Development of in vitro assays for measuring the relative potency of leptospiral bacterins containing serovars pomona and canicola

by

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DEDICATION

I would like to dedicate this work to those individuals who made as many sacrifices as I did in order for this work to be completed: my parents, my wife Susan, my son Nicholas James Ruby, my two step-sons Jason Brook and Nicholas Charles Sayre, and my step-daughter Christal Lynn Sayre. Without their continuous love, support, and understanding, I would not have been able to complete this research.

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I feel that it is also important to mention that this research would not have been possible without the veterinary biologics industry. Several individuals throughout the industry participated in the evaluation of the *L. pomona* ELISA and offered several comments and suggestions.

GENERAL ABSTRACT

The current testing of leptospiral bacterins licensed by the United States Department of Agriculture requires a hamster potency assay. The hamster potency assay involves vaccinating hamsters with a specified dilution of the bacterin. Two weeks post-vaccination, hamsters are challenged with virulent leptospires and the percent survival is determined. A bacterin is considered satisfactory if 80% or more vaccinated hamsters survive a virulent challenge. There is a need for a rapid, sensitive, and reliable alternate method that could be used in the routine testing of leptospiral bacterins. The large number of hamsters required for the manufacture and testing of these biologicals is expensive, and increasing concern for animal welfare and human health makes in vitro assays for measuring vaccine potency desirable. An enzyme-linked immunosorbent assay (ELISA) would be simple, safe, easily automated, inexpensive, and suitable for potency testing of large numbers of product serials. The objective of this research was to develop an ELISA-based detection system for measuring the relative potency of leptospiral bacterins as an alternative to the hamster potency assay.

GENERAL INTRODUCTION

The National Veterinary Services Laboratories (NVSL) are responsible for the laboratory support with regard to licensing of animal vaccines of viral and bacterial origin as well as veterinary diagnostic kits. Testing of vaccines and bacterins includes efficacy, potency, and safety. These procedures are well established for veterinary biologicals (1).

The current testing of leptospiral bacterins licensed by the United States Department of Agriculture requires a hamster potency assay (1). The hamster potency assay involves vaccinating hamsters with a specified dilution of the bacterin. Two weeks post-vaccination, hamsters are challenged with virulent leptospires and the percent survival is determined. There is a need to replace the in vivo assay with a rapid, sensitive, and reliable alternate in vitro method that could be used in the routine testing of leptospiral biologicals. The large number of hamsters required for the manufacture and testing of these biologicals is expensive, and increasing concern for animal welfare and human health makes in vitro assays for measuring vaccine potency desirable. An ELISA would be simple, safe, easily automated, inexpensive, and suitable for potency testing of large numbers of product serials. The objective of this study was to develop an in vitro assay for measuring the relative potency of leptospiral bacterins as an alternative to the current hamster potency assay.

Explanation of thesis format

Two papers are included in this thesis. The manuscript, "Assay for measuring the relative potency of leptospiral bacterins containing serovar *pomona*" by K. W. Ruby, M.A. Cardella, and W. U. Knudtson, has been accepted by *Biologicals*. The research was conducted at the NVSL in Ames. As principal investigator for the project, I was responsible for planning, coordination of activities, laboratory analysis, performing the work,

presentation and reporting of results. Additional work not included in the manuscript is also included in the Appendices.

A second manuscript is also included with this thesis entitled "Development of an in vitro assay for measuring the relative potency of leptospiral bacterins containing serovar *canicola*" by K. W. Ruby, C. L. Egemo, and D. M. Walden. This manuscript will be submitted to *Biologicals* for publication consideration. The work presented in this thesis on the development of the *L. canicola* in vitro assay is the first publication of this work for this serovar. Both manuscripts are in the journal format for *Biologicals*.

The papers are preceded by a general introduction and a literature review. There are four appendices following the first paper that include additional data that was not included in the original manuscript. A general summary and references cited in the literature review, appendices, and general summary follow the second paper.

LITERATURE REVIEW

Leptospiral Taxonomy

Leptospires are classified in the order of *Spirochetales* and in the family of *Leptospiracea*. Genus members include *Leptospira* and *Letponema*. The genus *Leptospira* is divided into two species: *L. biflexa* which are saprophytic and non-pathogenic, and *L. interrogans* which are pathogenic. The basic taxon is the serovar, with serovars having common surface antigens grouped into serogroups. Currently there are over 180 known pathogenic serovars assigned to 20 serogroups on the basis of cross-reacting antigens (2).

The various pathogenic leptospires are not readily distinguishable on the basis of morphological, biochemical, and cultural characteristics. They do, however, have distinct antigenic properties that can be demonstrated by a serological test called the microagglutination test (MAT) (3). These properties are important for serological diagnosis and provide the basis for classification.

Leptospiral Morphology and Structure

Leptospires are flexible, helical cells with dimensions of 0.1 μm in width by 5-30 μm in length. Leptospires are spirochetes with a coiled structure and an intracellular or periplasmic flagellum that is proton-driven. In the periplasmic space, the two flagella (axial filaments) are attached through an insertion pore at each end of the organism and is covered by outer protein sheath (2,4-6). These filaments are responsible for motility of the organism. The ends of the organism are hooked. When the organism moves there is a bidirectional, serpentine-like motion. When the organism moves forward, the anterior hook disappears and the trailing end retains the shape of a hook.

The three major components of the leptospiral cell are the outer membrane, cytoplasm, and axial filament (5-7). The structure and composition of the outer membrane of *Leptospira* resembles the outer membrane of other gram-negative bacteria (5,7,8). *Leptospira*, however, appear to have 46% protein, 27% carbohydrate, and 22% lipid as the major macromolecules present in the outer membrane (8-10). Like gram-negative bacteria, these substances are components of lipoproteins, phospholipids, and lipopolysaccharide (LPS) in leptospiral outer membranes (5,7-10). It is common among *Leptospira* to find phosphatidylethanolamine composed mainly of hexa- and octadecenoic acid (5). Leptospiral LPS differs from the classical LPS by its lack of 2-keto-3-deoxy-octulosonic acid (KDO) and L-glycero-D-manno-heptose (5,11-15). Sugars such as arabinose, rhamnose, xylose, galactose, mannose, eryhtrose, fucose, and glucose have been described as components of the O antigen in *Leptospira* (5,11,12,16).

Leptospira have a gram negative outer membrane composed of an LPS-like substance. Lipid A appears to be absent in the outer membrane (5,12,13,16,17). These differences may account for a number of leptospiral LPS characteristics including low cytotoxicity, pyrogenicity, mouse lethality, and mitogenicity (18-21).

The leptospiral axial filament is composed of a sheath and an inner core (10,22,23). At least six proteins have been identified as part of the axial filament (22) while other reports indicate that there are four axial-filament proteins (24). A doublet composed of a 33-34 kilodalton (kDa) band and a 37 kDa band were the most prominent proteins in polyacrylamide gels (24). Leptospiral axial filaments have protein electrophoretic profiles similar to those of treponemes (5,24). The cytoplasmic cylinder has an internal cell wall structure that is composed of a peptidoglycan layer under which the cytoplasmic membrane is present. These structures maintain the integrity of the organism (5).

Leptospiral Zoonosis

Leptospires occur naturally in a wide variety of feral and domestic mammals. In natural hosts, the spirochetes colonize the kidneys and are shed in the urine. Humans are accidental hosts, with most infections related to occupational or recreational activities involving either direct contact with animal urine (e.g., fish workers in rat-infested surroundings, abattoir workers, miners, or sewer cleaners) or contact with water and soil contaminated with animal urine (e.g., rice fields, cane fields, ponds and streams, etc).

Leptospires have very definitive maintenance host specificity. The organism is usually more parasitic than pathogenic and is shed in the urine. Maintenance hosts for *Leptospira* serovars are the rat (*Icterohaemorrhagiae*), dogs (*Canicola*), raccoon (*Grippotyphosa*), cattle and swine (*Pomona*), cattle and sheep (*Hardjo*), and sheep, swine and the hedge hog (*Bratislava*). A study performed at the University of Minnesota Veterinary Diagnostic Laboratory (25) indicated that the incidence of *Leptospira* serovars in descending order was as follows: Cattle (*pomona* > *icterohaemorrhagiae* > *hardjo* > *grippotyphosa* > *canicola*), swine (*icterohaemorrhagiae* > *pomona* > *grippotyposa* > *canicola* > *hardjo*), horses (*icterohaemorrhagiae* > *pomona* > *grippotyphosa* > *canicola*), and dogs (*icterohaemorrhagiae* > *pomona* > *canicola* > *grippotyphosa*). *Leptospira pomona* is the least adapted serovar. With the exception of the Minnesota study, *L. hardjo* has rarely been isolated from swine. Humans generally become infected with serovars *Icterohaemorrhagiae*, *Pomona*, and *Grippotypohsa*.

Leptospiral Pathogenesis

Leptospirosis is an acute, febrile, septicemic disease attributable to any one of the large number of serologically distinct members of

L. interrogans. The routes of infection are usually the eye, broken skin, mucous membranes, and oral ingestion. Transmission of disease may be via urine of an infected animal or semen/venereal. Transmission may also be horizontal. Known virulence factors of leptospires include motility, hemolysin and cytotoxin production, and factors contributing to the evasion of the host immune response (26-33). The pathogenesis of the disease following penetration of mucous membranes by actively motile leptospires begins with a bacteremia 7-10 days after infection. The organisms colonize the liver, spleen and kidneys. About 7 days after the start of the bacteremia, specific antibody titers to leptospiral antigens begin to increase and the organism disappears from the tissues and circulation, except in the kidneys, reproductive tract and some areas such as the central nervous system and the eye.

Clinical Manifestations

The clinical manifestations of leptospirosis are variable and depend on whether the infected animal is a definitive host or an incidental host. In general, symptoms of leptospirosis range from inapparent infections to flu-like illnesses to "aseptic" meningitis, and, less frequently, to an icteric-hemorrhagic form with severe hepatic and renal involvement. Leptospiral infections in a definitive host may result in a low serological response, chronic reproductive disease, long-term shedding, and a high frequency of intra-herd transmissions. Leptospiral infections in an incidental host may result in acute, severe disease, lack of detectable shedding, and a high serological response. All ages and sexes of incidental host may be affected with a low frequency of intra-herd transmissions. The acute phase of the disease presents with septicemia, pyrexia, anorexia, acute hemolytic anemia, hemoglobinuria, jaundice, and agalactia. The chronic phase may present with prolonged convalescence,

kidney and liver damage, and abortion and stillbirths. One of the most common signs of leptospirosis in animals is reproductive failure (abortions, stillbirths, and infertility) (34-37). In cattle infected with leptospires, calves borne to infected cows will be chronically infected with *L. hardjo* but are asymptomatic. In swine, *L. bratislava* produces reproductive disease. In horses, abortions are due to *L. pomona* (especially in vaccinated horses). In dogs, severe pulmonary hemorrhage is due to serovars *icterohaemorrhagiae* and *canicola*.

Leptospiral Immunity

Leptospirosis in animals may be controlled by vaccination. In general, immunity is measured by a rapid and strong antibody (agglutinating) response. Following an active infection, the duration of immunity is lifelong and is serovar-specific. Although cell-mediated immune responses are involved, immunity to leptospirosis is primarily antibody mediated (38-49). Current *L. interrogans* vaccines composed of heat-killed or chemically inactivated bacteria can protect animals against clinical signs of leptospiral infections (25,38,41,43,50,51).

Leptospiral Antigens

The primary targets for immunoglobulin-mediated killing of *L. interrogans* are located in the outer membrane (4,6,7,40,52,53). Leptospiral antigens include a lipopolysaccharide-like substance (LLS), outer membrane proteins, and flagellar proteins. To date, LLS is the only agglutinating and opsonic antigen to be identified (14,16,18-21,54-56). Recent studies indicate that immunization with LLS can confer protection from infection in hamsters (55-57).

The outer membrane of *L. interrogans* has been shown to contain a glycolipid antigen (5,15,57). High-titer agglutinating antiserum can be

obtained after immunization with purified leptospiral LLS (16,18,38,39,44,56). Leptospiral LLS, like gram-negative LPS, is thought to be important in evading the induction of the complement cascade (21,29,38,46-49). Leptospiral LLS is a glycolipid and is active in the limulus ameobocyte lysate assay although it lacks 2-keto-3-deoxyoctulosonic acid and the endotoxic properties of gram-negative LPS such as pyrogenicity, toxicity, mitogenicity, activation of the fibrinolytic and complement systems, adjuvanticity, and induction of interferon production (12,16,17,19,21,48,49,56).

The outer envelope of *L. interrogans* contains carbohydrates, lipids, and at least 9 different proteins (58). The outer envelope contains several potent immunogens; hamsters (41,50,59), dogs (41,51), guinea pigs (42), and cattle (43,38) have been vaccinated with outer envelope-enriched preparations and were shown to have a high antibody response based on the microagglutination test. These vaccinations were successful in preventing death and, frequently, the renal carrier state. There is very little information available about the ability of the outer envelope proteins to elicit a protective immune response, or about their function (31,58). Zuerner et al (9) have recently extracted a major cellular protein from the outer membrane. The major cellular protein was identified as a 31 kDa hydrophobic protein located in the outer membrane and is exposed on the surface of the cell. Sera from infected animals reacted with this immunogen on western blots. This is the first documentation of a major outer membrane protein that is immunogenic.

Leptospiral Monoclonal Antibodies

Most monoclonal antibodies which recognize antigens of *L. interrogans* are directed against LPS (54,55,60,61). This may be due to the fact that surface proteins from *L. interrogans* and other bacteria are closely

associated with, and difficult to separate from, immunodominant LPS (11,12,62,63). There have been few reports of the development of monoclonal antibodies directed to surface proteins of *L. interrogans* which provide passive protection against a virulent challenge (64,65).

Leptospiral ELISAs

Enzyme-linked immunosorbent assays (ELISA) have been used in many laboratories for diagnosis of leptospirosis in humans and other animals (66-76). Studies have shown that the ELISA is more sensitive than the MAT for detection of antibodies against *L. interrogans* (73-75,77). Both genus-specific and serogroup-specific antigens have been detected in ELISA. Genus-specific antigens were prepared using detergents (69). Serogroup-specific antigens have been prepared using phenol-water extraction (70) and sucrose gradient purification of the outer envelope (67). Crude antigen preparations have been prepared by sonication (65,75) and heating of whole cells (70). In 1990, Champagne et al (78) reported on the development of a sandwich ELISA system for detection and characterization of leptospiral antigens in clinical specimens. There are no reports of an ELISA-based detection system for measuring the relative potency of leptospiral bacterins.

Objectives and Rationale for Study

The objectives of this research were to develop in vitro assays for measuring the relative potency of leptospiral bacterins containing serovars *pomona* and *canicola* as an alternative to the current hamster potency test. These assays are sandwich ELISAs utilizing polyclonal hyperimmune rabbit sera to capture antigen in bacterins. Monoclonal antibodies are used to detect antigen in the captured bacterins, followed by an anti-mouse horseradish peroxidase-labeled antibody and subsequent addition of color

substrate.

Although LPS is the most protective leptospiral immunogen, I chose to use a "shot-gun" approach to obtain immune spleen cells with antigenic specificity for proteins as well as LPS. Because Triton X-100 is a non-ionic detergent and will solubilize outer membrane proteins (79), a Triton X-100 extract of serovars *pomona* type kennewicki and the NVSL challenge strain of *portlandvere* was used to immunize BALB/c mice. These serovars are the type strains used in leptospiral bacterins manufactured in the United States. The results of these studies will have a significant impact on the veterinary biologics industry as well as the NVSL with respect to testing leptospiral vaccines. In addition to leptospiral vaccine testing, the ELISA procedures developed in this study have the potential for use in diagnostic applications such as identification of *Leptospira* in semen and other biological samples.

PAPER I. ASSAY FOR MEASURING THE RELATIVE POTENCY OF LEPTOSPIRAL BACTERINS CONTAINING SEROVAR *POMONA*

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed for the quantitation of leptospiral antigen in bacterins containing *Leptospira interrogans* serovar *pomona* type kennewicki. A monoclonal antibody (MAb), 2D7, which is directed against a surface antigen on whole cells of *L. interrogans* serovar *pomona* type kennewicki, was used in the assay. The capture of antigen in bacterins by a polyclonal antiserum was followed by the addition of the 2D7 ascites fluid, an anti-mouse immunoglobulin conjugate, and substrate. Vaccines evaluated with this system included preparations containing type kennewicki antigen (homologous) and those not containing type kennewicki antigen (heterologous). Heterologous bacterins gave optical density (OD) values comparable to those of blank wells. Homologous bacterins yielded OD values equal to or greater than those of the National Veterinary Services Laboratories (NVSL) reference *pomona* bacterin. The relative potencies (RP) of 84 licensed commercial *L. pomona* bacterin serials were evaluated against the NVSL reference *pomona* bacterin using the NVSL Relative Potency computer program. Random samples of 1-, 2-, 3-, and 5-ml dose products were selected for evaluation with this system and had an RP of 1.0 or greater. All products tested passed the hamster potency assay required for leptospiral bacterins. This ELISA system enables detection of antigen in bacterins containing *L. interrogans* serovar *pomona* type kennewicki and demonstrates the potential for *in vitro* testing of leptospiral bacterins.

INTRODUCTION

Leptospirosis is a disease of domestic animals. *Leptospira interrogans* serovar *pomona* causes leptospirosis in cattle and swine with the cardinal sign of the disease being abortion. At the present time, the best preventive measure is vaccination with bacterins which, in many countries, contain chemically inactivated whole-cell leptospires.

