JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1987, p. 2094–2097
0095-1137/87/112094-04$02.00/0

Vol. 25, No. 11

Genetic and Antigenic Differences of Serologically Indistinguishable Leptospires of Serovar Hardjo

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Received 23 March 1987/Accepted 3 August 1987

Pathogenic leptospires of serovar hardjo isolated from North American cattle were compared genetically and antigenically to reference strain hardjoprajitno of the Sejroe serogroup. Guanine-plus-cytosine (G+C) contents were determined for the genomes, and microscopic agglutination, Western blotting (immunoblotting), and immunoprecipitation were used to characterize antigenic relatedness. Major differences were demonstrated between the isolates and the reference strain. The G+C content of the reference strain was calculated to be approximately 34 ± 1%, and those of the isolates were calculated at 39 ± 1%. Antigenic differences between the isolates and the reference strain were identified by using rabbit immune serum raised against a hardjo isolate exhaustively adsorbed with hardjoprajitno whole and sonicated cells. Western blot analysis and immunoprecipitation using this adsorbed serum revealed antigens apparently unique for the hardjo isolates. Microscopic agglutination with the adsorbed rabbit serum did not agglutinate hardjoprajitno when diluted 1:2 but agglutinated bovine isolates to a 1:32 dilution. Bovine antiserum raised against the isolates was also used to identify antigens by immunoprecipitation.

The spirochetes classified as *Leptospira interrogans* are pathogenic for humans and animals. Though it appears that there are preferred hosts for leptospires, with approximately 180 serovars and 19 serogroups, there is naturally a broad host range. North American cattle become infected with leptospires primarily of the hardjo serovar in the Sejroe serogroup. Symptoms of infection include abortion, agalactia, and reproductive disorders (2). More importantly, convalescent animals typically become chronic shedders of leptospires after the invasion of kidney tissue by the organism, thus serving as reservoirs, perpetuating the disease within a herd.

Diagnosis and classification of leptospires is usually performed by serology (2). The microscopic agglutination test (MAT) is the current method of choice. However, this technique often produces confusing or self-contradicting results. As an example, for years it was believed that the most prevalent infecting leptospire of North American cattle was hardjoprajitno, the reference strain of serovar hardjo. Isolates characterized by enzyme-linked immunosorbent assay (9) and monoclonal antibody typing (8) seemed to confirm the MAT results. In the present study, using methods such as guanine-plus-cytosine (G+C) content, microscopic agglutination using adsorbed serum, Western blotting (immunoblotting), and immunoprecipitation, we have provided new data indicating that though serologically similar, North American bovine leptospire isolates are genetically and antigenically distinct from the hardjoprajitno reference strain. These have been previously classified as hardjo-bovis strain A, B, or C on the basis of restriction enzyme analysis (10). We report the presence of several unique antigens of hardjo-bovis not represented in hardjoprajitno. Possibilities of development of specific diagnostic probes by using monoclonal antibodies against these unique antigens are discussed. The results presented here may explain, at least in part, why vaccines containing hardjoprajitno whole-cell antigens are not completely protective.

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MATERIALS AND METHODS

Leptospiral strains and isolates. Serovars hardjo (reference strain hardjoprajitno) and balcanica (reference strain 1627 Burgas) of the Sejroe serogroup were obtained from the National Leptospirosis Research Center (National Animal Disease Center, Agricultural Research Service, U.S. Department of Agriculture, Ames, Iowa). The origin and source of field isolates were: 15B033 (hardjo-bovis A), Colorado, urine; 15B089 (hardjo-bovis B), Florida, kidney; and 15B003 (hardjo-bovis C), Nebraska, urine. Hardjoprajitno isolates were isolated from cattle in Northern Ireland. They are designated HB15A-005 and HB15A-007.

High-molecular-weight chromosomal DNA was prepared according to a previously described method (11).

Determination of G+C content. Approximately 2 μg of genomic DNA from cells of hardjoprajitno reference strain and hardjo-bovis A was analyzed for G+C content in a Gilford System 2600 microprocessor-controlled UV-Vis spectrophotometer and a Gilford thermoprogrammer model 2527 (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). *Escherichia coli* DNA suspended in the same buffer was used as the control.

Antisera. Rabbits and cattle were bled and shown to be negative for the presence of leptospiral antibodies by the MAT (2). Hardjo-bovis A was the leptospire strain used to inoculate animals. The titer of rabbit antiserum was determined to be 1:10,000 by MAT, and that of bovine serum was determined to be 1:1,000. Absorbed antiserum was prepared by exposing rabbit anti-hardjo-bovis A serum to whole and sonicated hardjoprajitno cells and gently rocking it overnight, followed by centrifugation. The supernatant fluid was harvested, and the absorption was repeated three times.

