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Protein and Antigen Profiles of Prevalent Serovars of *Leptospira interrogans*

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Whole-cell and detergent-soluble proteins, enriched for outer membrane antigens, of the *Leptospira interrogans* serovars present in commercially available pentavalent vaccines (hardjo, pomona, icterohaemorrhagiae, grippotyphosa, and canicola) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting (immunoblotting). Protein and antigenic profiles of these serovars, representing several serogroups, were compared with similar profiles of the most common North American bovine pathogen, serovar hardjo type hardjo bovis. The reference strain of serovar balcanica and a hardjoprajitno bovine field isolate (serovar hardjo) were also assayed. Coomassie blue-stained gels revealed extensive protein similarities, and Western blots demonstrated antigenic relatedness throughout the low- and high-molecular-weight regions. Possible quantitative differences in protein expression among the strains were noted, as were similarities in the protein profiles of type hardjo bovis and the balcanica reference strain. A cocktail composed of these homologous antigens may serve as an efficacious subunit vaccine for leptospirosis.

Antigenic characterization of the members of the species *Leptospira interrogans* is a necessary step toward understanding the interactions between leptospires and the immune system. Classically, leptospires are categorized on the basis of their serological reactivity (12, 22). However, a failure to fully appreciate the antigenic composition of the most prevalent leptospire pathogens has resulted in the construction of vaccines that provide little or no protection against infection by organisms of the hardjo serovar (8, 9, 24). Furthermore, there is no convincing evidence that the vaccines protect other domestic animals and humans from subsequent asymptomatic infection and urinary shedding of organisms. Thus, there is a need to examine the antigenic character of these pathogens by methods other than the microscopic agglutination test (12) and the enzyme-linked immunosorbent assay (ELISA; 22).

Other studies have examined whole-cell lysates (10, 16) and outer envelope preparations (18) of only a few of the pathogenic leptospires in an effort to appreciate their antigenic characters. However, no study to date has compared the protein profiles and antigenic composition of whole-cell and detergent-soluble (outer envelope-enriched) preparations with regard to the serovars in commercially available bovine vaccines. In this study, we have subjected whole-cell and detergent-soluble proteins of the constituents in commercial pentavalent vaccines to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Antisera raised against detergent-soluble proteins from hardjo bovis and hardjoprajitno were used in the immunoassays. The results indicated extensive protein and antigenic relatedness among the represented serovars. Furthermore, the polyacrylamide gels suggested quantitative differences among serovars with regard to their protein expression. Such differences regarding other serovars and methodologies have been previously suggested by other investigators (10, 14, 21).

Of primary concern in leptospirosis research is the development of an effective vaccine to combat bovine leptospirosis. The failure of the currently available vaccine to provide complete protection (8) and the failure of a logical attempt to amend this vaccine (9) has left North American cattle vulnerable to infection from the hardjo serovar. Our data on the extensive antigenic relatedness among serogroups are discussed, particularly with regard to a potential subunit vaccine utilizing a common outer envelope antigen(s) represented in the detergent-soluble fraction.

MATERIALS AND METHODS

Leptospiral strains and isolates. All strains were obtained from Carol Bolin or David Miller at the National Leptospirosis Reference Center, the National Animal Disease Center, and the National Veterinary Services Laboratories, U.S. Department of Agriculture, Ames, Iowa (Table 1). (Serovars used in commercially available vaccines are denoted by superscripts numbered 1 to 5.) The cultures were grown to mid-logarithmic phase at 30°C in 10% EMJH leptospiral enrichment medium (Difco Laboratories, Detroit, Mich.) for at least 1 week before use. Ten milliliters of each culture was washed three times in sterile phosphate-buffered saline (PBS), and the pellet was resuspended to a final volume of 1 ml. To determine the quantity of protein present, 100 μl of the cell suspension was assayed with the BCA protein assay kit (Pierce, Rockford, Ill.) according to the manufacturer's directions.