Leptospires are classified into serovars based on agglutination reactions with rabbit antisera. The basic taxon for *Leptospira* is the serovar, each having one or more strains. Serovars having common surface antigens are grouped into serogroups.

The current testing of leptospiral bacterins licensed by the United States Department of Agriculture requires a hamster potency assay (1). The hamster potency assay involves vaccinating hamsters with a specified dilution of the bacterin. Two weeks post-vaccination, hamsters are challenged with virulent leptospires and the percent survival is determined. The large number of hamsters required for the testing of these products is expensive, and increasing concern for animal welfare and human health makes *in vitro* assays for measuring vaccine potency desirable.

Monoclonal antibodies (MAbs) have been produced by several laboratories in an attempt to determine the nature of those leptospiral antigens that stimulate production of protective antibodies (2-14). *Leptospira interrogans* serovar *pomona* type kennewicki is commonly used for preparing most serogroup Pomona bacterins in the United States. Monoclonal antibodies to antigens of this serovar would be useful for *in vitro* assays for quantifying antigen in *pomona*-containing bacterins. We chose to use a Triton X-100 extract of serovar *pomona* type kennewicki for the production of the 2D7 MAb for use in an ELISA-based detection system. This report describes an *in vitro* assay for use as an alternative to the hamster potency assay.

MATERIALS AND METHODS

Materials

Bacterial Strains

Leptospira interrogans organisms used in this study were obtained from the National Veterinary Services Laboratories (NVSL) collection. Strains virulent for hamsters included *L. pomona* 11000 (MLS), *L. hardjo* 11601 (SC1769), *L. grippotyphosa* 11808 (Oregon shrew), *L. canicola* 11203 (Moulton), and *L. icterohaemorrhagiae* 11403 (CF1). *Leptospira bratislava* 2a and *L. bratislava* jez were also used. Serovars within the Serogroup Pomona used in this study included *kunming* KS, *mozdok* 5621, *tropica* CZ 299U, *pomona* pomona, *tsaratsovo* B-52, and *proechymis* LT 796, as well as the type strain kennewicki 1026 of serovar *pomona*. Unless indicated, all organisms were heat inactivated by incubation in a 56° C water bath for 30 minutes. One-hundred microliter aliquots of the heat-inactivated cultures were used to inoculate medium to establish cell death.

Bacterins

Bacterins used in the antigen extinction studies and tested by the ELISA included licensed commercial adjuvanted bacterins (monovalent and multivalent). The NVSL reference *L. pomona* bacterin was also evaluated.

Animals

New Zealand white rabbits (Harlan-Sprague-Dawley, Inc., Indianapolis, IN) were used to prepare polyclonal, serovar-specific antisera.

Fifteen- to twenty-g male BALB/c mice (Harlan-Sprague-Dawley Inc., Indianapolis, IN) were used for MAb production. Antigen extinction studies were performed in 40- to 50-g male golden Syrian hamsters (Sasco, Inc., Omaha, NE).

Antisera Production

A hamster virulent culture of L. pomona was used to inoculate each of two tubes (1 ml each) of semi-solid P80-BA media. The tubes were then incubated at 37° C for 21 days. Sterility of the culture was determined by inoculating two 5% bovine blood agar plates, two tubes of fluid thioglycolate, and two tubes of trypticase soy broth with 200 µl each of the culture. One set of each inoculated media was incubated at room temperature (27° C) and one set at 37° C for seven days.

Two rabbits were inoculated with 1.0, 2.0, 4.0, and 5.0 ml of the P80-BA semi-solid culture. All inoculations were given intravenously in the marginal ear vein. The first three inoculations were given at 5 day intervals and the final inoculation was given at 7 day intervals. All inocula consisted of viable leptospires. After the final inoculation, the rabbits were observed for 10 days. On the tenth day, blood was obtained by cardiac puncture and the serum harvested by centrifugation at 3,000 x *g* for 15 min. The serum was then filtered with a 0.22 µ filter (Millipore Corp., Bedford MA) and stored at -70° C in 1 ml aliquots.

ELISA Reagents

Horseradish peroxidase (HRP) conjugated goat anti-mouse IgA (α) conjugate and 3, 3', 5, 5'-tetramethylbenzidine (TMB) (Kirkegaard-Perry Laboratories, Inc., Gaithersburg, MD) were used as the antibody conjugate and substrate respectively . Polyvinyl alcohol (PVA) was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI. Polyclonal rabbit anti-*pomona* serum was produced at the NVSL. Immulon-2 microtitration plates were obtained from Dynatech Laboratories, Inc., Alexandria, VA.

The capture antisera was diluted in coating buffer (0.05 M carbonate buffer, pH 9.6) containing 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ per liter of distilled water. The pH was adjusted to 9.6. This solution was stored at

4° C for no longer than 1 week. The antigen (bacterin) diluent was phosphate buffered saline (PBS) (0.15 M, pH 7.2) and contained 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 per liter of distilled water. The diluent was adjusted to pH 7.2 and filter sterilized (0.22 μm). The blocking solution consisted of a 1% solution of PVA (88% hydrolyzed, MW 10,000) in antigen diluent. Antibodies were diluted in blocking solution supplemented with 1% normal rabbit serum.

Methods

Antigen Extraction

Leptospira interrogans organisms were grown in 1 L P80 liquid media (10) for 7 days at 30° C. Cultures of *L. pomona* type kennewicki harvested after 7 days incubation contained approximately 1 x 10^6 leptospires/ml. The organisms were harvested by centrifugation (12,000 *X g* for 30 minutes) and were washed three times in PBS (pH 7.2). Pellets were resuspended in 6 ml of a tris-tricine, 5 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 8.6) containing 0.01% Triton X-100 (Sigma Chemical Co., St. Louis, MO). Suspensions were incubated overnight on a platform rocker at room temperature and then centrifuged as described above. Supernatants, which contained the outer envelope membranes (2,16,17), were filter sterilized (0.22 μm) and stored at 4° C. These supernatants were used to immunize BALB/c mice for the production of the MAb.

Monoclonal Antibody (MAb) Production

BALB/c mice were injected intraperitoneally (ip) with nonadjuvanted Triton X-100 extract of serovar *pomona* type kennewicki containing approximately 50 μg of protein. A booster injection containing 50 μg of protein was given intravenously 3 weeks later. The MAb (2D7) was produced by the method of Van Deusen and Whetstone (28,29) using the Triton X-100

extract of *L. interrogans* serovar *pomona* type kennewicki. Selection of positive clones was based on a) high ELISA optical density (OD) values with the homologous antigen and no cross reactivity with heterologous antigens, and b) western blot analysis. The selected clone, designated 2D7, was used to prepare ascites fluid. The ascites fluid was screened by ELISA, western blot analysis, and passive protection studies in hamsters. The antibody isotype of the 2D7 MAb was determined to be IgA using a Mouse Typer Sub-Isotype Kit (Bio-Rad Laboratories, Richmond, CA). The antibody concentration of the ascites fluid was determined using a radial immunodiffusion kit (Calbiochem, San Diego, CA).

Microscopic Agglutination Test (MAT)

The MAT was performed according to standard protocols (17). Briefly, serial 2-fold dilutions (beginning with 1:20) of the 2D7 MAb were prepared and incubated with viable cultures (with nephalometer readings of 20-30) of serovars *icterohaemorrhagiae, grippotyphosa, canicola, bratislava* 2a, and *pomona* type kennewicki. In addition to type kennewicki of serovar *pomona*, the following serovars of the Serogroup Pomona also served as antigen: *kunming* KS, *mozdok* 5621, *tropica* CZ 299U, *pomona* pomona, *tsaratsovo* B-52, and *proechymis* LT 796.

Passive Protection Studies

Five hamsters were injected ip with 0.5 ml of undiluted hybridoma culture fluid containing the 2D7 MAb. Five hamsters (control group) received an equal volume of sterile hybridoma culture medium. Both groups of hamsters were challenged 24 h later with 0.25 ml of liver suspensions from hamsters infected with type kennewicki (10 to 10,000 hamster LD_{50}'s as determined by titration) (1). In a separate study, five groups of hamsters (five per group) were each vaccinated ip with 0.5 ml of a serial 5-fold

(1:5 to 1:3125) dilution of the 2D7 MAb. Five hamsters were given an equal volume of hybridoma fluid prepared against *Pasteurella multocida* Type D dermonecrotic toxoid (NVSL). Challenge procedures were performed as outlined above.

Western Blotting

Triton X-100 extracted antigens of serovar *pomona* type kennewicki, and inactivated whole cells of serovars *canicola*, *icterohaemorrhagiae*, *grippotyphosa*, and *pomona* were separated on a 14% homogeneous separation gel (4% stacking gel) using a mini-electrophoresis system (Schleicher & Schuell, Keene NH). Extracted antigens were treated with sample buffer containing 0.05% 2-mercaptoethanol and 2% SDS. Samples were not boiled prior to electrophoresis. Electrophoresis was carried out at 30 mA for 1.5 h. Separated antigens were electro-transferred to nitrocellulose membrane (Millipore Corp., Bedford, MA) at 100 mA for 2 h in buffer containing 12 mM tris and 96 mM glycine (pH 8.3). After transfer, the membrane was rinsed in distilled water and placed into a 1:80 dilution (in PBS containing 0.05% Tween) of ascites fluid containing the 2D7 monoclonal antibodies. After rinsing with distilled water, the membrane was placed into a 1:1000 dilution (in PBST) of affinity purified peroxidase labeled goat anti-mouse IgA. After rinsing in distilled water, the membrane was placed in a solution of 4-chloronaphthol (Sigma Chemical Co., St. Louis, MO) for 10-15 min.

Antigen Extinction Studies

Antigen extinction studies were performed using selected bacterins in order to determine the end-point of protection. Hamsters were vaccinated intramuscularly with 0.25 ml of one of five 5-fold dilutions (in physiologic saline) of two commercial monovalent bacterins (bacterins 5649

and 9866) or the NVSL reference *L. pomona* bacterin. All three bacterins were 2-ml-dose products. Each 0.25 ml of the initial dilution contained 1/800 of the host-animal dose. There were 10 hamsters per dilution per bacterin. Fourteen days later, hamsters were challenged with virulent *L. pomona* organisms. Results were expressed as the protective dose at which a minimum of 80% of the hamsters survived an intraperitoneal challenge of 10 to 10,000 LD_{50}'s (1).

ELISA Procedure

Polyclonal hyperimmune rabbit antiserum (prepared against *L. interrogans* serovar *pomona* type kennewicki) was diluted 1:100 in coating buffer (pH 9.6). Immulon-2 96-well microtitration plates were coated with 100 µl of the diluted rabbit antisera per well, and the plates were incubated at 4° C overnight. The antiserum was aspirated, 300 µl of blocking solution was added to all wells, and the plates were incubated for 1 h at 37° C on an orbital shaker. The plates were washed once with distilled water, and 100 µl of 2-fold dilutions of bacterins and the test reference were added to duplicate wells beginning with a 1:2 dilution. The NVSL reference *pomona* bacterin served as the test reference and was diluted at 1:7, 1:14, 1:21, and 1:35 with distilled water. These dilutions were considered to be undiluted and were used in the ELISA protocol for 1-, 2-, 3-, and 5-ml dose products, respectively. Plates were incubated for 1 h at 37° C on an orbital shaker. After washing three times (with distilled water), 100 µl of a 1:2000 dilution of 2D7 ascites fluid was added to all wells, and the plate was incubated on an orbital shaker at 37° C for 1 h. The plate was washed (with distilled water) three times to remove unbound 2D7 MAb, 100 µl of a 1:1000 dilution of goat anti-mouse IgA (α) horseradish-peroxidase conjugate was added to all wells, and the plate was

incubated for 40 minutes at 37° C on an orbital shaker. After three washes (with distilled water), 100 µl of TMB was added to all wells, and the plate was incubated for 5 minutes at room temperature on an orbital shaker. The color reaction was stopped with 2.5 M sulfuric acid. The plate was read at a dual absorbance of 450/650 nm (Vmax Microtiter Reader, Molecular Devices, Inc., Menlo Park, CA).

Data Analysis

The mean OD for all blank wells was determined, and that value was subtracted from all sample OD values prior to data analysis. Linear regression and relative potency (RP) were calculated according to the NVSL RelPot Program (version 2.0). This program is designed to assign weight to linear regression lines for both a reference and a test sample. Slopes were checked for parallelism, and the relative potency calculations were made. Only the top three RP values were reported by the RelPot Program. For more information regarding the RelPot Program and availability, interested persons may contact the National Veterinary Services Laboratories, Veterinary Services, Animal and Plant Health Inspection Service, Ames, IA., 50010, USA. Criteria for a valid test were: a) a simple linear regression line must be fitted to a minimum of three contiguous dilutions, and b) lines must have a correlation coefficient (r) of at least 0.95. Any bacterin yielding an RP of 1.0 or greater is of equal or greater potency as compared to the reference bacterin.

RESULTS

The 2D7 hybridoma clone was shown to secrete IgA antibody (474 mg/ml) and was shown to react with a single antigen of approximately 14 to 16 kDa by western blot analysis using inactivated whole cells of serovar *pomona* type kennewicki (Figure 1). Monoclonal antibody 2D7 failed to react with antigens in whole cell preparations of the remaining leptospiral serovars. The 2D7 MAb also reacted with a single antigen of approximately 18 kDa in the Triton X-100 extract preparation.

The 2D7 MAb was specific for most but not all members of the serogroup Pomona when tested with inactivated whole cells of six *Leptospira* serogroups by ELISA. No cross reactivities were observed among serovars *canicola, icterohaemorrhagiae, grippotyphosa, hardjo* 11601, or *bratislava* 2a (Figure 2). Cross reactivity of the 2D7 MAb to six *pomona* serovars within the serogroup Pomona, including serovar *pomona* type kennewicki, is shown in Figure 3. Only two serovars, *mozdok* and *tropica*, failed to react with the 2D7 ascites fluid. Results from MAT studies were consistent with ELISA results using viable cells of seven hamster virulent *L. interrogans* serovars (Tables 1 & 2). In the hamster protection studies, the 2D7 MAb provided passive protection against a homologous challenge, but failed to protect hamsters from heterologous challenges (Table 3).

In order to determine the end-point of protection produced by licensed leptospiral bacterins in hamsters, the NVSL reference *L. pomona* bacterin, a monovalent adjuvanted product (bacterin 5649), and a monovalent nonadjuvanted product (bacterin 9866) were evaluated in hamsters. Results of the antigen extinction studies are shown in Table 4. The protective dose at which 50% of the hamsters died (PD_{50}) were 1:10,000, 1:35,000, and 1:40,000 for bacterin 5649, the NVSL reference *L. pomona* bacterin, and bacterin 9866, respectively.

Leptospiral bacterins evaluated by the ELISA represented 1-, 2-, 3-,

and 5-ml dose products (Table 5). All commercial bacterins tested at the recommended host-animal dose yielded relative potency (RP) values of 1.0 or greater. When tested at dilutions corresponding to antigen extinction values, no color development was noted. However, when doubling dilutions of these products were tested beginning with a 1:2 dilution, there was sufficient color development with an RP of $\geq$ 1.0 for all products tested.

Figure 1. Western blot using the 2D7 MAb to detect antigen in various cellular preparations. A 14% acrylamide gel was used to separate boiled antigens (50 μg of protein per lane). After electro-transfer, the membranes were probed with the 2D7 ascites fluid according to the protocol described in the Materials and Methods section. Lanes 1,2: *L. icterohaemorrhagiae* whole cells, Lanes 3,4: *L. grippotyphosa* whole cells, Lanes 5,6: *L. canicola* whole cells, Lanes 7,8: *L. pomona* Triton X-100 extract, Lane 9: *L. pomona* whole cells, Lane 10: pre-stained low molecular weight standards.

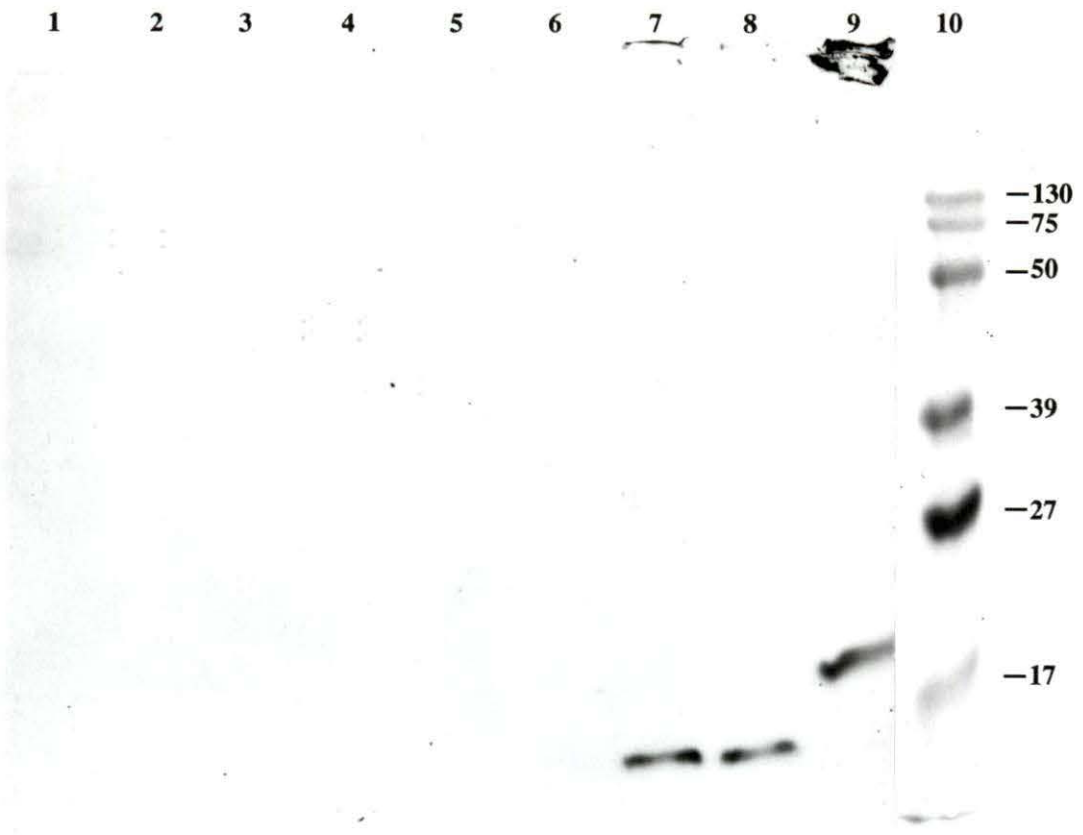
1
2
3
4
5
6
7
8
9
10
—130
—75
—50
—39
—27
—17

Figure 2. Reactivity of the ascites fluid containing MAb 2D7 with six inactivated whole cells of *Leptospira interrogans* serovars. Inactivated whole cells were diluted 1:100 and captured by the polyclonal anti-*pomona* rabbit serum as described in the Materials and Methods section. A 1:2000 dilution of the 2D7 ascites fluid was added followed by a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgA (a-chain specific). The substrate was 3, 3′, 5, 5′ -tetramethylbenzidine (TMB).