TABLE 1. Percent G+C

Reference strain or isolate[a]	% G+C
Hardjoprajitno	34
HB15A-005[b]	35
HB15A-007[b]	34
Hardjo-bovis A[b]	40
Hardjo-bovis B[b]	38
Hardjo-bovis C[b]	39

[a] All strains and isolates shown are serogroup Sejroe, serovar hardjo.
[b] Isolates from cattle.

Radiolabeling and immunoprecipitation of leptospiral antigens. Hardjoprajitno and hardjo-bovis A cells were labeled with [^{35}S]methionine and immunoprecipitated by the method of Nunes-Edwards et al. (6). To immunoprecipitate antigens bound to bovine antibodies, a modified procedure as outlined by Schmerr et al. (7) was used.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. Discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by the method of Laemmli (3) by using 10% gels. Whole-cell lysates from approximately 1×10^9 to 2×10^9 cells were added to each lane. Gels were stained with Coomassie blue. Fluorography was performed by immersing the gels in En^{3}Hance (New England Nuclear Corp., Boston, Mass.) for 30 min, followed by shaking in water containing 5% glycerol for 15 min. Gels were then dried and exposed to X-ray film.

Western blotting. Western blotting was performed by the methods of Towbin et al. (12) and Burnette (1). Strips of the nitrocellulose blots were cut representing individual lanes of hardjo-bovis A or hardjoprajitno. These were exposed to unadsorbed or adsorbed rabbit or bovine antiserum. Biotinylated antiserum (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) directed against the appropriate primary antibodies was added. Alkaline phosphatase conjugated to avidin (Vector Laboratories, Burlingame, Calif.) was mixed with the primary-secondary antibody complex. The enzyme substrate for the alkaline phosphatase for colorimetric visualization of the reacting antigen was 5-bromo-4-chloro-3-indolyl phosphate.

RESULTS

G+C content. Cesium chloride-purified genomic DNA from North American and European hardjo isolates and reference strain hardjoprajitno were assayed to determine their respective G+C contents. The DNAs were melted three times, and the results are listed in Table 1. The European isolates had G+C contents similar to that of the reference strain. These have been classified as pathogenic hardjoprajitno organisms. The North American isolates had a higher G+C content than did the reference strain.

Microscopic agglutination of rabbit anti-hardjo-bovis serum, before and after absorption. By using standard serological assays, it has not been possible to distinguish between hardjoprajitno reference strain and hardjo-bovis isolates. Rabbit antiserum raised against hardjo-bovis A gave the same titer of 1:10,000 by MAT using either hardjo-bovis A or hardjoprajitno live cells as antigens. However, because of the genomic disparity between these organisms, we looked more closely for differences in their antigenic determinants. To identify possible unique antigens on hardjo-bovis A, the rabbit antiserum was exhaustively adsorbed with whole and lysed hardjoprajitno cells. Titration of adsorbed serum by MAT revealed minimal reactivity to hardjoprajitno (1:1) (>75% of antigens agglutinated) but strong agglutination of hardjo-bovis A to a titer of 1:32. (At no dilution and dilutions of 1:2, 1:4, and 1:8, >75% of hardjo-bovis A antigens were agglutinated; at dilutions of 1:16 and 1:32, 25 to 50% of antigens were agglutinated.)

Western blot analyses of similar and unique antigens with rabbit anti-hardjo-bovis A serum. On the basis of the results of the MAT experiments, we investigated further the apparent unique antigens of the hardjo-bovis isolates. Whole cells were lysed and separated on sodium dodecyl sulfate-polyacrylamide gels, followed by transfer to nitrocellulose membranes and subsequent screening by various antisera. Figure 1 (left two lanes) shows antigens from hardjoprajitno and hardjo-bovis A whole-cell lysates reacting with anti-hardjo-bovis A rabbit serum. Though there appears to be a high degree of similarity of reacting antigens between the two leptospires, there are several antigens differing in molecular weight and intensity. In Fig. 1 (right two lanes), antigens from the same cell lysates were reacted with the anti-hardjo-bovis rabbit serum exhaustively adsorbed with hardjoprajitno. Here it is evident that though there are still some reactive antigens of hardjoprajitno, more importantly, there are a number of antigens of hardjo-bovis A which appear to be unique for the organism.