Preparation of leptospiral detergent-soluble fractions. The protocol for the preparation of the leptospiral detergent-soluble fractions was essentially that of Auran et al. (3), as modified by Nunes-Edwards et al. (18). Four hundred milliliters of each leptospiral culture was pelleted by centrifugation, washed three times in sterile PBS, and resuspended in 2 ml of distilled water. The cell suspension was then added to 30 ml of 1 M NaCl and rocked gently at room temperature. The suspension was checked periodically by dark-field microscopy until more than 90% of the cells became spherical (average time, 2 h). The cells were centrifuged at 20,000 × *g* at 4°C for 25 min and resuspended in 2 ml of distilled water. The cell suspension was added to 30 ml of 0.04% SDS for 15 min. A drop of this suspension was examined under dark-

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TABLE 1. Classification of organisms

Serogroup	Serovar[a]	Reference strain
Sejroe	Hardjo[1]	Hardjoprajitno
	Hardjo	Hardjoprajitno 083[b]
	Hardjo	Hardjo bovis
	Balcanica[2]	1627 Burgas
Pomona	Pomona	Pomona
	Kennewicki[c]	LT 1026
Icterohaemorrhagiae	Icterohaemorrhagiae[3]	Ictero I
Canicola	Canicola[4]	Hond Utrecht IV
Grippotyphosa	Grippotyphosa[5]	Moska V

[a] Superscripts 1 to 5 denote the serovars used in commercially available pentavalent vaccines.

[b] Field isolate.

[c] Kennewicki is no longer a recognized serovar, having been combined with serovar pomona. However, because of subtle restriction enzyme analysis differences and historical classification, it is referred to here as a distinct serovar.

field microscopy to ensure that the cells had achieved the characteristic low refractivity of SDS solubilization (10). The SDS-solubilized cells were centrifuged four times at 20,000 × *g* for 25 min at 4°C to remove protoplasmic cylinders and residual lipidlike material that seemed to cause distortions in the polyacrylamide gels. The SDS supernatant was subjected to ultracentrifugation at 100,000 × *g* for 90 min at 4°C. The pelleted detergent-soluble fractions were resuspended in 500 μl of PBS and frozen at −20°C until use. The total protein content of the detergent-soluble fractions was determined with the BCA protein assay kit according to the manufacturer's instructions (Pierce).

Antisera. All antisera were prepared in New Zealand White rabbits by using detergent-soluble preparations from reference strain hardjoprajitno and type hardjo bovis. One rabbit was used for each preparation. Detergent-soluble fractions were removed from the respective leptospires as described above. Four hundred milligrams of detergent-soluble proteins was mixed with Freund complete adjuvant for a final concentration of 400 mg/ml and injected intradermally into various sites along the back of the rabbit. After 2 weeks, the rabbit was again injected by the same method with another 400 mg of a detergent-soluble preparation in Freund incomplete adjuvant. All rabbits were bled by cardiac puncture 2 weeks after the booster dose, and the titers of the antisera were determined either by the microscopic agglutination test (12) or by ELISA. Each of the three detergent-soluble preparations elicited a titer of 1:10,000 in the rabbits, as determined by ELISA.

SDS-PAGE. Preparation of whole-cell and detergent-soluble proteins was performed by using the following lysis solution: 60 mM Tris-HCl (pH 6.8)–10% glycerol–2% SDS–5% mercaptoethanol–0.1% bromophenol blue. Whole-cell suspensions and detergent-soluble fractions were boiled for 10 and 5 min, respectively, in the lysis buffer. Discontinuous SDS-PAGE was performed by the method of Laemmli (15) by using 10 to 15% gradient gels. Twenty micrograms of whole-cell protein lysate or 10 μg of detergent-soluble protein of each strain was added to the appropriate gel. The gels were run at 60 mA for approximately 4.5 to 5 h, and upon completion the gels were stained with Coomassie blue.

