Bacillus piliformis Flagellar Antigens for Serodiagnosis of Tyzzer's Disease

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Purified flagella from multiple isolates of *Bacillus piliformis* were obtained and examined by electron microscopy. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analyses were used to assess the purity, antigenicity, and cross-reactivity of purified flagellar preparations. SDS-PAGE demonstrated a single, major protein band evident at approximately 53 to 56 kDa in all isolates tested. Results of Western blot analyses indicated a lack of cross-reactivity between flagellar antigens and heterologous isolates. Enzyme-linked immunosorbent assays (ELISAs) were used to compare the efficacies of flagellar preparations from the various isolates as antigens in detecting *B. piliformis* serum antibodies from several host species. ELISA results indicated that no single flagellar preparation could be relied on to consistently identify serum antibodies in all the host species tested; however, ELISAs that utilized a trivalent flagellar antigen preparation were shown to be specific and sensitive for the detection of antibodies to *B. piliformis*.

Bacillus piliformis, the etiologic agent of Tyzzer's disease, is a filamentous, weakly gram-negative, sporeforming, obligate intracellular bacterium which is peritrichously motile. It was first described by Ernest Tyzzer in 1917 as the etiology of fatal enterohepatitis of mice (15). Tyzzer designated *B. piliformis* on the basis of its morphology; it has never been biochemically characterized and may require reclassification. Tyzzer's disease has since been reported in a wide range of laboratory, domestic, and wild animals (7). Economic and scientific losses attributed to epizootic infections in laboratory animals have been devastating in terms of mortality and disruption of experimental studies (6). Although clinical Tyzzer's disease has not been reported in humans, spontaneous infection has occurred in a rhesus monkey (10) and antibodies to *B. piliformis* have been found in women (2), suggesting that *B. piliformis* infections may occur in humans.

Animals clinically infected with Tyzzer's disease may experience lethargy and a watery to pasty diarrhea. Death may occur within 2 to 3 days after the onset of illness, and no reliable treatment has been reported for clinical *B. piliformis* infections. Subclinical infections with *B. piliformis* are thought to be widespread (2). Animals latently infected may develop overt disease when subjected to stress (11, 16). The prevalence of infection in research animals has only been estimated, since accurate diagnosis of Tyzzer's disease has been difficult. The obligate intracellular nature of this pathogen has hindered in vitro propagation, so little work has been done to characterize the organism or improve diagnostic assays to detect clinical and subclinical infections. Histologically, the organism is difficult to detect in tissue sections routinely stained with hematoxylin-eosin; silver stains, such as Warthin-Starry, are usually required to demonstrate the organism in acutely infected liver or cecum (6). Serological diagnosis has been hampered because of the lack of cross-reactivity among isolates of *B. piliformis*. Antigenic heterogeneity among isolates has been well documented (3–5, 12). A recent study by Riley and colleagues identified immuno-

dominant antigens at a molecular mass of 56 to 59 kDa in isolates of *B. piliformis* examined (12). This molecular mass is consistent with that of the *B. piliformis* flagellar protein identified by Toriumi et al. (13).

The present study was undertaken to determine the feasibility of using flagellar preparations of *B. piliformis* as antigen sources for the serological diagnosis of Tyzzer's disease. Purified flagella from multiple isolates of *B. piliformis* were obtained and examined by electron microscopy. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analyses were used to demonstrate the purity, antigenicity, and cross-reactivity of purified flagellar preparations. Enzyme-linked immunosorbent assays (ELISAs) were used to compare the efficacies of flagellar preparations from the various isolates as antigens in detecting *B. piliformis* serum antibodies from several host species.

MATERIALS AND METHODS

Bacterial cultivation. The isolates of *B. piliformis* used in this study were recovered from naturally infected animals as previously described (12). Isolates from acutely infected animals included isolate R1 from a rat from Japan; isolate R2 from a rat from Indiana; isolates G and H from a gerbil and a hamster, respectively, from Missouri; isolate GP from a guinea pig from Wisconsin; isolate E from a horse from Kentucky; and isolate B from a rabbit from Maryland. Isolate M was collected from a latently infected mouse from New York. Isolate B was grown in a mouse embryonic fibroblast cell line, 17 clone 1 (Lawrence Sturman, Albany, N.Y.); all other isolates were grown in a rat hepatic cell line, BRL 3A (Buffalo rat liver; American Type Culture Collection, Rockville, Md.).

