<td>Horse, acute infection</td>
<td>Kentucky</td>
<td>BRL 3A</td>
</tr>
<tr>
<td>G</td>
<td>Gerbil, acute infection</td>
<td>Missouri</td>
<td>BRL 3A, 17</td>
</tr>
<tr>
<td>GP</td>
<td>Guinea pig, acute infection</td>
<td>Wisconsin</td>
<td>BRL 3A, 17</td>
</tr>
<tr>
<td>H</td>
<td>Hamster, acute infection</td>
<td>Missouri</td>
<td>BRL 3A, 17</td>
</tr>
<tr>
<td>M</td>
<td>Mouse, latent infection</td>
<td>New York</td>
<td>BRL 3A, 17</td>
</tr>
<tr>
<td>R</td>
<td>Rat, acute infection</td>
<td>Japan*</td>
<td>BRL 3A, 17, 17 clone 1</td>
</tr>
</tbody>
</table>

* Provided by K. Fujiwara, Fujisawa, Japan.
Serum absorption. *B. piliformis*, isolate R, was grown in mammalian cell cultures and harvested as described above. Bacteria were lysed and used to absorb sera by the method of Erwin and Kenny (5). Serum samples were incubated at 4°C with lysed bacterial pellets and then centrifuged at 43,000 × g for 20 min to remove antibody-antigen complexes. The absorption procedure was repeated two times with fresh bacterial pellets to remove antibody exhaustively. Each serum sample was absorbed for a total of 24 h. Absorbed antisera were diluted in 0.5% nonfat milk–PBS and used in Western blots at serum dilutions identical to analogous experiments with whole serum.

RESULTS AND DISCUSSION

Propagation and purification of *B. piliformis*. To isolate *B. piliformis* from infected animals, weanling gerbils were inoculated by either gavaging with homogenates of infected tissue or placing animals on infected bedding which served as a reservoir of bacterial spores. Weanling gerbils were utilized because they are particularly susceptible to acute disease (21). During the early stages of acute disease in gerbils, randomly arranged bundles of *B. piliformis* were typically observed within the cytoplasm of hepatic cells (Fig. 1). Infected hepatic tissue provided an inoculum free of other contaminating bacteria that was suitable for infection of mammalian cell cultures. Spencer and colleagues recently demonstrated that *B. piliformis* can be propagated in the mouse fibroblast cell line 3T3 (Spencer et al., in press). We extended these studies and showed that the organisms can also be cultivated on an embryonic mouse liver line, BNL/2; a normal rat liver line, BRL 3A; and an additional mouse fibroblast line, 17 clone 1. In diseased animals, *B. piliformis* are found primarily in intestinal and hepatic tissue, and occasionally organisms are seen in cardiac and brain tissue (9). Based on the tissue tropism observed in vivo, it was somewhat surprising that fibroblast lines supported bacterial growth.

Localization of *B. piliformis* in mammalian cell cultures was dependent on the host cell line. In BNL CL/2, 3T3, and 17 clone 1 cell lines, bacteria multiplied intracellularly, rapidly lysed the host cell, and existed primarily as motile bacteria within the extracellular medium (Fig. 2A). In the rat liver line, BRL 3A, the bacteria remained primarily within the intracellular confines of the eucaryotic cell in the “pick-up stick” arrays characteristic of in vivo growth (Fig. 2B). Few organisms were observed in the medium, and host cell lysis was minimal even in heavily infected cultures. In general, the ability to escape the host cell is a key component of the virulence of pathogens, allowing dissemination of the bacteria to new cells and continued proliferation. However, because *B. piliformis* often exists as a latent infection, the ability to hide from the normal host defenses by existing for
prolonged periods of time within mammalian cells may itself serve as a virulence determinant.

For preparation of bacterial lysates, *B. piliformis* were grown in either the 17 clone 1 or the BRL 3A cell line. Purification of bacteria by sonication to release intracellular bacilli and differential centrifugation yielded 70% of the total bacteria present in the infected culture. When uninfected monolayers were subjected to this procedure for isolation of *B. piliformis*, negligible amounts of protein were isolated, suggesting that little eucaryotic material was present in purified bacterial antigen preparations.