Immunoblotting. After SDS-PAGE, the gels were transferred to nitrocellulose (Bio-Rad, Richmond, Calif.) by using an LKB 2117 Multiphor II according to the manufacturer's directions (LKB Products, Bromma, Sweden). Proteins were transferred to the nitrocellulose sheets for exactly 1 h. The nitrocellulose sheets were soaked overnight in a blocking solution containing 2% bovine serum albumin (BSA) and then probed with a 1:5,000 dilution of the appropriate rabbit antiserum. The blots were washed twice with the 2% BSA solution and then screened with biotinylated goat anti-rabbit immunoglobulin G (IgG) antiserum (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). The blots were again washed twice and exposed to an alkaline phosphatase-avidin conjugate (Vector Laboratories, Burlingame, Calif.). 5-Bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium were used as the substrate for the alkaline phosphatase in order to visualize the antigen-antibody complexes.

RESULTS

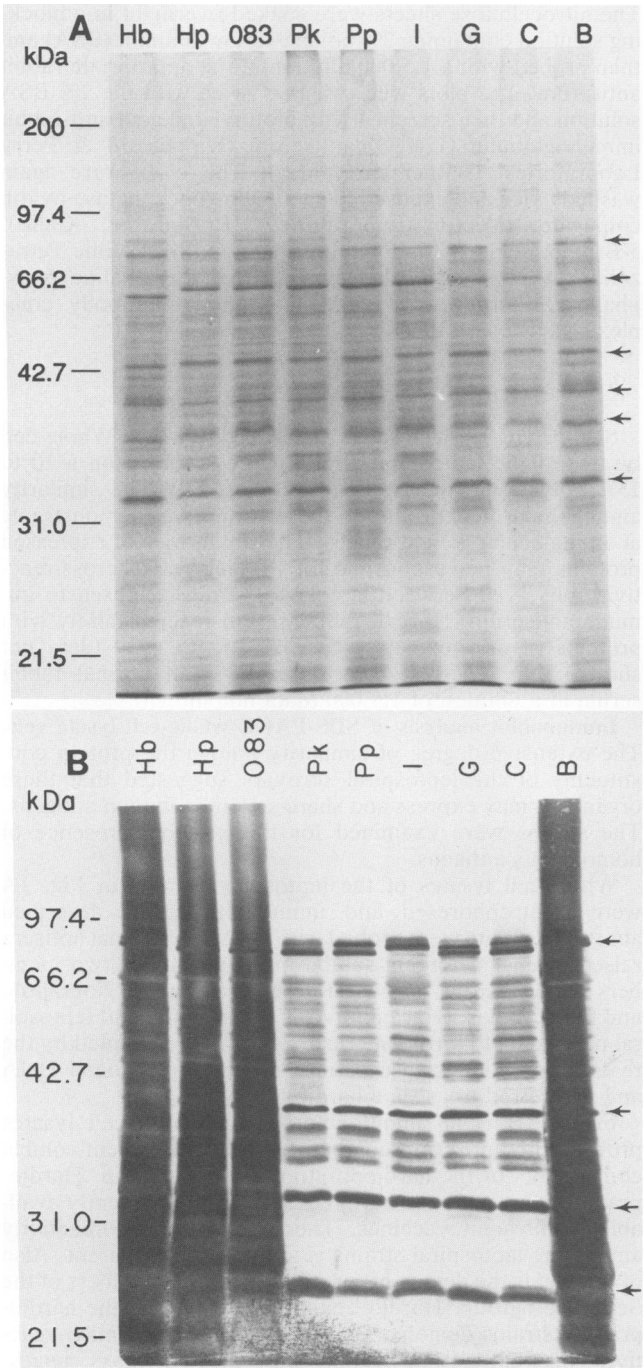
SDS-PAGE of leptospiral whole-cell lysates. Whole-cell lysates of the leptospiral strains were resolved on a 10 to 15% polyacrylamide gradient gel (Fig. 1A). The similarity among the protein profiles of these strains was demonstrable at all molecular weight ranges. The similarities of expressed proteins may be responsible for the degrees of cross-reactivity observed between leptospiral strains exposed to immune antiserum. Normal rabbit serum reacted faintly with proteins of approximately 63, 42, and 21 kDa (data not shown). No reactivity was observed with normal rabbit serum at a dilution of 1:5,000 (data not shown).