Cultures were grown at 37°C in Dulbecco's modified eagle's medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 10% Serum-Plus (Hazelton, Lenexa, Kans.) and 1% L-glutamine (Sigma) (12). Monolayers were scraped and harvested when *B. piliformis* concentrations were 10⁵ to 10⁶/ml. Bacterial suspensions were sonicated on ice for three 30-s bursts to lyse mammalian cells and release

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intracellular *B. piliformis*. Mammalian cells were removed by centrifugation at $200 \times g$ for 5 min at 4°C , and bacteria were pelleted by centrifugation of the supernatant at $12,000 \times g$ for 30 min at 4°C . Bacterial pellets were washed by resuspension in phosphate-buffered saline (pH 7.2) (PBS) and repetition of the final step. Unless otherwise noted, bacterial pellets were stored at -20°C .

Flagellar preparations. Flagellar preparations were collected by a modification of a technique previously described (1). Bacterial pellets were suspended in 5 to 10 ml of Tris buffer (0.1 M Tris, pH 8.0), and flagella were sheared with an Omni-Mixer (Omni-International, Inc., Waterbury, Conn.) on speed setting 5 (approximately 8,000 rpm) for 60 s on ice. The resulting suspensions were centrifuged at $12,000 \times g$ for 30 min at 4°C to pellet cell debris. Supernatants were recovered, and the centrifugation step was repeated twice to pellet remaining cell debris. Sheared flagella were sedimented by ultracentrifugation at $55,000 \times g$ for 2 h at 4°C . Pellets were resuspended in Tris buffer and again centrifuged at $12,000 \times g$ for 30 min at 4°C to remove residual cell debris. Supernatants were decanted, and the ultracentrifugation step was repeated. Flagellar pellets were resuspended in 0.5 ml of Tris buffer and stored at -20°C until used.

Electron microscopy. Sheared *B. piliformis* preparations pelleted at $12,000 \times g$ and flagellar preparations pelleted at $55,000 \times g$ were examined by electron microscopy. In brief, a small droplet of sample in a 4% solution of sodium phosphotungstate was deposited on a carbon-coated grid, and the negatively stained material was subjected to transmission electron microscopy.

SDS-PAGE and Western blotting. Five micrograms of *B. piliformis* flagellar preparations from the various isolates per lane was subjected to electrophoresis at a constant current of 40 mA on SDS-PAGE gels (8). Gels were stained with Coomassie blue to visualize protein-containing bands.

Flagellar preparations of *B. piliformis* were electrophoretically separated as described above and transferred to nitrocellulose membranes for Western blotting (14). Nitrocellulose membranes were blocked with a 5% nonfat dry milk solution in PBS and probed with hyperimmune sera. Antigen-antibody complexes were detected by sequential incubations with species-specific, affinity-purified, biotinylated secondary antibody (Vector Laboratories, Burlingame, Calif.) and avidin-biotin-horseradish peroxidase conjugate (Vector). Immunoreactive proteins were visualized with a solution of tetramethylbenzidine peroxidase substrate, peroxidase substrate solution B (0.02% hydrogen peroxide in citric acid buffer), and tetramethylbenzidine membrane enhancer (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). Staining reactions were terminated by exhaustive washing of the nitrocellulose in deionized water.

Hyperimmune sera. A *B. piliformis*-free New Zealand White rabbit was immunized subcutaneously and intramuscularly with Formalin-treated, *B. piliformis*-infected liver suspension emulsified in complete Freund's adjuvant as described previously (12). The rabbit was boosted with infected liver suspension in incomplete Freund's adjuvant 1 month later, and serum was collected 1 week after booster immunization.

Serum samples. Sera were obtained from animals routinely submitted for clinical evaluation to the University of Missouri Research Animal Diagnostic and Investigative Laboratory. Animals diagnosed with Tyzzer's disease via characteristic clinical signs and histopathological evidence of Tyzzer's bacillus in Warthin-Starry silver-stained tissue sections were found seropositive by an ELISA (9) with whole *B.*

piliformis isolate R1 as the antigen source. Animals from colonies with no history of outbreaks of Tyzzer's disease were found seronegative by this ELISA.