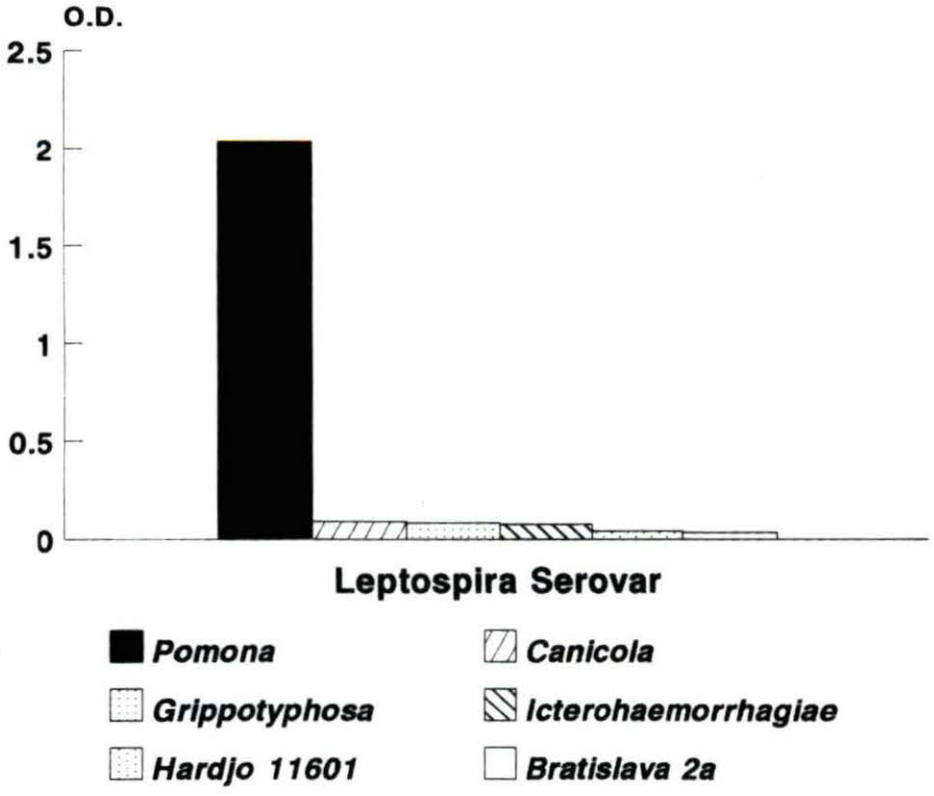
O.D.
2.5
2
1.5
1
0.5
0
Leptospira Serovar
Pomona
Canicola
Grippotyphosa
Icterohaemorrhagiae
Hardjo 11601
Bratislava 2a

Figure 3. Cross reactivity of ascites fluid containing MAb 2D7 to six *pomona* serovars including serovar *pomona* type kennewicki. Heat-inactivated whole cells were diluted 1:100 and captured by the polylconal rabbit anti-*pomona* serum as described in the Materials and Methods section. A 1:2000 dilution of the 2D7 ascites fluid was added followed by a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgA (a-chain specific). The subtrate was 3, 3′, 5, 5′ -tetramethylbenzidine (TMB).

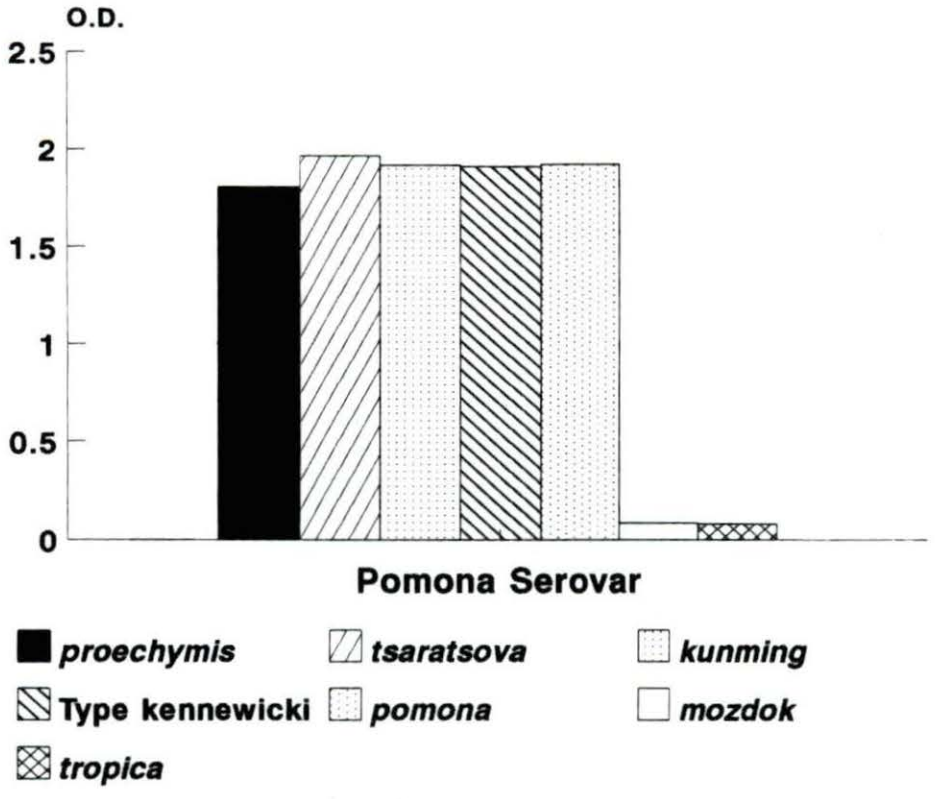
O.D.
2.5
2
1.5
1
0.5
0
Pomona Serovar
proechymis
tsaratsova
kunming
Type kennewicki
pomona
mozdok
tropica

Table 1. Microagglutination titers (MAT) of the 2D7 MAb for various serovars of *L. interrogans*

Antigen[a]	MAT Titer[b]
Pomona	10240
Canicola	<20
Grippotyphosa	<20
Icterohaemorrhagiae	<20
Hardjo	<20
Bratislava 2a	<20
Bratislava jez	<20

[a] Viable whole cells of hamster virulent serovars of *L. interrogans* were used as the antigen.

[b] Titer is expressed as the reciprocal dilution at which 50% of the leptospires were agglutinated.

Table 2. Specificity of the 2D7 MAb among *pomona* serovars of Serogroup Pomona by the microagglutination test

Antigen[a]	MAT Titer[b]
L. pomona type kennewicki 1026	10240
L. pomona pomona	10240
L. pomona proechymis LT 796	5120
L. pomona tsaratsovo B-52	640
L. pomona kunming KS	320
L. pomona mozdok 5621	<20
L. pomona tropica CZ 299U	<20

[a] Viable whole cells of each serovar were used as the antigen.

[b] Titer is expressed as the reciprocal dilution at which 50% of the leptospires were agglutinated.

Table 3. Passive protection of hamsters with the 2D7 MAb against a virulent challenge of serovar *pomona* type kennewicki, *Icterohaemorrhagiae*, *Canicola*, or *Grippotyphosa* [a]

Dilution of 2D7 MAb	Hamsters Surviving Homologous Challenge (type kennewicki)	Hamsters Surviving Heterologous Challenge[b] (I)	(C)	(G)	(DNT)
undiluted	5/5[c]	0/5	0/5	0/5	0/5
1:5	5/5	0/5	0/5	1/5	0/5
1:25	3/5	0/5	1/5	0/5	0/5
1:125	3/5	0/5	0/5	0/5	0/5
1:625	3/5	0/5	0/5	0/5	0/5
1:3125	0/5	1/5	0/5	0/5	0/5

[a] Hamsters were vaccinated with the 2D7 MAb and challenged 24 h later with virulent organisms. As a control, a MAb prepared against *Pasteurella multocida* Type D DNT was also used to vaccinate hamsters

[b] Results are expressed as survivors/total.

[c] Heterologous *L. interrogans* serovars *Icterhaemorrhagiae* (I), *Canicola* (C), and *Grippotyphosa* (G). Results using the *P. multocida* DNT MAb are also given.

Table 4. Comparison of PD_{50} values and RP values of three 2-ml-dose products

Bacterin	PD_{50}[a]	RP Range[b]
5649	10,000	8.23-9.98
NVSL	35,000	17.24-19.53
9866[c]	40,000	27.40-29.52

[a] The protective dose at which 50% of vaccinated hamsters survive a virulent challenge.

[b] ELISA relative potency (RP) values of each bacterin against the ELISA reference bacterin. Relative potencies were calculated with the NVSL ReiPot computer program. The top three RP values are shown.

[c] Non-adjuvanted product.

Table 5. Bacterins evaluated for relative potency by ELISA [a]

Total No. Products	Host Animal Dose	No. Firms
1	1 ml dose	1
30	2 ml dose	8
3	3 ml dose	1
50	5 ml dose	6
NVSL (TR)[b]	2 ml dose	-

[a] A total of 84 random bacterin samples were evaluated by the ELISA system described in Materials and Methods.

[b] The NVSL reference *L. pomona* bacterin was used as the test reference (TR) and was evaluated against itself in the ELISA system.

DISCUSSION

The utilization of an *in vitro* assay for measuring the potency of *L. interrogans* serovar *pomona* bacterins is advantageous in reducing animal costs and lowering the risks to human health associated with the current test methods for leptospiral bacterins. This ELISA system, which utilizes a MAb specific for serogroup Pomona, was developed for this purpose.

The anti-*Leptospira* MAb (2D7) utilized in this assay system is agglutinating (Tables 1 & 2) and protective (Table 3). This is advantageous for testing bacterin potency since agglutinating antibodies have been shown to be involved in the protective humoral response to infection (4,16,20-27). It is assumed that the outer envelope of the organism possesses epitopes against which protective antibodies are directed. Opsonization and agglutination would facilitate phagocytosis of the leptospires by macrophages and polymorphonuclear lymphocytes.

Antigen extinction studies in hamsters were utilized to determine the end-point of protection produced by the NVSL *L. pomona* reference bacterin and two monovalent bacterins (Table 4). Results from this study emphasize the high sensitivity of the hamster model. In the current hamster model, *L. pomona* bacterins are diluted to 1/800 of the host-animal dose and used to vaccinate hamsters. From the antigen extinction studies, many of these bacterins may conceivably be diluted 1:40,000 and still elicit a protective response against a virulent challenge in the hamster model. Although hamsters are extremely sensitive to leptospiral challenge, the sensitivity of this ELISA-based system closely parallels the hamster model. It is assumed that bacterins that fail the hamster potency test should also fail when tested by ELISA. It is noteworthy that the nonadjuvanted bacterin (9866) and the adjuvanted NVSL reference *L. pomona* bacterin yielded similar PD_{50} and RP values (Table 4). This suggests that adjuvant does not interfere with the performance of the ELISA-based system to detect

L. pomona antigen in these 2-ml-dose products.

The use of this ELISA provides a reproducible alternate method for testing the *L. pomona* component of bacterins. In addition, this test system could be used for diagnostic purposes, such as screening of master seed cultures used in the manufacture of leptospiral bacterins and for screening cultures for cross-contamination by other *L. interrogans* serovars. Other assays currently being developed in this laboratory to measure antigen(s) of serovars *canicola* and *grippotyphosa* will provide additional *in vitro* testing methods for the evaluation of leptospira bacterins and reduce even further the need for *in vivo* testing of these products.

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APPENDIX A: LABELING OF THE 2D7 MAB WITH HORSERADISH PEROXIDASE

Procedure

Reagents

Saturated ammonium sulfate [$(NH_4)_2SO_4$] was prepared by dissolving 550 g of pure $(NH_4)_2SO_4$ (reagent grade, Fisher Scientific, Fair Lawn, NJ) in 1 liter of distilled water. Sodium bicarbonate (0.3 M) was prepared by dissolving 1.26 g of $NaHCO_3$ in 50 ml distilled water. Fluorodinitrobenzene (1%) was prepared by adding 0.5 ml of 1-fluoro 2,4-dinitrobenzene $(FC_6H_3)NO_2)_2$ (Sigma Chemical Co., St. Louis, MO) to 49.5 ml of absolute alcohol. Sodium periodate (0.08 M) was prepared by dissolving 0.856 g of $NaIO_4$ in 50 ml distilled water. Ethylene glycol (0.16 M) was prepared by adding 0.45 ml of ethylene glycol to 49.55 ml of distilled water. A stock solution of 0.1 M carbonate-bicarbonate buffer (pH 9.5) was prepared by mixing 9 parts of a 0.1 M Na_2CO_3 solution to 28 parts of a 0.1 M $NaHCO_3$ solution and diluting the mixture 1:10 with distilled water. The 0.1 M Na_2CO_3 stock solution was prepared by dissolving 10.6 g Na_2CO_3 in 1 liter of distilled water and the pH was adjusted to 11.2. The 0.1 M $NaHCO_3$ stock solution was prepared by dissolving 8.4 g $NaHCO_3$ to 1 liter of distilled water and the pH was adjusted to 8.1. A 0.85% saline solution was prepared by dissolving 8.5 g NaCl in 1 liter of distilled water and the pH was adjusted to 8.0.

Horseradish peroxidase solution (HRP) was prepared by dissolving 50 mg HRP (Rz factor of 3.2, Sigma Chemical Co., St. Louis, MO) in 10 ml of a 0.3 M sodium bicarbonate solution. One milliliter of the 1% fluorodinitrobenzine (Sigma Chemical Co., St. Louis, MO) solution was added and allowed to incubate on a rotary shaker (10-20 rpm) for 1 h. Ten milliliters of a 0.8 M sodium periodate solution was added and incubated

for 30 min on a rotary shaker. Ten milliliters of 0.16 M ethylene glycol was added and incubated for 1 h on a rotary shaker. The HRP solution was dialized against 2 liters of 0.01 M carbonate buffer (pH 9.5) for 5 days with a total of eight changes of buffer. The HRP solution was filter sterilized with a 0.45 μm filter.

Delipidization of Ascites Fluid

Twenty milliliters of ascites fluid containing the 2D7 MAbs were filtered through a 0.45 μm filter (Millipore Corp., Bedford, MA) and 0.4 ml of a 10% solution of dextran sulfate was added in a drop-wise manner. The mixture was incubated at room temperature (25° C) for 30 min stirring occasionally. Two milliliters of a 1 M $CaCl_2$ solution was slowly added to the ascites fluid and incubated at room temperature for 15 min with occassional stirring. The mixture was centrifuged at 12,000 x g at 4° C for 30 min and the supernatant was collected without disturbing the pellet. The pellet was discarded.

Immunoglobulin Fractionation and HRP Labeling

The supernatant from delipidization was fractionated with three successive fractionations of 50%, 45%, and 45% saturated ammonium sulfate. For each fractionation, the ammonium sulfate was added in a drop-wise manner while stirring in an ice bath. The mixture was stirred for 30 min at room termperature and then centrifuged at 10,000 x g for 30 min at 4° C. The supernatant was discarded. The pellet was rinsed with distilled water and resuspended in 20 ml distilled water (pH 7.5). After the final fractionation, the pellet was resuspended to one-half the original volume (10 ml) with distilled water (pH 7.5).

The globulin-containing solution was placed in a dialysis bag and dialized against several changes of 0.85% saline until the dialysate was

free of sulfate ion (72 h). The dialysate was checked for the presence of sulfate ion by adding 1 ml of dialysate to 1 ml of saturated barium chloride (BaCl). When the dialysate was free of sulfate ions, the globulin-containing solution was centrifuged at 10,000 x g for 30 min at 4° C and filter sterilized with a 0.45 μm filter. A 1 ml sample of the globulin-containing solution was removed and used for protein determination using a Paragon Electrophoresis Kit SPE (Beckman, Palo Alto, CA). There were 16.4 mg protein/ml globulin present. The globulin-containing solution was dialyzed against eight changes of 0.01 M carbonate buffer (pH 9.5) for 5 days at 4° C.