**PAGE analysis of *B. piliformis* isolates.** Comparison of protein profiles among isolates of *B. piliformis* was performed by Coomassie blue staining of SDS-polyacrylamide gels on which bacterial isolates were electrophoretically separated. Results of this experiment are shown in Fig. 3. The polypeptide profiles for isolates G, H, and GP were similar (lanes 1 to 3). In these strains, the major protein bands were observed at 56 to 59 kilodaltons (kDa). Minor bands migrating at 74, 80, 100, and 110 kDa were present in each of the three strains. Similarities among other isolates, isolates M, E, B, and R, were less remarkable, although gel electrophoresis showed intensely stained proteins migrating at 56 to 59 and 110 kDa in each isolate (lanes 4 to 7). Strain E was distinct from all other isolates, as gel electrophoresis revealed a high-molecular-weight major polypeptide which barely migrated into the separating gel (lane 5). Control experiments with uninfected mammalian cell extracts showed banding patterns markedly different from those of purified Tyzzer’s disease antigens (data not shown).

**Western blot analysis of *B. piliformis* isolates.** Lack of cross-reactivity among *B. piliformis* isolates was first noted in serologic testing (8, 10, 13). In these reports, sera from animals diagnosed with Tyzzer’s disease, based on detection of the bacteria in stained histopathology sections, did not test positive in ELISA or fluorescent-antibody assay, using a heterologous strain of *B. piliformis* as the antigen source. To investigate the molecular basis for the apparent lack of cross-reactivity among isolates, we characterized the antigens of *B. piliformis* isolates by using Western blot analysis. Results of representative blots probed with hyperimmune antiserum produced against the various isolates are shown in Fig. 4. Probing of lysate from isolate R with the homologous antiserum revealed numerous antigens (Fig. 4A, lane 6). Immunodominant antigens were observed at *M*ₜ of 53,000, 110,000, and >200,000. When isolate R was reacted with heterologous antiserum produced against other isolates of *B. piliformis*, differences in antigenic profiles were noted (Fig. 4A, lanes 1 to 5). To confirm that immunoreactive bands were not caused by the binding of gerbil liver antibodies, present in hyperimmune sera, to mammalian cells in which bacteria were grown, control assays were performed. Control lanes contained 5 μg of uninfected mammalian cell extract. Blots revealed only one faint band (Fig. 4A, lanes 7 and 8), indicating that antigenic profiles observed in blots of bacterial extracts were due to bacterium-specific reactions and not to reactions of antiserum with cross-reacting material from mammalian cells in which *B. piliformis* were grown. Based on these findings, it appeared that use of infected gerbil liver as the immunogen for hyperimmune serum production against *B. piliformis* did not pose a problem in evaluation of bacterial antigens via Western blots. Figure 4B depicts the antigenic profile of isolate M probed with hyperimmune homologous antiserum (lane 1) and heterologous antisera (lanes 2 to 6). Immunodominant antigens were observed at *M*ₜ of 53,000 and 55,000, although neither the

![FIG. 4. Western blot analyses of *B. piliformis* isolates probed with hyperimmune antiserum. (A) A 5-μg portion of purified isolate R extract was electrophoretically separated in lanes 1 to 6. As a control, 5 μg of uninfected BRL 3A or 17 clone 1 extract was run in lane 7 or 8, respectively. After electrophoresis, proteins were transferred to nitrocellulose and reacted with the appropriate antiserum. Lane 1. Anti-isolate M; lane 2, anti-isolate G; lane 3, anti-isolate H; lane 4, anti-isolate E; lane 5, anti-isolate B; lanes 6 to 8, anti-isolate R. (B) A 5-μg portion of isolate M extract was electrophoretically separated in each lane and transferred as described above. Membranes were probed as described for lanes 1 to 6 in panel A. Molecular mass markers are indicated in kilodaltons (Kd) at the left.](http://iai.asm.org/)

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53- nor the 55-kDa antigen was recognized by all antisera tested. Similar antigenic profiles were observed in experiments in which bacteria were grown in vitro for multiple passages, for various lengths of time, or in alternative cell lines, either BRL 3A or 17 clone 1 (data not shown).