Serum was also obtained from rats and mice experimentally inoculated with *B. piliformis* (9). Rats were inoculated with either isolate R1 or isolate R2, and mice were inoculated with isolate M.

ELISAs. ELISAs were performed with a minor modification (9): *B. piliformis* flagellar preparations were used instead of whole bacterial lysates. Serum samples were determined to be positive for antibodies to *B. piliformis* when ELISA absorbance values were at least twice the baseline values for serum samples for that species. Baseline values were calculated as 3 standard deviations above the mean absorbance value for approximately 100 known negative serum samples. All assays were performed in duplicate and repeated three times for each sample, and the mean absorbance value was reported.

RESULTS AND DISCUSSION

Electron microscopy. Transmission electron microscopy confirmed that flagella were successfully sheared from *B. piliformis*. Numerous flagella up to $4 \mu\text{m}$ in length were seen attached to unsheared *B. piliformis* organisms (Fig. 1A). In contrast, most flagella were removed by shearing; only a few stubbles of flagella remained (Fig. 1B). Many *B. piliformis* organisms were completely denuded of flagella after shearing. Flagellar fragments of various lengths were seen with little contaminating cell debris in purified flagellar preparations (Fig. 1C). While flagella were successfully sheared from *B. piliformis* samples that had been frozen, fresh bacterial samples remained mostly covered with flagella after shearing. We determined by electron microscopic examination of frozen bacterial preparations that freezing itself did not cause flagella to break off of the organisms, but we believe that freezing made the flagella more susceptible to mechanical shearing.

SDS-PAGE and Western blotting. Flagellar preparations from several *B. piliformis* isolates were subjected to electrophoresis on polyacrylamide gels and stained with Coomassie blue. In all cases, a 53- to 56-kDa major protein band was evident (Fig. 2). This banding pattern confirmed that our flagellar preparations were composed of a major protein which electrophoretically migrated at a mass consistent with the reported mass of the major flagellar protein of *B. piliformis* (53 to 56 kDa) (13). Minor differences in the migration of the major protein band were noted and may be indicative of heterogeneity among isolates.

Western blotting was used to compare antigens from different flagellar preparations. Flagellar preparations from several *B. piliformis* isolates were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with hyperimmune sera raised against *B. piliformis*. Representative results of Western blot analyses are shown in Fig. 3. An immunodominant antigen was evident in all lanes at approximately 53 to 56 kDa. Flagellar preparations probed with anti-isolate H hyperimmune sera showed more intense staining in lanes with isolates G and GP, indicating greater cross-reactivity between flagellar preparations from isolates G and GP and anti-isolate H antibodies (Fig. 3A). Similarly, flagellar preparations probed with anti-isolate R1 hyperimmune sera showed more intense staining in the lane containing the homologous flagellar protein from isolate R1 than in lanes with heterologous ones (Fig. 3B). Western blots were overexposed to enhance the visualization of less cross-reactive

FIG. 1. Transmission electron microscopy of isolate E of *B. piliformis* (bar, 2 μ m) (A), isolate R1 of *B. piliformis* after shearing (bar, 2 μ m) (B), and a purified flagellar preparation (bar, 0.5 μ m) (C).

isolates. Results of our Western blot evaluations indicated that flagellar antigens might not have sufficient cross-reactivity among heterologous isolates to allow a single flagellar antigen to be used in the serological testing of animals infected with all isolates.

ELISAs. Flagellar preparations from *B. piliformis* isolates were used as antigens in ELISAs to test sera from several animal species. ELISAs were developed with flagellar preparations from individual *B. piliformis* isolates as the antigen sources. Serum samples from rats, mice, hamsters, and rabbits were tested in these flagellar antigen ELISAs, and the results were compared with those obtained in the *B. piliformis* R1 whole-antigen ELISA (9). Negative control

FIG. 2. Coomassie blue-stained SDS-PAGE gel of flagellar preparations from *B. piliformis* isolates. Lanes: 1, isolate R1; 2, isolate G; 3, isolate GP; 4, isolate M; 5, isolate B; and 6, isolate E.