Immunoglobulin Labeling

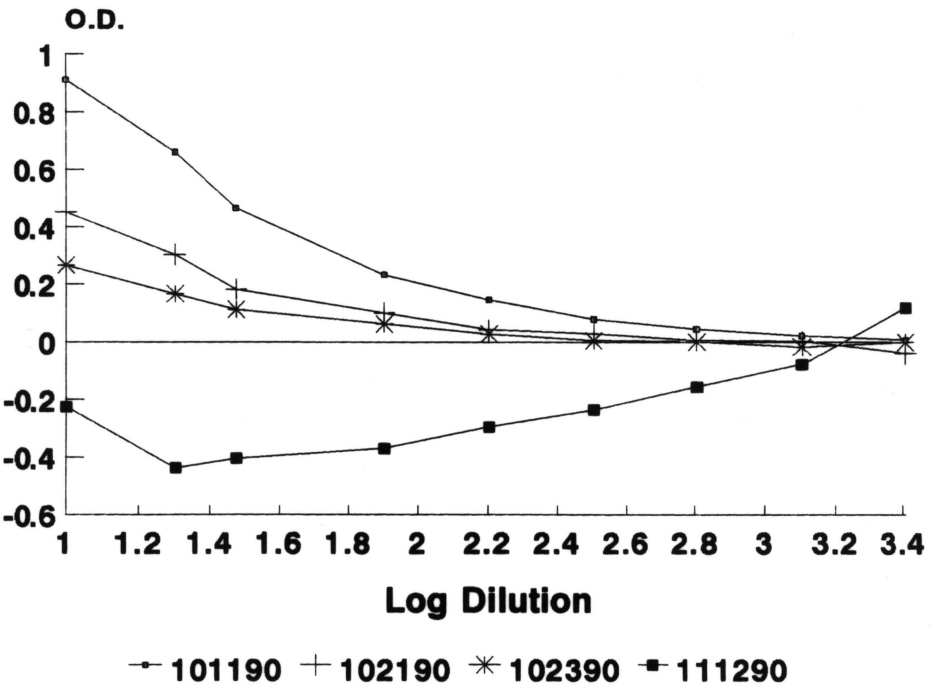
After the globulin-containing solution and the HRP solution were dialyzed, 3.5 ml of globulin-containing solution was added to 17.2 ml HRP solution. The mixture was allowed to rotate slowly at room temperature for 3 h. The 2D7 HRP-conjugated antibody was dispensed into 20 μl aliquots and stored at 4° C until use.

Results

Several attempts were made to obtain a stable 2D7 HRP conjugate. All attempts failed. We were able to conjugate the MAb, but the stability was poor. Initially, similar OD values were obtained using the 2D7 conjugate as compared to the two-step detection method (Figure A1). However, over a one month period, the OD values decreased until there was no difference between OD values obtained using the 2D7 conjugate and background values. Based on these results, no further attempts at conjugation of the 2D7 MAbs were made.

Figure A1. Stability of the 2D7 HRP conjugate. The 2D7 HRP conjugate was used in the ELISA protocol at a 1:1000 dilution. The assay was performed on four separate occassions using the NVSL reference *L. pomona* bacterin.

2D7 Conjugate Stability



Discussion

Antibodies can be tagged with a variety of labels. Each choice of label has advantages and disadvantages over the use of other labels. The most widely used labeling process involves covalently coupling the antibody to enzymes (79-83). The ideal result for any coupling reaction is a 1:1 ratio of antibody to enzyme with no loss of specific activity of either partner. This has not been achieved. Advantages with the use of enzyme-labeled antibodies include a) a long shelf life, b) high sensitivity, and c) the label is applicable at the electron microscopic level (84). Some of the disadvantages with using enzyme-labeled antibodies are: a) there are multiple steps involved in the labeling, b) some enzyme substrates are hazardous, and c) the presence of endogenous enzymes may interfere with the labeling process or enzyme-substrate reactions.

A large number of enzymes have been used to label antibodies. The most commonly used are horseradish peroxidase (HRP), alkaline phosphatase, and β-galactosidase. Two methods commonly used for the preparation of antibody-peroxidase conjugates are the two-step glutaraldehyde method and the periodate method. The two-step glutaraldehyde method has a relatively low coupling efficiency. This is due to the methodology in that glutaraldehyde is first coupled to pure horeseradish peroxidase (HRP) via the relatively few reactive amino groups available on the enzyme. After purification, the HRP-glutaraldehyde mixture is added to the antibody solution. This requires that the HRP-antibody conjugates be separated from unconjugated material for optimum sensitivity (85-87). As with any peroxidase labeling method, the HRP must be pure to minimize cross-linking of the enzyme molecules to contaminating proteins during the first step of the procedure. The periodate method involves treatment of carbohydrates on the antibody molecules. The periodate opens the ring structure and allows the periodate to bind to free amino groups. Coupling antibodies and HRP

with periodate linkage is an efficient method (88,89).

In this experiment, we labeled the 2D7 MAbs with HRP by the periodate method. However, we were not able to obtain a stable product. There are several reasons why the peroxidase label was not stable. First, monoclonal antibodies from ascites fluid usually contains contaminating mouse serum proteins. Between 2 and 10% of the antibodies from ascitic fluid will be from the mouse antibody repertoire and not from the hybridoma (90). These contaminating proteins may have interfered with the perioxidation and labeling of the 2D7 MAbs, resulting in less efficient labeling of the MAb.

Associated with the presence of antibodies from the mouse's antibody repertoire, is the possibility of varying immunoglobulin size and carbohydrate differences between the myeloma-derived and mouse-derived antibodies that are contained within the ascites fluid. The percentages of the ammonium sulfate fractionations may have precipitated a significant amount of mouse antibodies normally present in the ascites fluid. If this occured to any extent, these antibodies would have been labeled in the process as well. To my knowledge, there is no literature to indicate that myeloma-derived and mouse-derived antibodies differ in the ability to covalently bind an enzyme label.

A third explanation for the poor stabilization of the labeled MAb has to deal with the carbohydrate moieties present on immunoglobulins. Carbohydrate chains of variable length and shape are almost always associated with immunoglobulins (91). The chains can be either simple or branched and may consist of only two sugar residues. However, most immunoglobulins contain at least a dozen or more units (91). The number of carbohydrate chains attached to a single immunoglubin molecule varies, depending on the isotope of the antibody: IgG contains two carbohydrates chains per molucule, IgM 10, IgA 16, IgD 6, and IgE 12. It is possibile that even molcules of the same class and subclass can vary their

carbohydrate content.

A fourth explanation for the lack of stability is that under normal conditions, carbohydrate chains attach to the Fc portion of the antibody molecule, but under pathological conditions carbohydrates can also be found attached to light (L) chains and to variable (V) regions of heavy (H) chains. Carbohydrate groups present in the V region are inaccessible because they are buried in the interior of the molecule. Because the 2D7 MAb is derived from a myeloma cell, it is possible that the number and distribution of carbohydrate moieties are unusual. Because the periodate method was used to peroxidase label the 2D7 MAb, a change in the amount of available carbohydrate content would affect the labeling process and stability. A change in the amount of avaliable carbohydrate may be due to a portion of carbohydrate being buried in the interior of the molecule, or there may be very little carbohydrate present at all. There is also the possibility that the delipidization or any other stage involved in the labeling process may have destroyed potential reactive groups on the immunoglobulin molecule.

If labeling monolconal antibodies with any of the conventional techniques causes loss of activity, one convenient method for preparing tagged antibodies is to label the primary amino acid chain by growing the hybridomas in the presence of radioactive precursors (92). This can only be done with antibodies for which the hybridoma cells are available, but the resulting antibodies have a number of potentially important properties, the most of which is that the labeled antibodies will be essentially identical to the unlabeled proteins.

APPENDIX B: CHARACTERIZATION OF THE ANTIGEN RECOGNIZED BY THE *L. POMONA* 2D7 MAB

Procedure

SDS-PAGE

Whole cell preparations used for SDS electrophoresis and western blotting procedures were prepared by growing serovars *canicola*, *grippotyphosa*, *icterohaemorrhagiae*, and *pomona* to a density of 1 x 10^6 organisms/ml. Ten milliliters of each of these cultures were placed in a 56° C water bath for 30 min to inactivate the organisms. The cultures were centrifuged at 12,000 x *g* for 30 min at 4° C and the pellets were washed three times with sterile PBS (pH 7.2). After the final wash, the pellets were resuspended in 2 ml PBS (pH 7.2). These suspensions were used in SDS-PAGE and immunoblot studies. Protein concentrations were determined using a BCA protein determination kit (Pierce, Rockford, IL).

Inactivated whole cells and/or Triton X-100 extracted antigens (50 μg protein) of each of serovars *canicola*, *grippotyphosa*, *icterohaemorrhagiae*, and *pomona* type kennewicki were separated using a mini-electrophoresis system and prepared gels (Schleicher & Schuell, Keene, NH). Samples were treated with sample buffer containing 0.05% 2-mercaptoethanol and 2% SDS. Samples were boiled for 5 min at 100° C prior to electrophoresis. Electrophoresis was carried out on a 14% homogeneous gel with a 4% stacking gel at 30 mA per gel for 1.5 h. Gels were stained with either a silver nitrate stain for lipopolysaccharide (LPS) or with Coomassie Brilliant Blue for protein. The migration distances of the bands contained within the gel matrix were determined using a laser densitometer (Pharmacia-LKB). Molecular mass markers (Bio-Rad) were used to calculate the apparent molecular mass of the antigens.

Silver Stain for Lipopolysaccharide (LPS)

Polyacrylamide gels were stained for LPS using a modified silver stain (93). Briefly, LPS in the gels was oxidized without prior fixation with a 0.7% (w/v) periodic acid in 40% ethanol and 5% acetic acid for 20 min at 22° C. After three washes (5 min each) in distilled water, the gels were incubated in fresh staining solution for 10 min at 22° C. The staining solution was prepared by adding 4 ml of concentrated ammonium hydroxide (29.4%) to 56 ml of a 0.1 M sodium hydroxide solution. After adding 200 ml of distilled water, 10 ml of a 20% (w/v) solution of silver nitrate (in distilled water) was added dropwise while stirring. After washing the gel three times (5 min each) with distilled water, the color development solution was added. The color development solution was prepared by adding 0.1 ml of a 37% formaldehyde solution to 200 ml of distilled water containing 10 mg of citric acid. The color development was allowed to proceed for approximately 10 min at 22° C and the reaction was stopped by exposure of the gel to 10% acetic acid for 1 min followed by several washings in distilled water.

Western Blotting

After separation, gel-bound antigens were transfered to nitrocellulose membrane (Millipore Corp., Bedford, MA) overnight (16-20 h) at 20 mA in buffer containing 12 mM tris and 96 mM glycine (pH 8.3). After transfer, the membrane was placed directly into a 1:80 dilution of ascites fluid containing the 2D7 monoclonal antibodies. After incubating at 37° C for 1 h on an orbital shaker, the membrane was rinsed 3 times with distilled water and incubated as before in a 1:1000 dilution of affinity purified peroxidase labeled goat anti-mouse IgA. After rinsing the membrane in distilled water 4 times, the membrane was incubated in 4-chloronaphthol (Sigma Chemical Co., St. Louis, MO) for 10-15 min at room

temperature.

Biochemical Determination of the Epitope by Proteinase K Digestion

In this experiment, heat-inactivated whole cells of serovars *canicola, grippotyphosa, icterohaemorrhagiae*, and *pomona* (50 µg of protein each) were treated with proteinase K prior to electrophoresis. Paired non-treated samples were also used. Samples were separated into 2 identical 8-16% SDS-PAGE grandient gels with a 4% stacking gel (Schleicher and Schuell). After electrophoresis, one gel was stained with Coomassie Brilliant Blue for protein. The antigens contained in the second gel were electro-transfered to nitrocellulose membrane and then probed with the 2D7 ascites fluid as described previously. Treated and non-treated samples were also evaluated in the *L. pomona* ELISA.

Results

The 2D7 hybridoma clone was shown to secrete IgA antibody using a commercial isotype ELISA kit. Heat-inactivated whole cells and Triton X-100 extracts of serovars *canicola, grippotyphosa, icterohaemorrhagiae*, and *pomona* type kennewicki were separated by SDS gel electrophoresis. The gels were either stained for protein, LPS, or were subsequently electro-transfered to nitrocellulose membranes. The membranes were then probed with the 2D7 ascites fluid as previously described. Results demonstrated that the 2D7 ascites fluid contained monoclonal antibodies specific for a 14-16 kDa antigen in the Triton X-100 extract of serovar *pomona* (Figure B1). The monoclonals also reacted to a single antigen of approximately 18 kDa in the whole cell preparation. The monoclonal antibodies contained within the 2D7 ascites fluid failed to recognize antigen in whole cell preparations of the remaining leptospiral serovars.

Although LPS-silver stained gels indicate that the 2D7 MAbs detect an

epitope on a 16 kDa Triton-extracted antigen that is predominantly LPS-like in nature, this LPS-like antigenic moiety may be associated with protein. This antigen is faintly stained by both LPS-silver stain (Figure B2) as well as by Coomassie stain (Figure B3). The epitope on the 18 kDa antigen detected by the 2D7 MAbs in inactivated whole cell preparations shows similar staining properties. When samples were treated with proteinase K, there was a lack of staining by Coomassie Brilliant Blue in treated samples except for one faintly staining antigen of approximately 16 to 18 kDa (Figure B4). In Figure B5, the 2D7 MAbs detected the same epitopes on inactivated whole cells and the Triton extracted antigens whether they were treated or not treated with proteinase K. The 2D7 MAb detected an epitope on the same antigenic moiety in proteinase digested samples as well as in non-treated samples. When tested by the ELISA, proteinase-treated samples yielded slightly higher OD values as compared to non-treated samples (data not shown).

Figure B1. Immunoblot using the 2D7 MAb to detect antigen in various antigen preparations. A 14% homogeneous gel was used to separate boiled antigens (50 μg of protein per lane). After electro-transfer, the membranes were probed with the 2D7 ascites fluid according to the protocol described in the Materials and Methods section. Lanes 1,2: *L. icterohaemorrhagiae* whole cells, Lanes 3,4: *L. grippotyphosa* whole cells (25 μl), Lanes 5,6: *L. canicola* whole cells, Lanes 7,8: *L. pomona* Triton X-100 extract, Lane 9: *L. pomona* whole cells, Lane 10: pre-stained low molecular mass stanndards (8 μl).

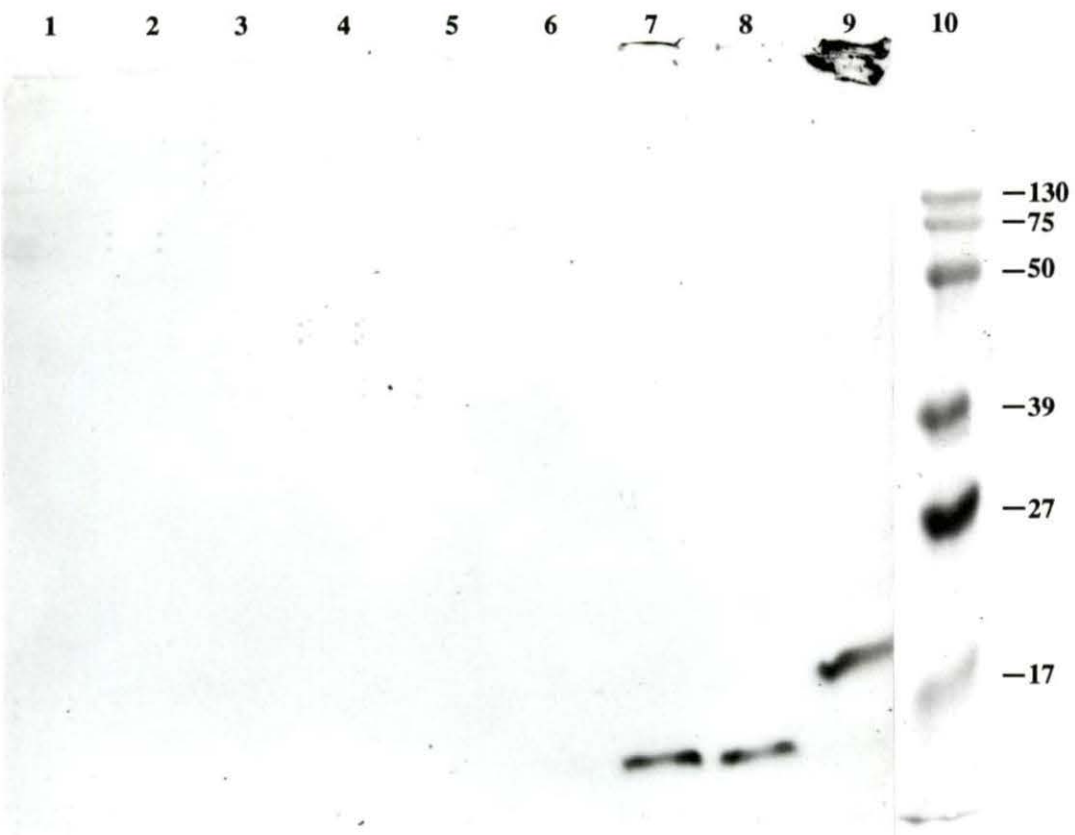

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Figure B2. LPS silver stained gel containing inactivated whole cells and the Triton X-100 extract of serovar *pomona*. Boiled samples (50 μg of protein per lane) were separated into a 14% SDS gel with a 4% stacking gel. After separation, the gel was stained for LPS using a modified silver stain. Lane 1: pre-stained low molecular mass markers, Lane 2: *L. pomona* Triton X-100 extract, and Lane 3: *L. pomona* inactivated whole cells.