Immunoblot analysis of SDS-PAGE whole-cell lysate gels. The extensive degree of similarity among the protein constituents of the leptospiral serovars suggested that these organisms may express and share several common antigens. The strains were examined for the possible presence of homologous antigens.

Whole-cell lysates of the leptospires shown in Fig. 1A were electrophoresed and immunoblotted as described above. The blots were probed with rabbit polyclonal antisera raised against detergent-soluble preparations of two members of the hardjo serovar, reference strain hardjoprajitno and hardjo bovis. (Hardjo bovis is the predominant leptospiral pathogen of North American cattle. It is identified by the restriction endonuclease pattern of its chromosomal DNA and is referred to as type hardjo bovis [23].)

Figure 1B is an immunoblot of the whole-cell lysates probed with antisera prepared against the detergent-soluble components of the hardjoprajitno reference strain. Hardjoprajitno represents the hardjo component of currently available pentavalent vaccines. The extensive cross-reactivity among the leptospiral strains is immediately apparent. Also of interest is the degree of reactivity with the members of the Sejroe serogroup. Hardjo bovis, balcanica, and the hardjoprajitno strains demonstrated a high degree of similar reactivity that extends throughout all molecular mass ranges. The strongest-reacting antigens shared by all of the organisms are at approximate molecular masses of 25, 32, and 35 kDa and a doublet at 80 kDa (Fig. 1B, arrows). The intense staining background material evident in the hardjo bovis, hardjoprajitno, and balcanica lanes is characteristic for members of the Sejroe serogroup when antiserum raised against one of the members of this group is used to react with the blot. On the basis of the use of lipid-specific stains, the material appears to be lipid in nature (data not shown) and is not due to overloading of the gel, as is evident by the Coomassie blue-stained gel (Fig. 1).

An immunoblot of the whole-cell lysates exposed to antiserum generated against components of hardjo bovis detergent-soluble proteins is shown in Fig. 1C. Here again, it



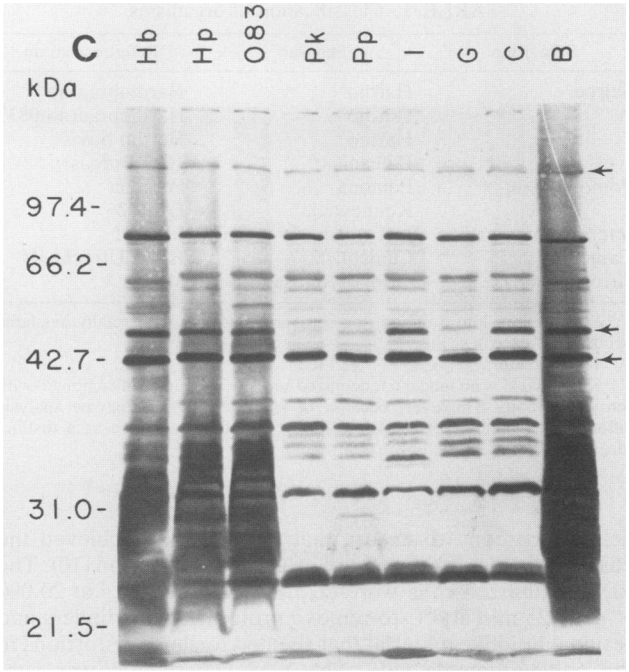


FIG. 1. (A) Whole-cell lysates of selected pathogenic leptospires resolved on a 10 to 15% SDS gradient gel. Twenty micrograms of whole-cell lysate was added to each lane. Gels were run at 60 mA for approximately 4.5 to 5 h and stained with Coomassie blue. Samples loaded in each of the figures are identified as follows: Hb, type hardjo bovis; Hp, hardjoprajitno reference strain; 083, hardjoprajitno field isolate; Pk, Pomona kennewicki; Pp, Pomona pomona; I, Icterohaemorrhagiae icterohaemorrhagiae; G, Grippotyphosa grippotyphosa; C, Canicola canicola; and B, Sejroe balcanica. Arrows mark proteins of similar molecular masses (70, 64, 42, 37.5, 35, and 32.5 kDa) shared among the serovars. (B) Immunoblot of whole-cell leptospiral lysates resolved on 10 to 15% SDS gradient gels and probed with antiserum raised against hardjoprajitno reference strain detergent-soluble antigens. Twenty micrograms of whole-cell lysate was added to each lane. Gels were run and lanes were loaded as described for panel A. Arrows mark similar antigens expressed by the serovars recognized by the hardjoprajitno outer envelope antiserum. (C) Immunoblot of whole-cell lysates probed with antiserum raised against type hardjo bovis detergent-soluble antigens. Twenty micrograms of whole-cell lysate was added to each lane. Gels were run and samples were loaded as described for panel A. Arrows mark antigens of the same molecular masses of each of the serovars which were recognized by the hardjo bovis outer envelope antiserum.