FIG. 3. Western blots of flagellar preparations from *B. piliformis* isolates probed with hyperimmune sera. (A) Lanes: 1, isolate R1; 2, isolate G; 3, isolate GP; 4, isolate M; 5, isolate B; 6, isolate E. Anti-isolate H hyperimmune sera were used for probing. (B) Lanes: 1, isolate R1; 2, isolate B; 3, isolate G; 4, isolate GP; 5, isolate M; 6, isolate H. Anti-isolate R1 hyperimmune sera were used for probing.

TABLE 1. Comparison of ELISA absorbance values obtained with whole *B. piliformis*, a single flagellar preparation, or a trivalent flagellar preparation as the antigen source

Animal	Absorbance value ^a obtained with:							
	Whole organism preparation (R1)	Single flagellar preparation						Trivalent flagellar preparation (R1, G, and M)
		R1	GP	G	E	M	B	
Rat	2.82	2.79	1.5	0.96	0.09	1.44	0.16	2.14
Mouse	0.90	0.25	0.07	0.07	0.38	0.36	0.37	0.47
Hamster	0.55	0.12	0.73	0.55	0.24	0.11	0.14	0.40
Rabbit	1.26	0.90	1.09	0.91	0.33	0.40	0.34	0.97

^a Boldfacing indicates absorbance values that were seropositive, as explained in Materials and Methods.

serum samples were obtained from animal colonies historically free of Tyzzer's disease, as determined by repeated histopathological monitoring. Serum samples which were negative in the *B. piliformis* R1 whole-antigen ELISA were also negative in ELISAs with flagellar antigens. Representative ELISA results for serum samples which were positive in the *B. piliformis* R1 whole-antigen ELISA are shown in Table 1. No single flagellar preparation could be relied on to consistently identify serum antibodies in all the host species tested. The lack of cross-reactivity between flagellar preparations and serum samples from heterologous species is consistent with the results of Western blot analyses. These differences may be due to genetic drift among isolates and may indicate that the organisms have become adapted to different host species. While ELISAs based on two flagellar preparations were evaluated (data not shown), a combination of three flagellar preparations was needed to consistently serodiagnose *B. piliformis* antibodies in a wide variety of host species. A trivalent ELISA with R1, G, and M flagellar antigen preparations detected serum antibodies in 17 of 17 serum samples from naturally infected animals with histopathologically confirmed Tyzzer's disease.

Relatively few serum samples were available from animals which were naturally infected, had clinical signs of Tyzzer's disease, and also had histopathological evidence of *B. piliformis*. Animals clinically ill and with histopathological confirmation of Tyzzer's disease are often at or below weaning age and are usually too young to have serum antibodies. Adult animals seropositive for *B. piliformis* are usually subclinically infected, and disease cannot be confirmed histopathologically. Because a limited number of serum samples from animals naturally infected with *B. piliformis* were available, we tested serum samples from rats and mice experimentally infected in other studies with *B. piliformis*. Fifty-seven rats were inoculated with isolate R1, 21 rats were inoculated with isolate R2, and 25 mice were inoculated with isolate M. All postinoculation serum samples were positive in the *B. piliformis* R1 whole-antigen ELISA. In the trivalent flagellar antigen ELISA, 55 of 57 serum samples from rats inoculated with isolate R1 were positive, 21 of 21 samples from rats inoculated with isolate R2 were positive, and 25 of 25 samples from mice inoculated with isolate M were positive. The trivalent flagellar antigen ELISA was less sensitive than the whole-antigen ELISA, as indicated by false-negative reactions obtained in two of the rat serum samples that were positive in the whole-antigen ELISA but negative in the trivalent flagellar antigen ELISA. The trivalent flagellar antigen ELISA had a calculated sensitivity of 98% and a calculated specificity of 100%.