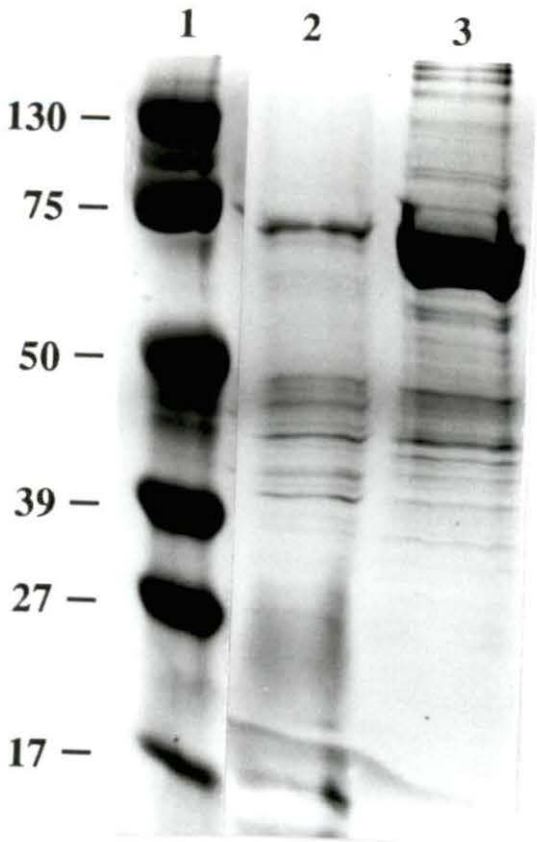
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Figure B3. Coomassie Brilliant Blue stain of Triton X-100 extracts of serovars *canicolá* and *pomona*. Boiled samples (50 μg of protein per lane) were separated into a 14% homogenous SDS polyacrylamide gel with a 4% stacking gel. Lane 1: pre-stained low molecular mass markers, Lane 2: *L. canicola* Triton X-100 extract, Lane 3: *L. pomona* Triton X-100 extract, Lane 4: blank, Lane 5: *L. canicola* inactivated whole cells, and Lane 6: *L. pomona* inactivated whole cells.

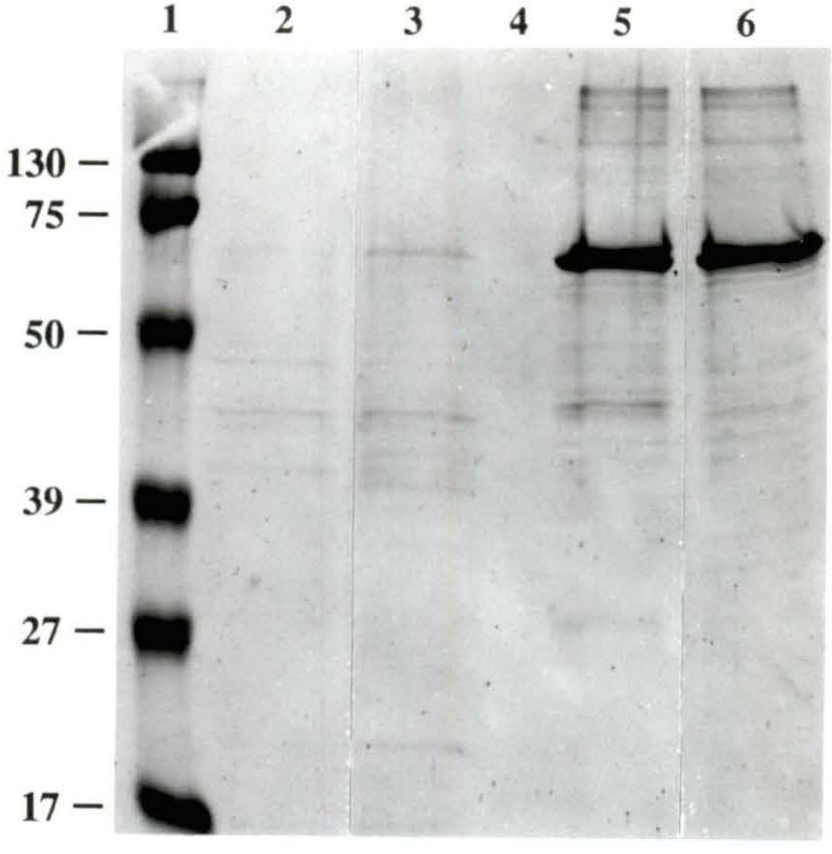
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Figure B4. Comassie Brilliant Blue stain of inactivated whole cells of serovars *canicola*, *grippotyphosa*, *icterohaemorrhagiae*, and *pomona*. Boiled samples were treated with proteinase K prior to electrophoresis and were loaded (50 μg of protein per lane) in the same order onto identical 14% SDS polyacrylamide gels. Paired, non-treated control samples were also used. After electrophoresis, one gel was stained with Coomassie Brilliant Blue. Lane 1: *L. pomona* inactivated whole cells, Lane 2: *L. pomona* Trtion X-100 extract, Lane 3: *L. canicola* inactivated whole cells, Lane 4: *L. canicola* Trtion X-100 extract, Lane 5: pre-stained low molecular mass markers. Samples in lanes 1-4 were not treated with proteinase K. Lanes 6-9 contain samples in the order given for lanes 1-4 but were treated with proteinase K prior to electrophoresis.

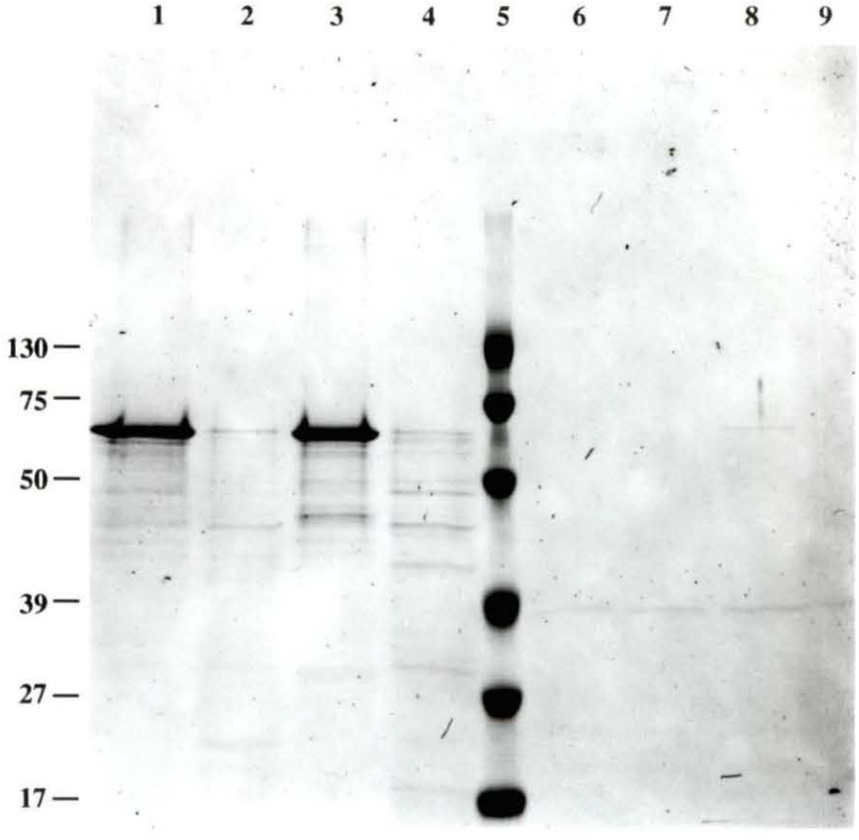
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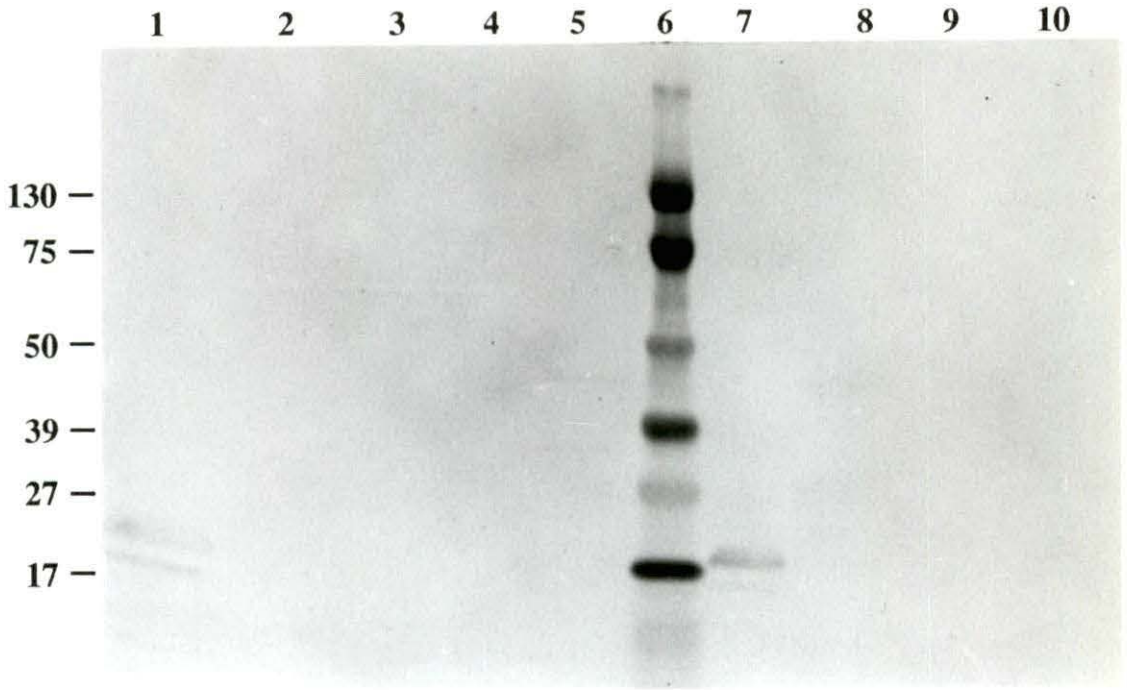
Figure B5. Western blot of inactivated whole cells of serovars *canicola*, *grippotyophosa*, *icterohaemorrhagiae*, and *pomona*. Boiled samples (50 μg of protein per lane) were treated with proteinase K prior to electrophoresis and were separated (50 μg of protein per lane) into a 14% polyacrylamide gel with a 4% stacking gel. Paired, non-treated control samples were also used. After electrophoresis, antigens in the gel were electro-transfered to nitrocellulose membrane and probed with the 2D7 ascites fluid as described in Materials and Methods. Lane 1: *L. pomona* inactivated whole cells, Lane 2: *L. icterohaemorrhagiae* inactivated whole cells, Lane 3: *L. grippotyphosa* inactivated whole cells, Lane 4: *L. canicola* inactivated whole cells, Lane 5: blank, Lane 6: pre-stained low molecular mass markers. Samples in lanes 1-4 were not treated with proteinase K. Lanes 7-10 contain samples as ordered in lanes 1-4 and were treated with proteinase K.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Discussion

Based on the results of these studies, it is speculated that the epitope recognized by the 2D7 MAbs is associated with an antigen that is predominantly LPS-like in nature, although some protein may be associated with an LPS-like moiety. The 2D7 MAbs detected an epitope present on a Triton-extracted 16 kDa LPS-like moiety that may be associated with protein as well as an epitope present on an 18 kDa LPS-like moiety present on inactivated whole cells of serovar *pomona* type kennewicki (Figures 1, 2, and 3). The difference in the mass of the epitope-associated antigenic moieties detected in the two preparations may be partially explained by the extraction process itself. The antigenic moieties may be associated with other molecules (protein) present on the membrane surface. During extraction, a portion of this molecule is removed resulting in a lower molecular mass antigen. The 2 kDa difference between the two antigenic preparations may represent the removal of a membrane-anchoring molecule. The presence of the protein, as shown in Coomassie stained gels, may represent that part of the protein that is normally associated with this LPS-like moiety in the intact molecule. When the proteinase K-treated and non-treated samples were separated and transfered to nitrocellulose membrane and then probed with the 2D7 ascites fluid, the 2D7 MAbs detected epitopes on the same LPS-like moieties regardless of treatment (Figure 4). A second explanation is that the 18 kDa band may represent the 14-16 kDa in complex with an aggregation of post-translational gene products in response to the increased temperature used to inactivate the organisms. One must be cognizant of the fact that although the 2D7 MAbs react with a single epitope, more than one epitope is probably recognized during the immune response to *L. pomona* infection. Therefore, protection must be thought of in terms of more than one epitope.

Although these results are not conclusive for an LPS-like epitope, the presence of an LPS-like substance is consistent with previous reports which indicate that leptospiral LPS differs from classical LPS (5, 11-15). Further work needs to be done in this area. The LPS silver stain technique used in this study probably stained some protein in addition to the LPS. Staining for LPS using a silver stain is unpredictable. Protein may have been stained due to either: a) fluctuations in room temperature during staining, or b) too much ammonium hydroxide in the color development solution. In order to better define this LPS-like substance, radioisotopic precursors could be included in the growth media. This would allow labeling of the sugars and proteins during growth and would provide a more precise method of determining if the epitopes are a combination of an LPS-like substance and protein. Additional work that may be performed include labeling the 2D7 MAb with colloidal gold and use this conjugate to determine whether the antigen is part of the flagella or the outer sheath.

APPENDIX C: *L. POMONA* ELISA EVALUATION

Procedure and results

ELISA Evaluation by Independent Laboratories

Veterinary biological firms marketing leptospiral bacterins were invited to participate in the evaluation of this assay using six coded licensed bacterins. Evaluation kit components included the test reference bacterin, two 3-ml dose bacterins (C1 and C2), two 5-ml dose bacterins (D1 and D2), and two negative bacterins containing no leptospires. Reagents included in the kit were ascites fluid containing the 2D7 monoclonal antibodies, and the hyperimmune rabbit capture serum. Participating firms were asked to assay each coded bacterin in triplicate according to the ELISA protocol and to provide their results and any comments to the NVSL. The submitted relative potencies, geometric means of the relative potencies, variances of the relative potencies, and standard deviations of the relative potencies were determined. Variances measuring each firm's in-house reproducibility were tested for homogeneity using Chi-squares.

Ten biologics firms marketing leptospiral bacterins expressed interest in participating in an industry-wide evaluation of the L. pomona ELISA. Six of those ten firms have completed their evaluation and have returned data and comments. The mean RP values for each coded bacterin from each firm was determined and the natural log of that value used to graphically represent the data. Data generated by the NVSL on three separate occasions were treated in a similar manner. Relative potencies between the NVSL and firms were within reasonable values (Figure C1). In general, results indicate that there was more variation due to bacterin than due to firm. Although a significant Chi-square resulted, the variation was due to one firm's results for two of the coded bacterins. This firm had large variances between replicates when assaying the 3-ml

dose bacterins (Figure C2). Variances of the remaining firms were low. With the exception of the one firm, most firms had nearly consistent standard deviations for the bacterins tested (Figure C3). Comments regarding the ELISA that were supplied to the NVSL include:

1) "The ELISA is very specific for *L. pomona*."

2) "The ELISA is easy to follow and straight-forward."

3) "We feel very comfortable with the protocol."

4) "The data is reproducible."

5) "The 5-ml dose products are more consistent as compared to the 3-ml dose products."

6) "Is there a possibility of an expanded dilution scheme?"

7) "We would like to refrain from the use of TMB in our laboratory."

8) "Antigen elution is detrimental to the antigen being detected."

Figure C1. Relative potency values for coded bacterins C1, C2, D1, and D2 obtained by the NVSL and participating firms.

Relative Potency Means
(in Natural logs)

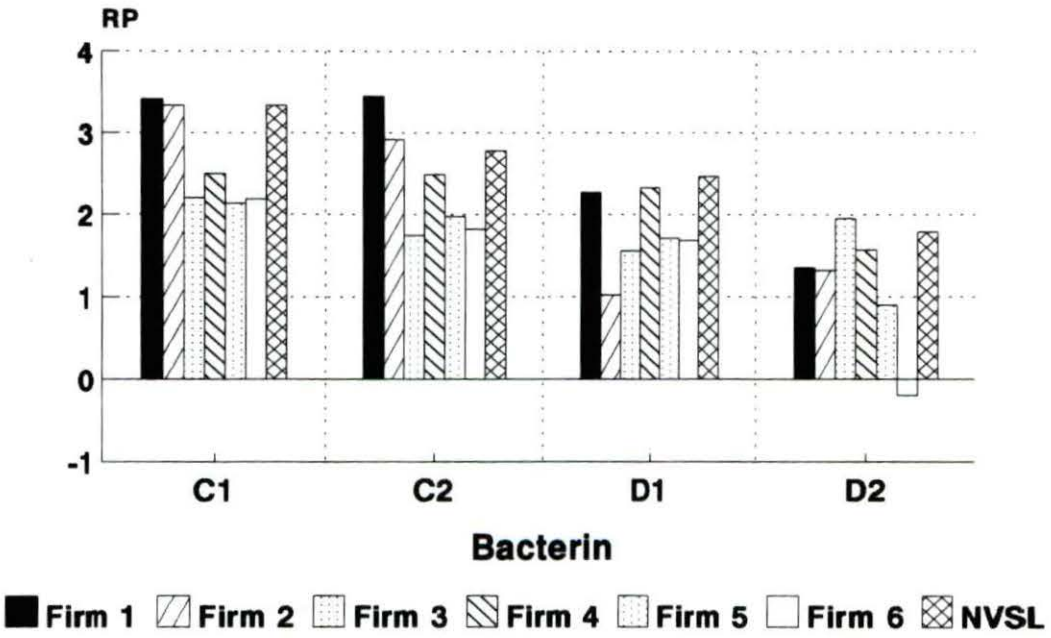


Figure C2. Variances of relative potency values for coded bacterins C1, C2, D1, and D2 obtained by each participating firm. Variances were determined using Chi-squares.