is evident that the leptospiral strains share several similar antigens of the same approximate molecular mass and degree of reactivity to the antiserum. The 25-, 32-, and 35-kDa antigens recognized by hardjo bovis antiserum were similar to those recognized by hardjoprajitno antiserum (Fig. 1B). However, there were some significant differences. Two strongly reacting antigens at approximately 42.7 and 44 kDa were bound by the hardjo bovis antiserum (Fig. 1C, arrows) but were not as strongly bound by the hardjoprajitno serum. Of interest was the apparent reduced reactivity or lack of the 44-kDa antigen in the pomona and grippotyphosa strains (Fig. 1C, lanes Pk, Pp, and G). There appeared to be only one antigen in the 80-kDa range which reacted strongly with hardjo bovis serum, whereas there was a doublet of this size with the hardjoprajitno serum. Another antigen recognized by the hardjo bovis antiserum at approximately 150 kDa (Fig. 1C, top arrow) was not bound at all by the hardjoprajitno antiserum.

SDS-PAGE of leptospiral detergent-soluble preparations. Detergent-soluble proteins, representing enriched fractions of outer membrane proteins, from each of the leptospiral strains were resolved by SDS-PAGE on 10 to 15% gradient gels and stained with Coomassie blue (Fig. 2A). As with the results for whole-cell proteins shown in Fig. 1A, the detergent-soluble proteins displayed a striking homogeneity extending from the high- to the low-molecular-weight regions. The arrows in Fig. 1A highlight the major proteins shared among the serovars (approximate molecular masses of 95,