Identification of radiolabeled leptospire antigens reacting

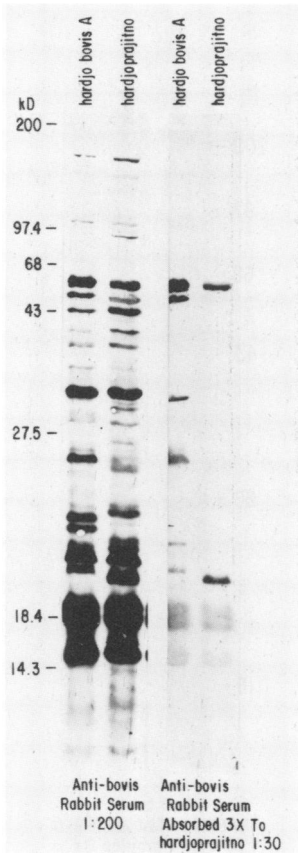

FIG. 1. The lanes represent a Western blot of whole-cell lysates of hardjo-bovis A and the hardjoprajitno reference strain. The left two lanes were reacted with rabbit antiserum raised against hardjo-bovis A. The right two lanes were reacted with the same rabbit antiserum adsorbed three times to hardjoprajitno reference strain cells. kD, Kilodaltons.

with immunoglobulin G antibodies of rabbit and bovine antisera. Antigens of hardjoprajitno and hardjo-bovis radiolabeled with [^{35}S]methionine were characterized with rabbit and bovine antisera raised against hardjo-bovis A. Figure 2 shows several (hardjo-bovis A) antigens immunoprecipitated with adsorbed rabbit anti-hardjo-bovis A serum which appear to be unique. A few low-level-reacting antigens are also represented in the hardjoprajitno lane.

Figure 3 demonstrates hardjo-bovis A antigens immunoprecipitated with either anti-hardjo-bovis A bovine or rabbit antiserum. This comparison of antigens immunoprecipitated by the two different host sera reveals many proteins of the same approximate size, indicating that rabbits and cattle respond immunologically to a similar repertoire of hardjo-bovis A antigens.

DISCUSSION

Serology has long been the accepted method for diagnosing leptospirosis and classifying organisms of the genus *Leptospira*. The results of such tests have dictated which organisms should be used in vaccine development. It was by serological findings that hardjoprajitno of serovar hardjo was believed to be the primary leptospiral pathogen of North American cattle. The first clue that a different leptospire was involved was provided by restriction endonuclease analysis of field isolates (5, 10). Through the use of this technique, it has become apparent that hardjoprajitno has yet to be identified as a pathogen of North American cattle. Hence, it is conceivable that the presence of hardjoprajitno in the available vaccines, instead of hardjo-bovis, could be part of the reason for a lack of complete protection of animals against this infection. Further studies illustrated the differences in the genomes of hardjoprajitno and hardjo-bovis by DNA hybridization (4) and G+C content. It should be noted, however, that most of the pathogenic leptospires tested in our laboratory from this and other serogroups had G+C contents in the 35% range, similar to that of hardjoprajitno (unpublished results). We have also shown that a high degree of homology exists between the genomes of hardjoprajitno and several other pathogenic leptospires of North America (4). The hardjo isolates from North America have very little homology with hardjoprajitno but do show a high degree of hybridization with serovar balcanica, another member of the Sejroe serogroup. The balcanica strain also has a G+C content of 39%, resembling that of the hardjo isolates.

The lack of sequence homology between the genomes of hardjoprajitno and hardjo-bovis led us to look more closely at their respective antigens. Nunes-Edwards et al. (6) have shown similarities between these organisms by immunoprecipitation using rabbit antiserum. Here, we have also shown evidence of antigenic similarity by Western blot analysis with anti-hardjo-bovis antibody of both rabbit and bovine origins. This is the first report of leptospiral antigens identified by either Western blots or immunoprecipitation with bovine antiserum raised against hardjo-bovis A. Results

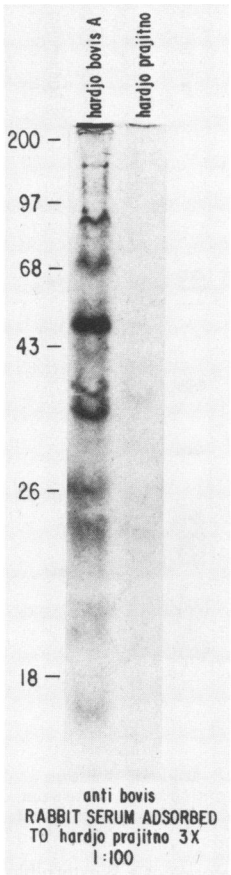

FIG. 2. Autoradiograph illustrating antigens and [^{35}S]methionine-labeled hardjo-bovis A and reference strain hardjoprajitno immunoprecipitated with rabbit anti-hardjo-bovis A serum adsorbed three times to reference strain hardjoprajitno cells. The numbers indicate molecular size in kilodaltons.