Relative Potency Variances
(in Natural logs)

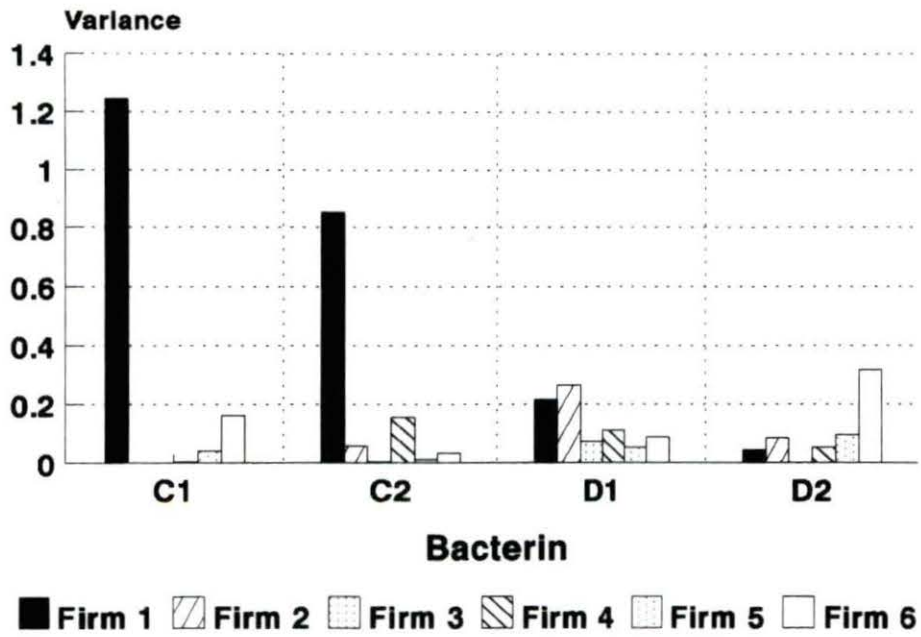
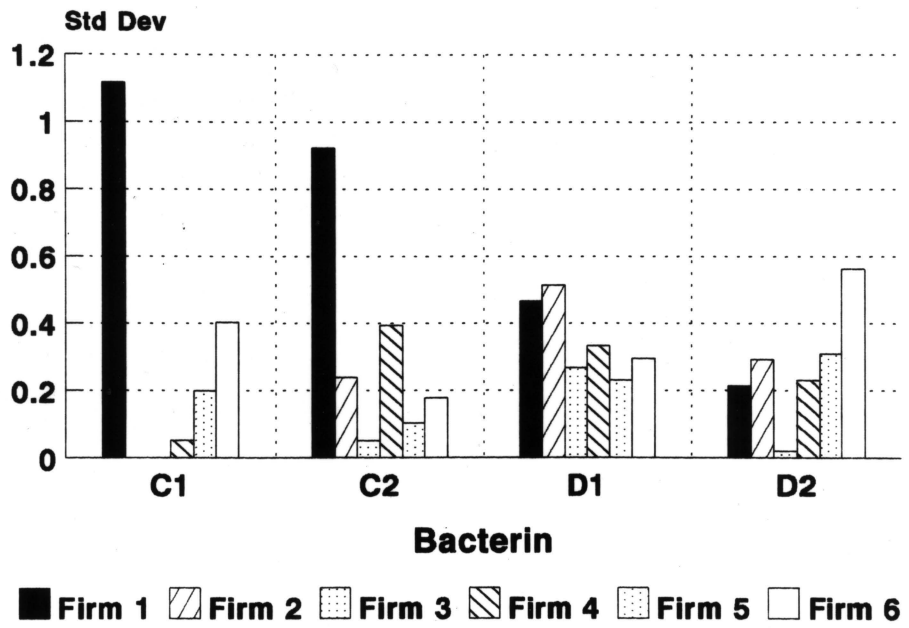


Figure C3. Standard deviations of relative potency values for coded bacterins C1, C2, D1, and D2 obtained by each participating firm.

Standard Deviations
(in Natural logs)



Discussion

Whenever one is dealing with a large industry such as the veterinary biologics industry, one must be cognizant that new advances are constantly being made in relation to antigen delivery and antigen processing. In regards to the development of in vitro assays (i.e., ELISA), a major concern is the type of adjuvants that are used in the production of a bacterin. Some biologics firms use a single adjuvant. Others use a combination of adjuvants. Some adjuvants are superior to others and bind the antigen more tightly than others. By far the most common adjuvant used for leptospiral bacterins is aluminum hydroxide. When using a superior adjuvant, a smaller proportion of a given leptospiral serovar may be used. Not all biologics firms use the same amount of antigenic material in their products for a given serovar. Given this background, it is not unusual for there to be more variation due to bacterin than to firm.

Dilutions of the test reference bacterin that are used in the ELISA protocol will protect hamsters against a virulent challenge. However, there is no color change when bacterins are used in the ELISA protocol at the dilutions set forth in the Code of Federal Regulations (1). Because of the use of different adjuvants, it is difficult to develop an in vitro assay that correlates perfectly with the host animal. The best that we can do at this time is to approximate what the host animal recognizes as foreign in the bacterins.

APPENDIX D: CORRELATION OF THE *L. POMONA* ASSAY WITH THE IN VIVO ASSAY

Procedure

Ten serial two-fold dilutions (beginning with 1:3) of the NVSL reference *L. pomona* bacterin were prepared and diluted to 1/800th of the host animal dose according to established protocols (1). The diluted bacterins were used to vaccinate hamsters by intramuscular injection. The NVSL reference *L. pomona* bacterin was also evaluated undiluted. There were five hamsters per bacterin dilution as well as 10 nonvaccinated control hamsters. Two weeks later, hamsters were challenged (ip) with 0.25 ml of liver homogenates from *L. pomona*-infected hamsters (10 to 10,000 LD50's as determined by titration (1)). Results were recorded as the number of survivors per total number of animals challenged. Dilutions of the NVSL reference *L. pomona* bacterin used in hamsters were evaluated by the *L. pomona* ELISA without diluting to 1:800th of the host animal dose. Results of the ELISA evaluation were expressed as the highest RP.

Results and discussion

The purpose of this experiment was to determine whether the *L. pomona* ELISA would detect an unsatisfactory bacterin. Serial two-fold dilutions of the NVSL reference *L. pomona* bacterin were prepared and used to vaccinate hamsters according to established protocols (1). The NVSL reference *L. pomona* bacterin was also evaluated without prior dilution. In the hamster potency assay, all bacterins tested for potency against *L. pomona* are diluted so that each hamster receives 1/800th of the host animal dose. The undiluted and diluted NVSL reference bacterins were also evaluated in the ELISA protocol without prior dilution to 1/800th of the host animal dose.

Results indicate that the ELISA approximates the hamster potency assay (Table D1). Eighty percent of the hamsters were protected with a 1:24 dilution (1.224×10^5 organisms), whereas a 1:12 dilution (5.878×10^6 organisms) yielded an RP of 1.03. Dilutions of 1:48 and greater failed to protect hamsters. Dilutions of 1:24 and greater failed to yield an RP of 1.0 or greater in the ELISA. There are four reasons why protection was provided by more dilute bacterins as compared to the ELISA results: a) the hamster is not a natural reservior for leptospirosis and may be too sensitive for potency testing of leptospiral bacterins, b) there are other processes involved in the host immune response to vaccination that are not accounted for by the ELISA, c) the adjuvant (aluminum hydroxide) allows for a slower release of antigen in the hamster as well as serving as a mild immunostimulant, and d) since the antigen is not eluted from the adjuvant, not all of the epitopes were available to bind to the 2D7 MAb. Because it is not possible to mimic all aspects of the host immune response to leptospiral vaccination in vitro, it should not be suprising that the ELISA does not parallel the hamster potency assay exactly.

Table D1. Comparison of the hamster potency assay and the ability of the *L. pomona* ELISA to detect an unsatisfactory bacterin [a]

Bacterin Dilution	No. Organisms Injected	No. Survivors[b]	No. Organisms Represented[c]	ELISA Results[d]
Undiluted	5.875×10^7	5/5(100%)	7.050×10^7	9.13
1:3	1.958×10^6	5/5(100%)	2.351×10^7	5.45
1:6	9.792×10^5	5/5(100%)	1.176×10^7	2.77
1:12	4.896×10^5	5/5(100%)	5.878×10^6	1.03
1:24	2.448×10^5	4/5(80%)	2.939×10^6	0.41
1:48	1.224×10^5	0/5(0%)	1.470×10^6	NVC
1:96	6.119×10^4	0/5(0%)	7.347×10^5	NVC
1:192	3.060×10^4	0/5(0%)	3.673×10^5	NVC
1:384	1.530×10^4	0/5(0%)	1.837×10^5	NVC
1:768	7.648×10^3	1/5(20%)	9.184×10^4	NVC

[a] The NVSL reference *L. pomona* bacterin was diluted to 1/800th of a 2-ml dose and used to vaccinate hamsters followed by a virulent *L. pomona* challenge two weeks later. The same bacterin was evaluated according to the ELISA protocol without prior dilution to 1/800th of a 2-ml dose.

[b] Number of hamsters surviving a virulent *L. pomona* challenge. Results are expressed as the number of survivors per total number of animals challenged.

[c] Number of organisms used in the ELISA at a 1:2 dilution.

[d] Results are expressed as the highest RP values as determined by the RelPot computer program. NVC, no valid combinations.

PAPER II. DEVELOPMENT OF AN IN VITRO ASSAY FOR MEASURING THE RELATIVE POTENCY OF LEPTOSPIRAL BACTERINS CONTAINING SEROVAR *CANICOLA*

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ABSTRACT

Licensing of leptospiral bacterins is currently based on a hamster potency assay in which a minimum of 80% of vaccinated hamsters must survive a virulent challenge. The development of an in vitro assay would reduce the number of hamsters required for testing, would decrease the potential for human exposure, and would be less expensive to the manufacturer and the National Veterinary Services Laboratories. Recently, an in vitro assay was developed for measuring the relative potency of leptospiral bacterins containing *Leptospira pomona*. This report describes the development of a similar assay for *Leptospira canicola*. Hybridomas were prepared and screened by established protocols using a non-adjuvanted extract of the NVSL challenge strain of serovar *canicola* (Moulton strain) of the Serogroup canicola. A *canicola*-specific clone, designated as 4DB, was chosen for further study based on the specificity of hamster protection and reactivity to the homologous serovar and no reactivity to heterologous serovars by western blot studies. Monoclonal antibody 4DB was not cross-reactive by ELISA with *L. interrogans* serovars *grippotyphosa, hardjo, icterohaemorrhagiae, pomona*, or *bratislava*. The potency assay is a sandwich ELISA and involves capturing antigen from bacterins with anti-*canicola* polyclonal rabbit sera, followed by the 4DB ascites fluid, anti-mouse IgM peroxidase-labeled antibody, and color substrate (ABTS). Bacterins adjuvanted with aluminum hydroxide or oil require treatment to elute antigen from the adjuvant prior to use in the ELISA protocol. Non-adjuvanted bacterins and bacterins containing adjuvants other than aluminum hydroxide or oil require no treatment prior to use in the ELISA protocol. The potency of 185 leptospiral bacterins, relative to a reference bacterin, were evaluated with this ELISA. In order to be considered satisfactory, all bacterins must have a relative potency of 1.0 or greater. All bacterins tested passed the ELISA assay.

INTRODUCTION

Leptospires occur naturally in a wide variety of feral an domestic mammals. In natural hosts, the spirochetes colonize in the kidneys and are shed in the urine. Humans are accidental hosts, with most infections related to occupational or recreational activities involving either direct contact with animal urine or contact with water and soil contaminated with animal urine. Leptospirosis in animals may be controlled by vaccination. In general, immunity includes a rapid and strong agglutinating response. The duration of immunity is lifelong and is serovar-specific. Although cell-mediated immune responses are involved, immunity to leptospirosis is primarily humorally mediated and antibodies involved in opsonization and agglutination of leptospires are of prime importance (1-7). Current *L. interrogans* vaccines composed of heat-killed or chemically inactivated bacteria can protect animals against many signs of leptospirosis (7-10).

The National Veterinary Services Laboratories (NVSL) are responsible for the laboratory support and licensing of vaccines of viral and bacterial origin as well as veterinary diagnostic kits and reagents. Efficacy, potency, and safety testing of vaccines and bacterins are important components of pre-license assessment as well as quality control of each serial (lot) of product produced. These procedures are well established for veterinary biologicals.

The current testing of leptospiral bacterins licensed by the United States Department of Agriculture requires a hamster potency assay (11). The hamster potency assay involves vaccinating hamsters with a specified dilution of the bacterin. Two weeks post-vaccination, hamsters are challenged with liver homogenates from the appropriate serovar-infected hamster and the percent survival is determined. There is a need to replace the in vivo assay with a rapid, sensitive, and reliable alternate in vitro method that could be used in the routine testing of leptospiral

biologicals. The large number of hamsters required for the manufacture and testing of these biologicals is expensive, and increasing concern for animal welfare and human health makes in vitro assays desirable. An enzyme-linked immunoassay (ELISA) would be simple, safe, easily automated, inexpensive, and suitable for potency testing of large numbers of product serials. The NVSL has recently reported on the development of an ELISA test for measuring the relative potency of leptospiral bacterins containing serovar *pomona* (12). The objective of this study was to develop a similar assay for measuring the relative potency of leptospiral bacterins containing serovar *canicola*.

MATERIALS AND METHODS

Materials

Bacterial Strains

Leptospira interrogans strains used in this study were obtained from the National Veterinary Services Laboratories (NVSL) collection. Strains virulent for hamsters included *L. pomona* 11000 (MLS), *L. hardjo* 11601 (SC1769), *L. grippotyphosa* 11808 (Oregon shrew), *L. canicola* 11203 (Moulton), and *L. icterohaemorrhagiae* 11403 (CF1). *Leptospira bratislava* 2a and *L. bratislava* jez were also used. Serovars within the Serogroup canicola used in this study included *banfani* (strain banfani), *benjamin* (strain benjamin), *bindjei* (strain bindjei), *broomi* (strain patone), *canicola* (strain Hond U IV), *galtoni* (strain 1014), *jonsis* (strain jones), *kamituga* (strain kamituga), *malaya* (strain H6), *portlandvere* (strain 63-69), *schueffneri* (strain vleermuis), and *sumneri* (strain sumner). Unless indicated, all organisms were heat inactivated by incubation in a 56° C water bath for 30 min.

Bacterins

Bacterins used in the antigen extinction studies and tested by the ELISA included licensed commercial adjuvanted and non-adjuvanted bacterins. The NVSL reference *L. canicola* bacterin was also used. The test reference bacterin for the ELISA was a non-adjuvanted bacterin containing approximately 4.7×10^9 organisms per ml using a Petroff-Houser counting chamber. In order to determine whether the *L. canicola* ELISA would detect an unsatisfactory bacterin, ten serial two-fold dilutions of the NVSL reference *L. canicola* bacterin (beginning with 1:3) were prepared in PBS (pH 7.2). These dilutions were then used to vaccinate hamsters (5 hamsters per dilution) and challenged two weeks later according to the Code of Federal Regulations (11). These dilutions were also evaluated in the

L. canicola ELISA.

The specificity of the ELISA was evaluated using preparations containing no *L. canicola* organisms (heterologous) as well as those containing *L. canicola* organisms (homologous). Heterologous commercial licensed bacterins evaluated include: Pasteurella Haemolytica, Escherichia coli, Leptospira Hardjo-Icterohaemorrhagiae-Pomnona, Salmonella Choleraesuis, Campylobacter Fetus, Actinobacillus Pleuropneumoniae-Haemophilus Parasuis-Pasteurella Multocida, and Actinobacillus Pleuropneumoniae. Homologous commercial licensed bacterins evaluated include: Parvovirus-Erysipelothrix Rhusiopathiae-Leptospira Canicola-Grippotyphosa-Icterohaemorrhagiae-Hardjo-Pomona, Trichomonas Fetus-Campylobacter Fetus-Leptospira Canicola-Grippotyphosa-Icterohaemorrhagiae-Hardjo-Pomona, Campylobacter Fetus-Leptospira Canicola-Grippotyphosa-Icterohaemorrhagiae-Hardjo-Pomona, and the NVSL L. canicola reference bacterin. Inactivated whole cells evaluated by the ELISA include: *Leptospira* serovars *grippotyphosa, pomona, icterohaemorrhagiae*, and *hardjo*, *Bordetella bronchiseptica*, *Salmonella typhimurium*, *Salmonella choleraesuis*, *Escherichia coli*, and *Pasteurella multocida* type D.

Animals

Eight- to nine-pound New Zealand white rabbits (Harlan-Sprague-Dawley, Inc., Indianapolis, IN) were used to prepare serovar-specific antisera.

Fifteen- to twenty-gram male BALB/c mice (Harlan-Sprague-Dawley Inc., Indianapolis, IN) were used for the monoclonal antibody (MAb) production. Antigen extinction studies were performed in 40- to 50-gram golden Syrian hamsters (Sasco, Inc., Omaha, NE). Correlation with in vivo studies was performed in 40- to 50-g golden Syrian hamsters (Sasco, Inc., Omaha, NE).

Antisera Production

A hamster virulent culture of *L. canicola* was used to inoculate each of two tubes (1 ml each) of semi-solid P80-BA medium (13). The tubes were then incubated at 37° C for 21 days. Sterility of the culture was determined by inculating two 5% bovine blood agar plates, two tubes of fluid thioglycolate, and two tubes of trypticase soy broth with 200 µl of the culture. One set of each inoculated media were incubated at room temperature (27° C) and one set at 37° C for seven days.