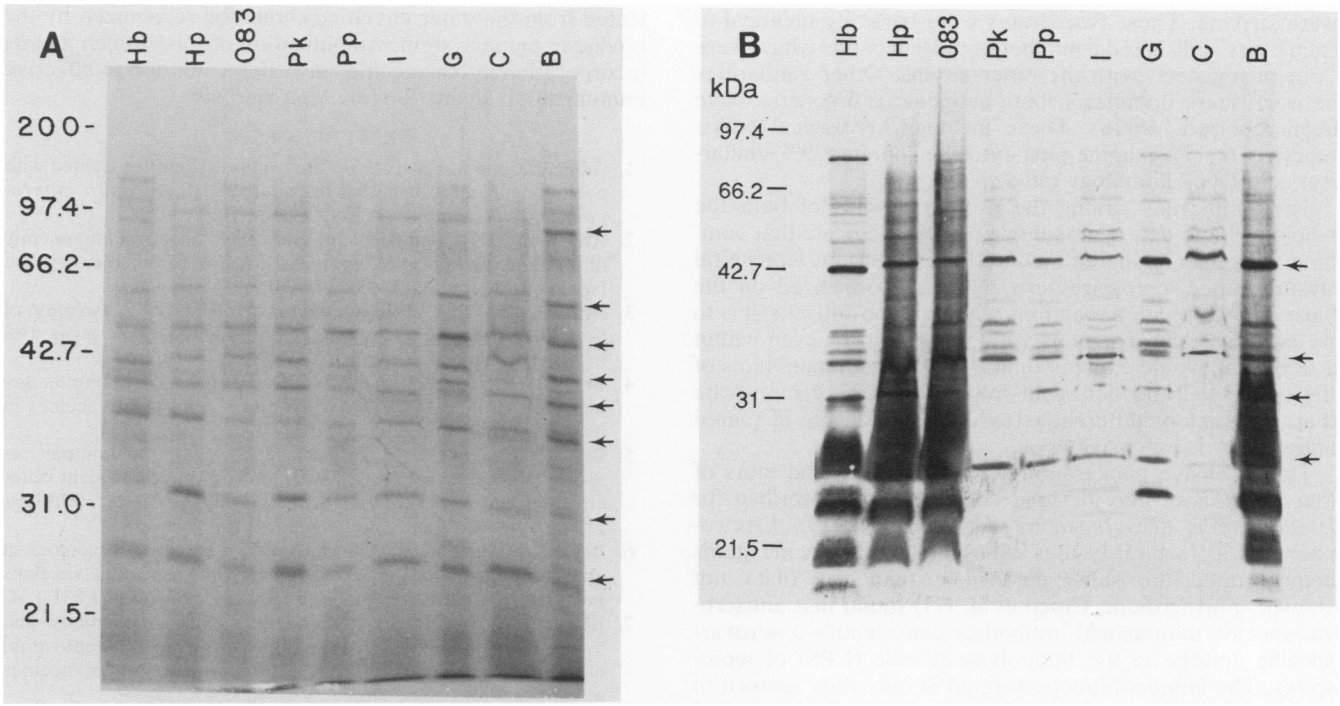


FIG. 2. (A) Leptospiral detergent-soluble proteins resolved on a 10 to 15% SDS gradient gel. Ten micrograms of protein was added to each lane. The gel was run and the samples were loaded as described in the legend to Fig. 1A. Arrows mark proteins of similar molecular masses (kilodaltons) expressed by the different serovars. (B) Immunoblot of leptospiral detergent-enriched proteins probed with antiserum raised against type hardjo bovis detergent-soluble antigens. Ten micrograms of protein was added to each lane. The gel was run and the samples were loaded as described in the legend to Fig. 1A. Arrows mark similar antigens recognized by the hardjo bovis antiserum.

60, 50, 42.7, 40, 38, 32, and 25 kDa), although some qualitative differences were noted.

Immunoblot analysis of leptospiral detergent-soluble proteins. As in the case of the whole-cell lysates, the degree of similarity among the detergent-soluble proteins suggests that they may have many antigens in common. In an effort to identify these antigens, an identical gel to that shown in Fig. 2A was blotted and probed with antiserum raised against hardjo bovis detergent-soluble proteins. The detergent-soluble proteins from the various strains have many antigens in common (Fig. 2B). In particular, the 25-, 32-, 35-, and 42.7-kDa antigens (Fig. 2B, arrows) are conserved in these strains. As expected, the antiserum reacted extensively with the members of the Sejroe serogroup. This immunoblot demonstrates that antigenic determinants of hardjo bovis are widespread among several serogroups of *L. interrogans* and suggests an explanation for the high degree of cross-reactivity in agglutination reactions.

DISCUSSION

Examination of selected leptospiral whole-cell lysates and detergent-soluble preparations, representing enriched outer membrane proteins, by PAGE has been previously reported (10, 16, 18). The analyses described in this report, however, represent the first description and comparison of whole-cell and detergent-soluble outer envelope-enriched proteins and antigens prepared from the *L. interrogans* serovars commonly involved in pathogenesis which are components of commercially available vaccines. This report also describes for the first time the study of the detergent-soluble antigens by using antisera raised specifically against this cellular component of hardjo bovis and hardjoprajitno. The similarity among the protein profiles of these strains is immediately apparent in both the whole-cell and detergent-soluble gels. In the SDS-PAGE whole-cell lysate gel, a number of possible quantitative differences in the distribution of proteins among the strains were noted. This observation is important, particularly since several investigators have concluded that some interactions between immune antisera and different leptospiral strains suggest quantitative and not qualitative differences in the expressed proteins. Johnson and Muschel (14) found that differences in leptospiral sensitivity to antibody and complement were probably a function of antigen quantity, while Thiermann and Garrett (22) stated that in experimentally infected animals immune antisera often show higher titers to heterologous serovars than to homologous ones. Furthermore, the Coomassie blue-stained gels of Chapman et al. (10) showed that differences between serovar profiles can be attributed to the relative abundance of the proteins. In light of these observations and the fact that humoral factors typically attack the outer surface of intact cells, these quantitative differences should be apparent in detergent-soluble fractions enriched for outer envelope antigens. We did observe these differences in our SDS-PAGE gel on which detergent-soluble antigens were analyzed (Fig. 2).