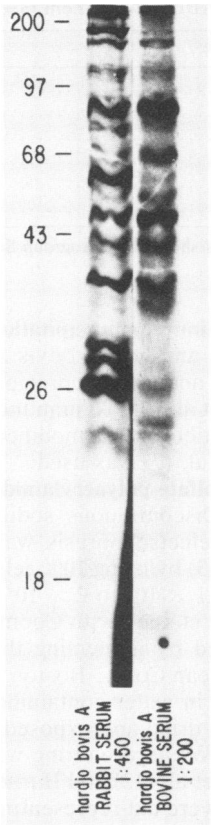

FIG. 3. Autoradiograph illustrating similarities of immunoprecipitated antigens of hardjo-bovis A using rabbit antiserum (left lane) and bovine antiserum (right lane), both raised against hardjo-bovis A. The numbers indicate molecular size in kilodaltons.

from Western blot and immunoprecipitation experiments illustrated that rabbit and bovine immune responses to hardjo-bovis antigens produce antibodies to similar or identical antigens. Such information is important in choosing the rabbit as an animal model for use in the development of a more protective vaccine. It appears that rabbits will serve as a good source for leptospiral antibody production in the study of important and protective antigens.

In this study, we report the presence of unique antigens on hardjo-bovis not heretofore described. It is possible that these antigens represent the difference in titer of adsorbed serum between hardjoprajitno and hardjo-bovis A in the MAT. Whether these antigens turn out to be important is currently under investigation; however, the fact that they were recognized by immunoglobulin G antibodies in the adsorbed rabbit antiserum indicates that they may be important in long-term immunity of the animal. It should be noted that the strain of hardjoprajitno which is currently used in vaccines and which was used to absorb the anti-bovis rabbit serum was a nonpathogenic laboratory-adapted organism. It is conceivable that the unique antigens in hardjo-bovis represent virulence or pathogenic factors. We are investigating this possibility in our laboratory by performing similar experiments using a recent isolate of hardjoprajitno from cattle in Northern Ireland. We are also looking at the possibility of using these unique antigens for monoclonal antibody development for diagnostic purposes.

We have shown, using several methodologies, major and important differences between two leptospires which until recently were thought to be one and the same organism. It is of interest that hardjoprajitno and hardjo-bovis are very different genetically and similar antigenically, especially in light of a generally accepted rule that serologically related organisms also exhibit a relative degree of genetic homology. Other work comparing the DNA homologies of several leptospiral serovars (4) has also raised serious concerns regarding the current classification of leptospires solely on the basis of serology.

ACKNOWLEDGMENT

We thank A. Handsaker for her expert technical assistance.

LITERATURE CITED

1. **Burnette, W. N.** 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112**:195–203.
2. **Cole, J. R., Jr., C. R. Sulzer, and A. R. Pursell.** 1973. Improved microtechnique for the leptospiral microscopic agglutination test. Appl. Microbiol. **25**:976–980.
3. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227**:680–685.
4. **Le Febvre, R. B., and A. B. Thiermann.** 1986. DNA homology studies of leptospires of serogroups Sejroe and pomona from cattle and swine. Am. J. Vet. Res. **47**:959–963.
5. **Marshall, R. B., B. E. Wilton, and A. J. Robinson.** 1981. Identification of *Leptospira* serovars by restriction endonuclease analysis. J. Med. Microbiol. **14**:163–166.
6. **Nunes-Edwards, P. L., A. B. Thiermann, P. J. Bassford, Jr., and L. V. Stamm.** 1985. Identification and characterization of the protein antigens of *Leptospira interrogans* serovar *hardjo*. Infect. Immun. **48**:492–497.
7. **Schmerr, M. J. F., J. M. Patterson, M. J. Van Der Maaten, and J. M. Miller.** 1985. Conditions for binding bovine IgG_1 to protein A Sepharose. Mol. Immunol. **22**:613–616.
8. **Terpstra, W. J., H. Korver, J. Van Leeuwen, P. R. Klatser, and A. H. J. Kock.** 1985. The classification of Sejroe group serovars of *Leptospira interrogans* with monoclonal antibodies. Zentralbl. Bakteriol. Mikrobiol. Hyg. Ser. A **259**:498–506.
9. **Thiermann, A. B., and L. A. Garrett.** 1983. Enzyme-linked immunosorbent assay for the detection of antibodies to *Leptospira interrogans* serovars *hardjo* and *pomona* in cattle. Am. J. Vet. Res. **44**:884–887.
10. **Thiermann, A. B., A. L. Handsaker, J. W. Foley, F. H. White, and B. F. Kingscote.** 1986. Reclassification of North American leptospiral isolates belonging to serogroups Mini and Sejroe by restriction endonuclease analysis. Am. J. Vet. Res. **47**:61–66.
11. **Thiermann, A. B., A. L. Handsaker, S. L. Moseley, and B. F. Kingscote.** 1985. New method for classification of leptospiral isolates belonging to serogroup pomona by restriction endonuclease analysis: serovar *kennewicki*. J. Clin. Microbiol. **21**:585–587.
12. **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.