Two rabbits were inoculated with 1.0, 2.0, and 4.0 ml of the P80-BA semi-solid culture. A fourth inoculation of 4.0 ml was given to each rabbit 7 days after the last 5-day interval inoculation. The first three vaccinations consisted of heat-inactivated cultures that were prepared by incubating the inocula in a 56° C water bath for 25 min. The last vaccination consisted of viable cultures. All inoculations were given intravenously in the marginal ear vein. After the final inoculation, the rabbits were observed for 10 days. On the tenth day, blood was obtained by cardiac puncture and the serum harvested by centrifugation at 3,000 x *g* for 15 min. The antibody titer of the serum was determined by the microscopic agglutination test (MAT) as described below. A titer of 1:1600 was considered to be satisfactory. The serum was filtered with a 0.22 µm filter (Millipore Corp., Bedford, MA) and stored at -70° C in 2-ml aliquots.

Methods

Antigen Extraction

Leptospira interrogans organisms were grown in 1 L P80 liquid media (13) for 7 days at 30° C. Cultures of the Moulton strain of *L. canicola* harvested after 7 days incubation contained approximately 1 x 10^6 leptospires/ml. The organisms were harvested by centrifugation (12,000 X *g*

for 30 minutes) and were washed three times in PBS (pH 7.2). Pellets were resuspended in 6 ml of a tris-tricine, 5 mM ethylenediaminetetraacetic acid (EDTA) solution (pH 8.6) containing 0.01% Triton X-100. Suspensions were incubated overnight on a platform rocker at room temperature and then centrifuged as before. Supernatants containing the outer envelope antigens were filter sterilized and stored at 4° C until used to immunize BALB/c mice for the production of the 4DB MAb. Total protein was determined by the BCA method (Pierce Chemical Co., Rockford, Il).

Monoclonal Antibody Production

The MAb 4DB was produced by the method of Van Deusen and Whetstone (14) using the Triton X-100 extract of the NVSL challenge strain (Moulton) of *L. canicola*. BALB/c mice were injected intraperitoneally (ip) with nonadjuvanted Triton X-100 extract of serovar *canicola* containing approximately 50 μg of protein. A 50-μg booster injection was given intravenously 3 weeks later. Selection of clones was based on a) high ELISA optical density (OD) values (e.g., $\geq$ 0.400) with the homologous antigen and no cross-reactivity with heterologous antigens, and b) passive protection studies in hamsters. The selected clone, designated 4DB, was used to prepare ascites fluid. The antibody isotype of the 4DB MAb was determined to be IgM using a Mouse Typer Sub-Isotype Kit (Bio-Rad Laboratories, Richmond, CA). The total antibody concentration in the ascites fluid was determined to be 275 mg/ml using a radial immunodiffusion (Calbiochem, San Diego, CA).

Microscopic Agglutination Test

The MAT was performed according to established protocols (15). Briefly, serial 2-fold dilutions (begining with 1:25) of the 4DB MAb were incubated with viable serovars *canicola*, *grippotyphosa*, *hardjo*,

icterohaemorrhagiae, *pomona*, and *bratislava*. The following serovars of the Serogroup canicola also served as antigen: *banfani* (strain banfani), *benjamin* (strain benjamin), *bindjei* (strain bindjei), *broomi* (strain patone), *canicola* (strain Hond U IV), *galtoni* (strain 1014), *jonsis* (strain jones), *kamituga* (strain kamituga), *malaya* (strain H6), *portlandvere* (strain 63-69), *schueffneri* (strain vleermuis), and *sumneri* (strain sumner). The ability of the 4DB MAb to agglutinate viable cells was assessed.

Passive Protection Studies

Four groups of five hamsters each were injected ip with 0.5 ml of undiluted hybridoma culture fluid containing the 4DB MAb. Five non-vaccinated control hamsters received an equal antibody concentration of ascites fluid prepared against *Pasteurella multocida* type D dermonecrotic toxin (NVSL). Both groups of hamsters were challenged 24 h later with 0.25 ml of liver suspensions from hamsters infected with serovars *canicola*, *icterohaemorrhagiae*, *grippotyphosa*, *and pomona* (10 to 10,000 LD_{50}'s as determined by titration (11)). Results were recorded as the number of survivors per total number of animals challenged.

SDS-PAGE

Heat-inactivated whole cells of serovars *canicola*, *grippotyphosa*, *hardjo*, *icterohaemorrhagiae*, and *pomona* as well as the Triton X-100 extract of serovar *canicola* were used for SDS-PAGE analysis. The samples were separated on a 14% polyacrylamide gel with a 4% stacking gel using a Profile System (Schleicher & Schuell, Keene, NH). Antigens were treated with sample buffer containing 0.05% 2-mercaptoethanol and 2% SDS. Samples were boiled for 5 min at 100° C prior to electrophoresis at 30 mA per gel for 1.5 h. Gels were either stained for protein with Commassie Brilliant

Blue (G-250), or stained for lipopolysaccharide with a modified silver stain.

Silver Stain for Lipopolysaccharide (LPS)

Polyacrylamide gels were stained for LPS using a modifed silver stain (16). Briefly, LPS in the gels was oxidized without prior fixation with a 0.7% (w.v) periodic acid in 40% ethanol and 5% acetic acid for 20 min at 22° C. After three washes (5 min each) in distilled water, the gels were incubated in fresh staining solution for 10 min at 22° C. The staining solution was prepared by adding 4 ml of concentrated ammonium hydroxide (29.4%) to 56 ml of a 0.1 M sodium hydroxide solution. After adding 200 ml of distilled water, 10 ml of a 20% (w.v) solution of silver nitrate (in distilled water) was added dropwise while stirring. After washing the gel three times (5 min each) with distilled water, the color development solution was added. The color development solution was prepared by adding 0.1 ml of a 37% formaldehyde solution to 200 ml of distilled water containing 10 mg of citric acid. The color development was allowed to proceed for approximately 10 min at 22° C. The color development solution was decanted and the reaction was stopped by adding 10% acetic acid (in distilled water) for 1 min followed by several washings in distilled water.

Western Blotting

After fractionation, gel-bound antigens were electro-transfered to nitrocellulose membranes (Millipore Inc., Bedford, MA) at 125 mA for 2.5 h. Electro-transfer was carried out in buffer containing 12 mM tris and 96 mM glycine (pH 8.3). After transfer, the membrane was incubated in PBS (pH 7.2) containing 0.15% Tween 20 (PBST) for 10 min followed by rinsing in distilled water for 1 min. The membrane was then incubated in 20 ml of a 1:100 dilution (in PBST containing 1% normal rabbit sera) of 4DB ascites

fluid for 1 h at 37° C. After rinsing in distilled water, the membrane was incubated in a 1:2000 dilution (in PBST containing 1% normal rabbit sera) of goat anti-mouse IgM peroxidase-labeled antibody conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) for 1 h at 37° C. The membrane was washed in PBST for 2 min followed by incubation in color development solution for 10-15 min. The color development solution consisted of two solutions (A & B) that were mixed prior to use. Solution A contained 75 mg of 4-chloronaphthol dissolved in 25 ml of cold methanol. Solution B consisted of 125 ml tris-buffered saline (TBS) containing 76 µl of 37% hydrogen peroxide (Fisher Scientific, Fair Lawn, NJ).

Biochemical Determination of Epitope by Proteinase K Digestion

In this experiment, heat-inactivated whole cells of serovars *canicola, grippotyphosa, icterohaemorrhagiae*, and *pomona* (50 µg protein each) were treated with proteinase K prior to electrophoresis. Paired non-treated samples were also used. Samples were separated into 2 identical 8-16% gradient gels with a 4% stacking gel (Schleicher & Schuell, Keene, NH). After electrophoresis, one gel was stained with Coomassie Brilliant Blue for protein. Antigens contained in the second gel were electro-transfered to nitrocellulose membrane and then probed with the 4DB ascites fluid as previously described. Proteinase K-treated samples were also tested in the *L. canicola* ELISA protocol (Phase II).

ELISA Protocol

Reagents

Horseradish peroxidase (HRP) labeled goat anti-mouse IgM conjugate and 2,2'-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) (Kirkegaard-Perry Laboratories, Inc., Gaithersburg, MD) were used as the antibody conjugate and substrate, respectively. Polyvinyl alcohol (PVA) was obtained from

Aldrich Chemical Co., Inc, Milwaukee, WI. Polyclonal rabbit antisera prepared against *L. canicola* was produced at the NVSL. Immunlon-2 microtitration plates were obtained from Dynatech Laboratories, Inc., Alexandria, VA.

The capture antiserum was diluted in coating buffer (0.05 M carbonate buffer, pH 9.6) containing 1.59 g Na_2CO_3 and 2.93 g $NaHCO_3$ per liter of distilled water. The antigen (bacterin) diluent was filter sterilized phosphate buffered saline (PBS) (0.15 M, pH 7.2) containing 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, and 0.2 g KH_2PO_4 per liter of distilled water. The blocking solution consisted of a 1% solution of PVA (MW 13,000) in antigen diluent. Antibodies were diluted in blocking solution supplemented with 1% normal rabbit serum.

Antigen Elution Procedure

Only bacterins that were adjuvanted with aluminum hydroxide or oil (and their derivatives) required elution treatment prior to use in the ELISA protocol. Bacterins containing adjuvants other than aluminum hydroxide or oil (and their derivatives) required no treatment prior to use in the ELISA protocol. As an adjuvant control, an aluminum hydroxide solution (2% Al_2O_3) was diluted to correspond to the final concentration contained in the product being tested. Serial two-fold dilutions of the adjuvant control (begining with 1:4) was used in the ELISA protocol. Sodium citrate and potassium phosphate antigen elution methods were evaluated for removing antigen from aluminum hydroxide adjuvants. The sodium citrate elution method consisted of adding 1 ml of the bacterin to 0.171 g of sodium citrate crystals (Fisher SCientific, Fair Lawn, NJ) and incubating overnight (16-20 h) at 37° C on an orbital shaker (120 RPM). The treated bacterin was used in the ELISA protocol without centrifugation. The potassium phosphate elution buffer was prepared by dissolving 8.2 g of

KH_2PO_4 (Mallinckrodt, Inc., Paris KY) in 94 ml distilled water. After the pH was adjusted to 7.25, 1.55 ml of bacterin and 0.45 ml of buffer were mixed together and incubated overnight at 37° C on an orbital shaker (120 RPM), the samples were used in the ELISA protocol.

Two antigen-elution methods were evaluated for removing antigen from bacterins containing oil as an adjuvant. One method was a sodium desoxycholate buffer which was prepared by dissolving 0.50 g of sodium desoxycholate (Difco Laboratories, Detroit, MI) in PBS (pH 7.2). Equal amounts of buffer and bacterin were incubated overnight at 22° C. The mixture was centrifuged (10,000 x *g*) for 5 min, and the aqueous phase below the oil layer was aspirated with a tuberculin syringe by insertion of the needle through the tube. The aqueous phase was used in the ELISA protocol. The second antigen elution method involved incubation of the bacterin with a 0.5% (w/v) solution of sodium dodecylsulfate (SDS) (Biorad Laboratories, Richmond, CA). The buffer was prepared by dissolving 0.5 g of SDS in 100 ml distilled water. One-and-one-half milliliters of bacterin was added to 0.5 ml of buffer and incubated overnight at 22° C on a rotary shaker at 120 RPM followed by centrifugation (10,000 x *g*) for 10 min. The aqueous phase immediately beneath the oil layer was removed with a tuberculin syringe as previously described. This phase was used in the ELISA protocol. Serial two-fold dilutions of a commercial oil adjuvant were also used as an adjuvant control.

Bacterins containing a combination of adjuvants such as aluminum hydroxide and oil, either alone or in combination with other adjuvants, required adding 1.5 ml of the bacterin to 0.5 ml of the potassium phosphate (pH 7.25) and 0.5 ml of the 0.5% SDS solution. The mixture was incubated at room temperature (22° C) overnight on a rotary shaker (120 RPM). After centrifugation (10,000 x *g*), the aqueous phase was removed as previously described. This phase was used in the ELISA protocol.

ELISA Procedure

The ELISA procedure consisted of two phases. In phase I, a 1:20 dilution (in PBS) of the bacterin was made and used in the ELISA protocol. Bacterins that did not pass in phase I are then evaluated in phase II in which the bacterins were not diluted prior to use in the ELISA protocol.

Polyclonal hyperimmune rabbit antiserum (prepared against *L. canicola*) was diluted 1:100 in coating buffer (pH 9.6). All wells of an Immulon-2 microtitration plate were coated with 100 μl of the diluted rabbit antisera prepared against *L. canicola*, and the plate was incubated at 4° C overnight (16-20 h). Bacterins containing aluminum hydroxide or oil (and their derivatives) as an adjuvant were treated as previously described. A non-adjuvanted *L. canicola* bacterin served as the test reference and was not pre-treated. Non-adjuvanted bacterins and bacterins containing adjuvants other than aluminum hydroxide or oil were assayed without prior elution treatment.

The reference bacterin was a non-adjuvanted bacterin containing approximately 4.7×10^9 organisms per ml. The ELISA dilutions of the reference bacterin were 1:50, 1:100, 1;150, 1:250, and 1:500 diluted in 10 ml of PBST for 1-, 2-, 3-, 5-, and 10-ml dose products, respectively, regardless of the adjuvant contained in the product. These dilutions were considered to be undiluted and were used in the ELISA protocol without prior diluting. The pre-diluted test reference, the treated bacterins, and all other bacterins (non-adjuvanted and those not containing aluminum hydroxide or oil) are assayed at serial 2-fold dilutions (in PBST) beginning with a 1:2 dilution. All dilutions were made in a Falcon 96-well transfer plate (Becton Dickinson and Co., Lincoln Park, NJ).

Following aspiration of the capture polyclonal sera, 250 μl of the PVA blocking solution was added to all wells, and the plate was incubated for 1 h at 37° C on an orbital shaker. The plate was washed once with

PBST (PBS containing 0.05% Tween 20, pH 7.2), and 100 µl of each dilution of the bacterins and the test reference was transfered to duplicate wells beginning with the highest dilution. The plate was incubated for 1.5 h at 37° C on an orbital shaker. After washing five times with PBST, 100 µl of a 1:14,000 dilution of the 4DB ascites fluid was added to all wells, and the plate was incubated at 37° C for 1 h. After the plate was washed four times to remove unbound 4DB MAb, 100 µl of a 1:2000 dilution of goat anti-mouse IgM(µ) peroxidase labeled antibody conjugate was added to all wells, and the plate was incubated for 1 h at 37° C. After four washes, 100 µl of ABTS was added to all wells, and the plate was sealed and incubated for 30 min at 37° C on an orbital shaker (120 RPM). The color reaction was stopped with an equal volume of 10% SDS. The plate was read at a dual absorbance of 405/490 nm (Vmax Microtiter Reader, Molecular Devices, Inc., Menlo Park, CA).

Data Analysis

The mean OD for all blank wells was determined, and that value was subtracted from all sample OD values prior to data analysis. Linear regression and relative potency (RP) were calculated according to the NVSL RelPot computer program (version 3.0). This program is designed to assign weight to linear regression lines for both a reference and a test sample. Slopes were checked for parallelism, and the relative potency calculations were made. Criteria for a valid test were: a) a simple linear regression line must be fitted to a minimum of three contiguous dilutions, and b) lines must have a correlation coefficient (r) of at least 0.95. For more information and availability of the RelPot Program, interested persons may contact the National Veterinary Services Laboratories, Ames, Iowa 50010, USA.

In vitro vs In Vivo Correlation Study

Ten serial two-fold dilutions (beginning with 1:3) of the NVSL reference *L. canicola* bacterin were prepared and used to vaccinate hamsters by intramuscular injection. These preparations were considered to be undiluted and were diluted to 1/80th of the host animal dose according to established protocols (11). The NVSL reference *L. canicola* bacterin was also evaluated without prior dilution. There were 5 hamsters per bacterin dilution as well as 10 non-vaccinated control hamsters. Two weeks later, hamsters were challenged (ip) with 0.25 ml of liver homogenates from *canicola*-infected hamsters (10 to 10,000 $LD_{50's}$ as determined by titration (11)). Results were recorded as the percent of survivors per total number of animals challenged.

RESULTS

A Triton X-100 extract of the NVSL challenge strain of serovar *canicola* was used to prepare hybridomas secreting monoclonal antibodies. The selected twice-cloned hybridoma, designated as 4DB, was shown to secrete IgM antibody and was shown to react with an epitope present on three moieties of 30, 20, and 15 kDa in the Triton X-100 extract and inactivated whole cells by western blot analysis (Figure 1). The 4DB MAb did not detect antigen in whole cell preparations of the heterologous serovars.

When inactivated whole cells were treated with proteinase K prior to SDS-PAGE, protein digestion appeared to be complete as demonstrated by the lack of staining by Coomassie Brilliant Blue (Figure 2). When these samples were transferred to nitrocellulose membranes, the 4DB MAbs detected the same epitope on the three major moieties, as well as other minor LPS-like moieties (Figure 3). The additional bands detected by the 4DB MAb in Figure 3 were very similar, if not identical, to the epitope present on the LPS-like moieties that may have been masked by protein. Following proteinase digestion, these epitopes became available to bind to the 4DB MAb. The presence of more than one LPS-like moieties, (containing the 4DB epitope), detected by the MAb may be related to the growth phase of the organism at the time of antigen extraction. It is possible that the culture that was used for antigen extraction and heat-inactivation may have contained more than one population of *canicola* cells. One population may have predominantly expressed the 4DB epitope on a 30 kDa LPS-like moiety, another population may have predominantly expressed the epitope on a 20 kDa moiety, etc. When proteinase K-treated and non-treated samples of serovar *canicola* were evaluated by the ELISA, the OD values were slightly higher as compared with the OD values of nontreated samples (data not shown). The same three major moieties, (containing the 4DB epitope), were also stained

with an LPS silver stain using proteinase K-treated and non-treated inactivated whole cells and the Triton X-100 extract (Figure 4). Based on these results, it is speculated that the 4DB binds to an epitope contained within LPS-like moieties. This is advantageous for testing bacterin potency since LPS-like antigens have been shown to be responsible for the reaction in the agglutinating MAT and are involved in the protective humoral response to leptospiral infection (2-6).