A careful comparison of the protein profiles revealed some interesting results. In regard to serovar hardjo, several differences exist between the whole-cell and detergent-soluble proteins of the hardjo bovis and the hardjoprajitno reference strains. Differences between these two organisms have been reported by using other assays in previous studies (16, 18).

The similarities of proteins and reacting antigens between hardjo bovis and the reference strain of serovar balcanica

were striking. These two strains were basically identical to each other and shared a number of protein bands which were "out of register" with the other strains. Other similarities between these organisms, both antigenic and genetic, have been reported (16–18). These included serological cross-reactivities (17), guanine-plus-cytosine content (39% similarity), and DNA homology (16).

The similarities among the protein profiles of both the whole-cell and detergent-soluble proteins indicate that common antigens are shared among these important leptospiral strains. Since leptospires are classically organized on the basis of agglutination reactions with immune antisera, it is to be expected that organisms within a serovar or even within a serogroup would express similar antigens. Immunoblots of the whole-cell and detergent-soluble antigens demonstrate that, even among different serogroups, the degree of shared antigenicity is extensive.

The extensive background seen in the gels and blots of these organisms may be lipid complexes, as described for *Pseudomonas aeruginosa* by Poxton et al. (19). Electrophoresed leptospiral lysates exposed to lipid-specific stains demonstrated the same pattern of reactivity (data not shown). Furthermore, Cinco et al. (11) found that antiserovar-specific monoclonal antibodies can identify a serovar-specific epitope in the lipopolysaccharide (LPS) of leptospires. The immunoblots performed in our study seemed to suggest that LPS in the membranes of hardjo bovis and hardjoprajitno may contain a serogroup-specific epitope(s), since the anti-hardjo antisera reacted not only with members of the hardjo serovar but with balcanica as well.

Studies involving the structure and immunogenicity of the leptospiral outer envelope are of particular interest; because this membrane surrounds the exterior of the cell, it provides the primary interaction between the leptospire and the immune system. Antibody reactivity and complement fixation are known to occur on the outer envelope (2). In addition, the outer envelope is a potent immunogen; hamsters (3, 4, 7), dogs (6, 7), guinea pigs (20), and cows (5, 24) have been vaccinated with outer envelope-enriched preparations, and these were successful in preventing death and, for most vaccines, the renal carrier state. Experimental immunization of hamsters with leptospiral LPS antigens has also been shown to be efficacious (13). Interest in different methods of vaccination against bovine leptospirosis continues because of the lack of protection afforded by commercially prepared vaccines (8). An attempt to incorporate pathogenic hardjo bovis in place of the nonpathogenic hardjoprajitno in a pentavalent vaccine has proven futile in combatting bovine leptospirosis (9). The low immunogenicity of serovar hardjo is a persistent problem. One investigator has suggested that the immune response to this serovar may be more complicated than the response to the other serovars (1). Thus, new approaches to vaccination against leptospirosis are necessary.

Our study demonstrates that a variety of IgG-specific antigens are held in common by constituent strains of leptospiral pentavalent vaccines. This suggests that an efficacious vaccine could be constructed by using one or a number of these common antigens as an immunogen. The proposed antigen(s) should be selected from the outer envelope because of the previously described interaction between outer envelope proteins and immune system components. Furthermore, LeFebvre et al. (16) have demonstrated that the leptospiral proteins recognized by rabbit antiserum constitute the majority of proteins recognized by the bovine humoral immune response. Therefore, the antigen(s) selected from the outer envelope should be recognized by the bovine immune system. Amplification of this antigen and its incorporation into a vaccine may offer a solution to effective immunization against bovine leptospirosis.

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