To confirm that immunologically reactive bands revealed with hyperimmune serum did not reflect epitopes generated by hyperimmunization, Western blots with sera from spontaneously infected animals were performed. Blots were prepared by electrophoresing 5 μg of extract from each isolate in SDS-PAGE, transferring the proteins to nitrocellulose, and reacting them with antisera from naturally infected animals (Fig. 5). Sera were obtained from animals histopathologically diagnosed with the disease or from animals originating in facilities in which Tyzzer’s disease had been documented. All sera used had high titers of anti-B. piliformis antibodies in ELISAs. With serum from an infected rat, isolates G, H, and GP showed similar banding patterns (Fig. 5A, lanes 2 to 4). Obvious differences existed between these three isolates and isolates M, E, B, and R (lanes 1 and 5 to 7, respectively). Immunodominant antigens appeared at 53 to 56 and approximately 100 kDa. Differences in the migration of the 53- to 56-kDa immunodominant antigens may represent genetic drift among isolates. Controls in which 5 μg of uninfected mammalian cell extract were probed showed a single faint band, indicating that the spectrum of antigens detected in lanes loaded with bacterial lysates represented bacterium-specific reactions (Fig. 5A, lanes 8 and 9).

Additional evidence of the antigenic relatedness of isolates G, H, and GP was revealed by probing identical blots with sera from an infected hamster and an infected rabbit (Fig. 5B and C, respectively). These three isolates exhibited a large number of intensely reactive bands when probed with the hamster serum (Fig. 5B, lanes 2 to 4), whereas reactivity with other isolates was much more limited (Fig. 5B, lanes 1 and 5 to 7). Blots reacted with the infected rabbit serum also showed similar banding patterns for isolates G, H, and GP (Fig. 5C, lanes 2 to 4). Immunoreactive profiles of the other four isolates examined were distinct from each other and from the three related isolates. Blots performed with other infected rat and hamster serum samples exhibited banding patterns similar to those shown for that species in Fig. 4.

To determine whether the differences in antigenic profiles among isolates reflected electrophoretic variants of similar proteins or unique antigens not shared between strains, Western blots were performed with serum preabsorbed with a homologous or heterologous isolate to remove cross-reactive antibodies. The effectiveness of serum absorption was demonstrated by comparison of blots probed with whole anti-isolate R serum with those probed with serum preabsorbed with isolate R antigen (cf. lanes 6 in Fig. 4A and 6A). The absorption procedure removed nearly all of the antibodies. Analogous experiments in which serum from a rat infected with a different isolate of B. piliformis was preabsorbed and used to probe a Western blot are shown in Fig. 6B. Comparison of this blot with an identical blot reacted with unabsorbed whole serum (Fig. 5A) showed that almost all immunologically reactive bands were removed when serum was preabsorbed. Bands representing unique proteins were expected to be visible, whereas similar proteins with shared epitopes should not be detected. These findings indicate that, although electrophoretic mobilities of antigens differ among isolates, common epitopes must be present.

Results of this study have shed new light on the problems of diagnostic testing to detect anti-B. piliformis antibodies. There appear to be common antigens among isolates of B. piliformis. Differences in electrophoretic migrations of cross-reactive antigens may be due to genetic drift which has occurred as the organisms have adapted to a particular host species. ELISAs and immunofluorescence assays currently used for serologic testing may not be sensitive enough to detect antibodies to heterologous cross-reactive antigens, unless the isolates are antigenically closely related. It is possible that more sensitive testing systems, such as the biotin-streptavidin amplification system utilized in our Western blots, could be used to improve the sensitivity of immunoassays, allowing detection of less closely related isolates. Alternatively, common or shared antigens identified in this study may be good candidates for development of
improved serologic tests, allowing detection of animals infected with a wide range of isolates.

There remain many unanswered questions about this unusual pathogen. How does this obligate intracellular organism gain entry into mammalian cells? And once inside the host cell, what mechanisms does the pathogen utilize to evade host intracellular killing mechanisms? What mechanisms are responsible for clinical manifestations of the disease? Because *B. piliformis* has such a broad host range, including most animal species, primates, and perhaps humans, finding answers to these questions may yield valuable information on the control and prevention of Tyzzer's disease.

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LITERATURE CITED


FIG. 6. Western blot analyses of six *B. piliformis* isolates probed with absorbed sera. Blots were prepared as described in the legend to Fig. 5 and probed with absorbed hyperimmune serum produced against isolate R (A) or absorbed serum from a naturally infected rat (B). Lanes 1. Isolate M; lanes 2. isolate G; lanes 3. isolate H; lanes 4. isolate E; lanes 5. isolate B; lanes 6. isolate R. Molecular mass markers are indicated in kilodaltons (Kd) at the left.