Our laboratory recently reported that serum antibodies to *B. piliformis* could be detected in subclinically infected rats

and mice with an ELISA (9). However, we surmised that antigenic heterogeneity among *B. piliformis* isolates might hamper the ability of a test relying on an antigen from a single isolate to diagnose infection in a wide variety of host species. An additional disadvantage of the ELISA that we describe is that the antigen source is a lysate of *B. piliformis* organisms which, being obligately intracellular, must be grown in cell cultures. The laboriousness of growing and harvesting the bacillus hampers the production of large quantities of an antigen for diagnostic testing and precludes the widespread availability of the test. A more economical antigen preparation is needed for diagnostic use. Shared immunodominant antigens, such as the flagellar antigens identified, might prove useful in the improvement of serological tests to diagnose *B. piliformis* infections. Studies are currently under way in our laboratory to identify and clone the genes coding for these flagellar proteins; expressing them in *Escherichia coli* will allow the production of large quantities of highly specific antigens which will be used for the development of an improved ELISA and for providing a reliable supply of bacterial components. These advancements will facilitate the diagnosis of Tyzzer's disease and an estimation of its prevalence and will assist in the characterization of the organism.

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REFERENCES

1. DePamphilis, M. L., and J. Adler. 1971. Purification of intact flagella from *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. 105:376-383.
2. Fries, A. 1980. Antibodies to *Bacillus piliformis* (Tyzzer's disease) in sera from man and other species, p. 249-252. In A. Spiegel, S. Erichsen, and H. A. Utrecht (ed.), Animal quality and models in biomedical research: 7th ICLAS Symposium, Utrecht, The Netherlands. Gustav Fischer Verlag, New York.
3. Fujiwara, K., H. Kurashina, T. Magaribuchi, S. Takenaka, and S. Yokoiyama. 1973. Further observation on the difference between Tyzzer's organisms from mice and those from rats. Jpn. J. Exp. Med. 43:307-315.
4. Fujiwara, K., M. Nakayama, H. Nakayama, W. Toriumi, S. Oguihara, and A. Thunert. 1985. Antigenic relatedness of "*Bacillus piliformis*" from Tyzzer's disease occurring in Japan and other regions. Jpn. J. Vet. Sci. 47:9-16.
5. Fujiwara, K., A. Yamada, H. Ogawa, and Y. Oshima. 1971. Comparative studies on the Tyzzer's organisms from rats and mice. Jpn. J. Exp. Med. 41:125-133.
6. Ganaway, J. R., A. M. Allen, and T. D. Moore. 1971. Tyzzer's disease. Am. J. Pathol. 64:717-732.

7. Harkness, J. E., and J. E. Wagner. 1989. The biology and medicine of rabbits and rodents, 3rd ed., p. 198–201. Lea and Febiger, Philadelphia.
8. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680–685.
9. Motzel, S. L., J. K. Meyer, and L. K. Riley. 1991. Detection of serum antibodies to *Bacillus piliformis* in mice and rats using an enzyme-linked immunosorbent assay. *Lab. Anim. Sci.* **41**:26–30.
10. Niven, J. S. F. 1968. Tyzzer's disease in laboratory animals. *Z. Versuchstierkd.* **10**:168–174.
11. Peace, T., and O. A. Soave. 1969. Tyzzer's disease in a group of newly purchased mice. *Lab. Anim. Dig.* **5**:8–9.
12. Riley, L. K., C. Besch-Williford, and K. S. Waggle. 1990. Protein and antigenic heterogeneity among isolates of *Bacillus piliformis*. *Infect. Immun.* **58**:1010–1016.
13. Toriumi, W., N. Okada, S. Kawamura, and K. Fujiwara. 1986. Production and characterization of monoclonal antibodies to Tyzzer's organism '*Bacillus piliformis*.' *Jpn. J. Vet. Sci.* **48**:977–988.
14. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354.
15. Tyzzer, E. E. 1917. A fatal disease of the Japanese waltzing mouse caused by a spore-bearing bacillus (*Bacillus piliformis*, N. Sp.). *J. Med. Res.* **37**:307–338.
16. Yamada, A., Y. Osada, S. Takayama, T. Akimoto, H. Ogawa, Y. Oshima, and K. Fujiwara. 1969. Tyzzer's disease syndrome in laboratory rats treated with adrenocorticotrophic hormone. *Jpn. J. Exp. Med.* **39**:505–518.