Results from MAT studies indicate that the 4DB MAb agglutinated serovar *canicola* but failed to agglutinate heterologous *L. interrogans* serovars (Table 1). When 12 Canicola serovars were evaluated by MAT, only *canicola* serovars *bafani, broomi,* and *portlandvere* as well as *canicola* strain Hond U IV were agglutinated by the 4DB MAb (Table 2). When tested by ELISA, the 4DB MAb detected antigen in serovars *bafani, bindjei, jonsis,* and *portlandvere* as well as the *canicola* strain Hond U IV, but failed to detect significant amounts of antigen in the remaining serovars (Figure 5). The Moulton strain of serovar *canicola* was also evaluated, and results were comparable to the Hond U IV and portlandvere strains (data not shown). Because most leptospiral biologicals manufactured in the United States contain either the serovar *portlandvere* or the *canicola* strains Hond U IV or Moulton, it is not important that the 4DB MAb failed to detect other serovar strains within the Serogroup of canicola. No cross-reactivities were observed among serovars *grippotyphosa, icterohaemorrhagiae, hardjo,* or *bratislava* (Figure 6). In the hamster protection studies, the 4DB provided passive protection against a homologous challenge; none of the hamsters were protected from heterologous challenges (Table 3).

Results from specificity studies indicated that the ELISA is specific for *Leptospira interrogans* serovar *canicola* (Tables 4 & 5). The 4DB MAb failed to detect antigen in members of other bacterial genera or in heterologous leptospiral serovars. Antigen was detected in all

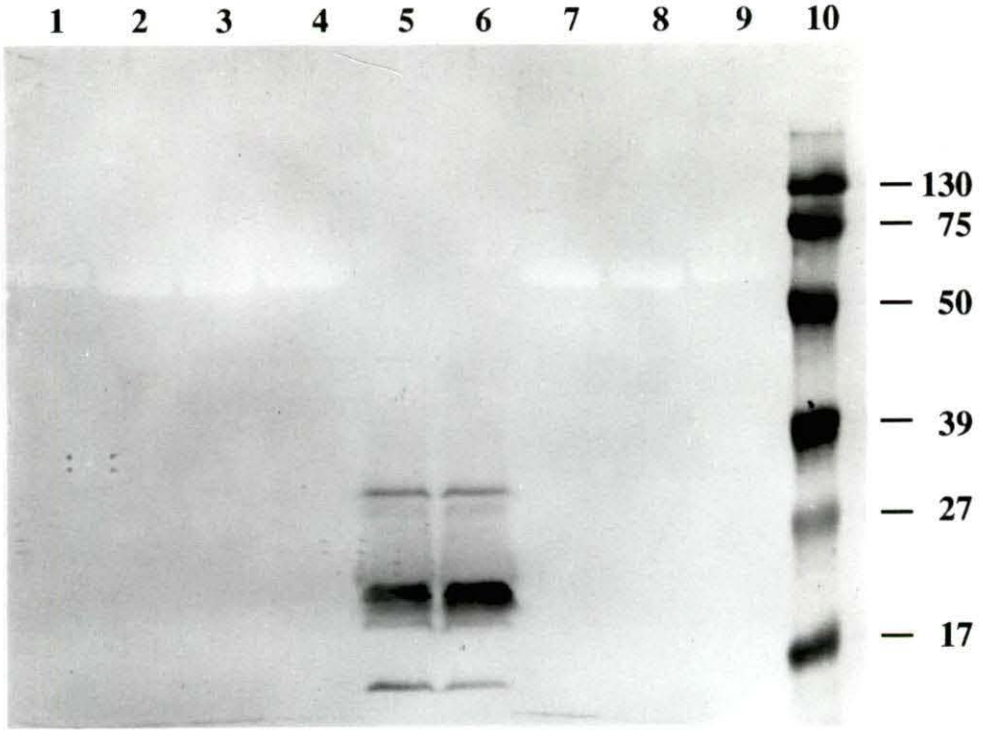
preparations that contained serovar *canicola* (data not shown).

Results from initial ELISA studies using the 4DB ascites fluid indicated that non-adjuvanted bacterins and those containing adjuvants other than aluminum hydroxide or oil did not require antigen elution treatment prior to use in the ELISA protocol. Bacterins containing aluminum hydroxide as an adjuvant required treatment to elute antigen prior to use in the ELISA protocol. Although the nontreated bacterin resulted in higher OD values, a portion of these values was attributable to the effect of the aluminum hyrdoxide. Therefore, products adjuvanted with aluminum hydroxide required treatment to elute antigen form the adjuvant. A potassium phosphate buffer yielded optimal OD readings. Figure 7 shows ELISA results of a non-treated aluminum hydroxide-adjuvanted product and the same product treated with either sodium citrate or potassium phosphate. Pre-treatment with sodium citrate yielded OD values that were comparable to blank wells. Only the potassium phosphate treatment yielded satisfactory OD values. ELISA results also indicated that bacterins containing oil as an adjuvant required treatment to elute antigen prior to use in the ELISA protocol. Two antigen elution methods were evaluated. Treatment with a 0.5% SDS solution dramatically increased the OD values. Figure 8 shows ELISA results using a non-treated oil-adjuvanted bacterin and the same bacterin treated with either a 0.5% SDS solution or sodium desoxycholate. The adjuvant control was also evaluated. A 0.5% SDS solution yielded optimal OD values as compared to the use of sodium desoxycholate as an antigen elution buffer. Wells containing the adjuvant control resulted in OD values comparable to blank wells. Although sodium desoxycholate treatment resulted in OD values comparable to the non-treated bacterin, these values were considered to be too low for use in calculation of relative potency of the bacterins.

A total of 185 leptospiral bacterins representing 1-, 2-, 3-, 5-, and 10-ml dose products (Table 6) were evaluated by the ELISA (Table 7). All bacterins evaluated by this system have met the minimum requirement of a relative potency of 1.0 or greater.

Results of the in vitro and in vivo correlation study indicated that the ELISA approximates the sensitivity of the hamster potency assay (Table 8). When serial two-fold dilutions of the NVSL reference *L. canicola* bacterin were used to vaccinate hamsters according to established protocols (11), a dilution of 1:24 (1.823×10^6 organisms) provided 80% protection, whereas a 1:48 dilution (9.115×10^5 organisms) provided only 40% protection against a virulent challenge. When these bacterin dilutions were evaluated by the ELISA (phase II), the 1:48 dilution (4.380×10^6 organisms) resulted in an RP of 1.08, whereas the 1:96 dilution (2.190×10^6 organisms) resulted in an RP of 0.61. The 1:24 dilution used to vaccinate hamsters contained 83% of the organisms (1.823×10^6) used in the ELISA and provided 80% protection.

Figure 1. Western blot using the ascites fluid containing the 4DB MAb to detect antigen in the Triton X-100 extract of serovar *canicola* and inactivated whole cells of serovars *canicola*, *icterohaemorrhagiae*, *hardjo*, *grippotyphosa*, and *pomona*. Samples were standardized to 50 μg of protein per lane. After eletro-transfer, the membrane was incubated in a 1:100 dilution of the 4DB ascites fluid followed by incubation in a 1:2000 dilution of horseradish peroxidase-conjugated anti-mouse IgM. The substrate was 4-chloronaphthol. Lane 1: *L. pomona* whole cells, Lane 2: *L. grippotyphosa* whole cells, Lane 3 *L. hardjo* whole cells, Lane 4: *L. icterohaemorrhagiae* whole cells, Lane 5: *L. canicola* whole cells, Lane 6: Triton X-100 extract of *L. canicola*, Lane 7: *L. bratislava* whole cells, Lanes 8 & 9: blank, Lane 10: pre-stained low molecular mass markers.


1
2
3
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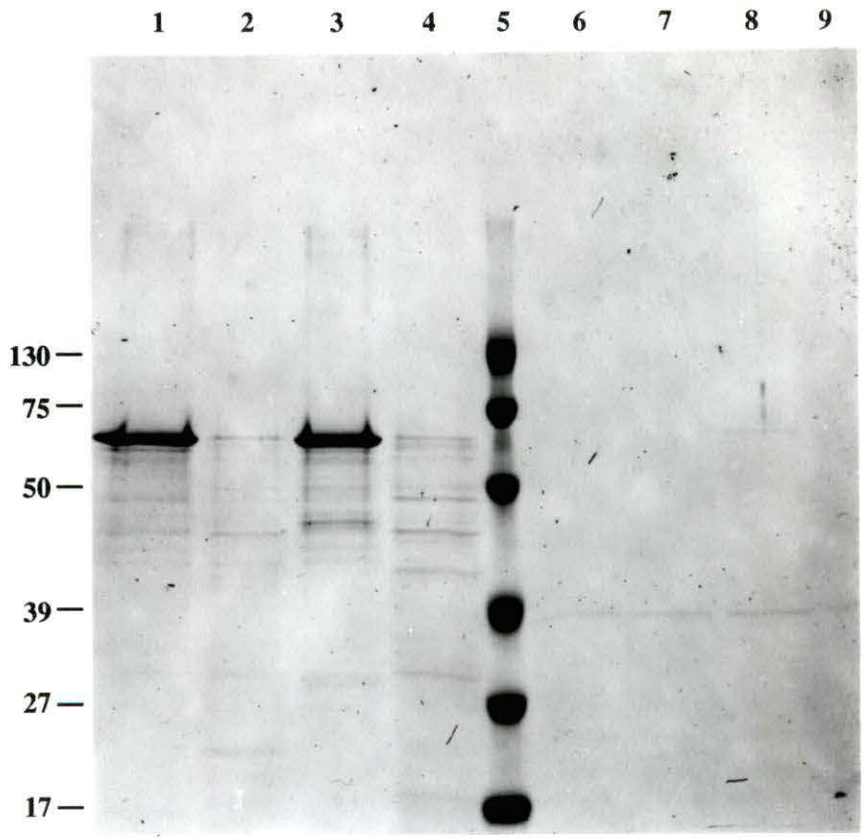
5
6
7
8
9
10
130
75
50
39
27
17

Figure 2. Coomassie Brilliant Blue stained SDS-PAGE gel containing boiled Triton X-100 extracts and boiled inactivated whole cells of serovars *canicola* and *pomona*. Samples were standardized to 50 μg of protein per lane and were either treated or not treated with proteinase K prior to electrophoresis into a 4-20% acrylamide gradient gel. Lanes 1&6: *L. pomona* inactivated whole cells, Lanes 2&7: *L. pomona* Triton X-100 extract, Lanes 3&8: *L. canicola* inactivated whole cells, Lanes 4&9: *L. canicola* Triton X-100 extract, Lane 5: pre-stained low molecular mass markers. Samples in lanes 1-4 were not treated with proteinase K. Lanes 6-9 were identical to lanes 1-4 except that these samples were treated with proteinase K.


1
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130
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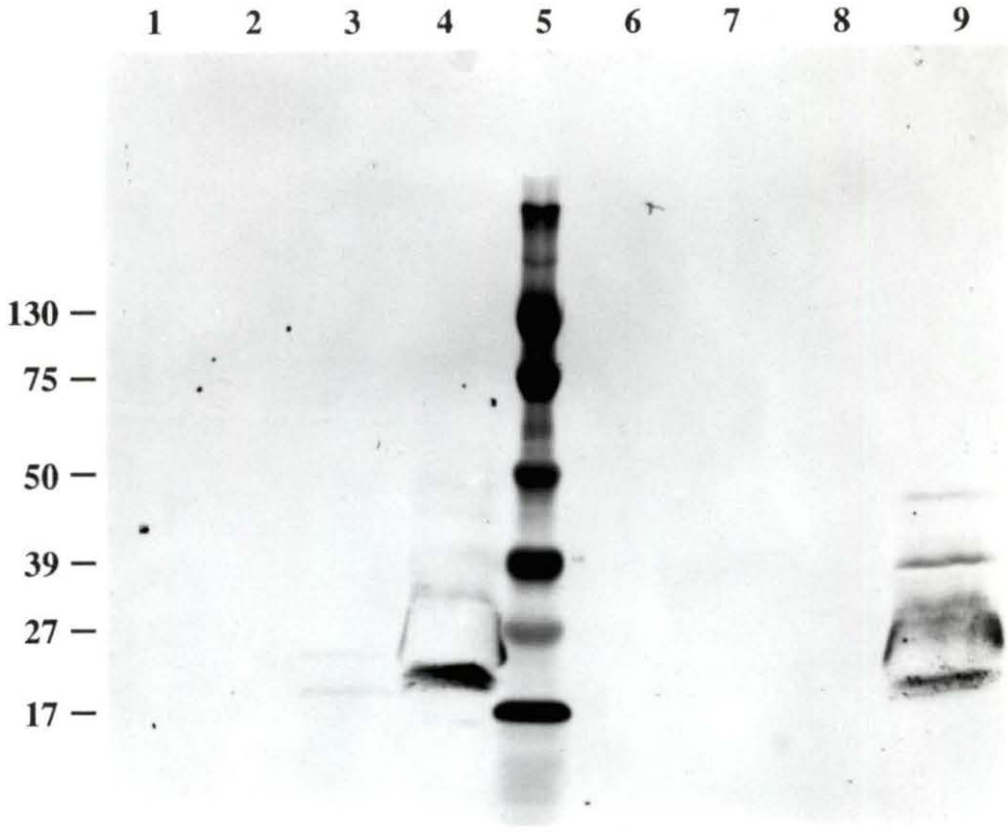
27
17

Figure 3. Western blot using boiled inactivated whole cells of serovars *canicola, grippotyphosa, icterohaemorrhagiae*, and *pomona*. The boiled Triton X-100 extract of serovar *canicola* was also used. Samples were standardized to contain 50 μg of protein per lane and were either treated or not treated with proteinase K prior to electrophoresis into a 4-20% SDS gradient gel. Lanes 1&6: *L. grippotyphosa* inactivated whole cells, Lanes 2&7: *L. icterohaemorrhagiae* inactivated whole cells, Lanes 3&8: *L. canicola* inactivated whole cells, Lanes 4&9: *L. canicola* Triton X-100 extract, Lane 5: pre-stained low molecular mass markers. Lanes 1-4 were treated with proteinase K. Lanes 6-9 were not treated with proteinase K.


1
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3
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130
75
50
39
27
17

Figure 4. A 14% SDS gel containing the Triton X-100 and inactivated whole cells of serovar *canicola*. Both samples were boiled prior to electrophoresis. The gel is stained for lipopolysaccharide using a modifed silver stain. The samples were standardized to contain 50 μg of protein per lane. Lane 1: pre-stained low molecular mass markers, Lane 2: *L. canicola* Triton X-100 extract, Lane 3: *L. canicola* inactivated whole cells.

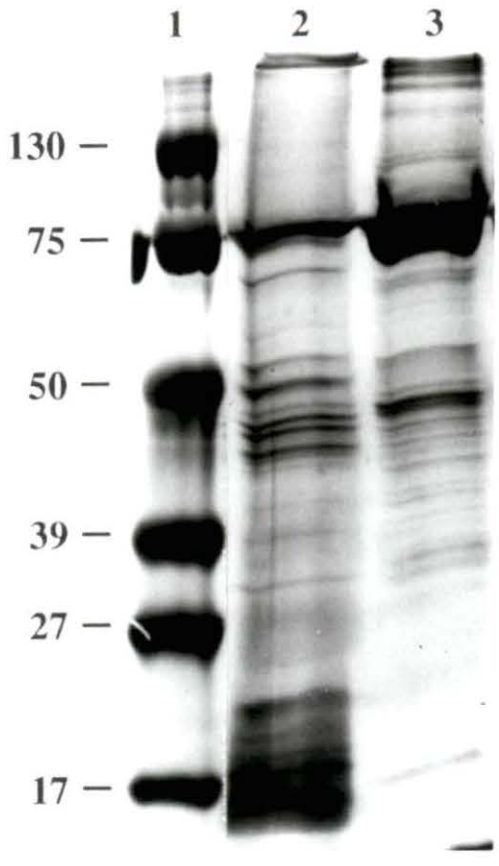
1
2
3
130
75
50
39
27
17

Table 1. Microagglutination titers of the 4DB MAb for various serovars of *L. interrogans*

Antigen[a]	MAT Titer[b]
Pomona	<20
Canicola	10240
Grippotyphosa	<20
Icterohaemorrhagiae	<20
Hardjo	<20
Bratislava 2a	<20
Bratislava jez	<20

[a] Viable whole cells of hamster virulent serovars of *L. interrogans* were used as the antigen.

[b] Titer is expressed as the average reciprocal dilution at which 50% of the leptospires were agglutinated.

Table 2. Specificity of the 4DB MAb among *canicola* serovars by the microagglutination test (MAT)

Antigen[a]	MAT Titer[b]
L. canicola bafani bafani	2560
L. canicola benjamin benjamin	<20
L. canicola bindjei bindjei	<20
L. canicola schueffneri vleermuis	<20
L. canicola sumneri sumner	<20
L. canicola broomi patone	15360
L. canicola canicola Hond U IV	20480
L. canicola galtoni 1014	<20
L. canicola jonsis jones	80
L. canicola kamituga kamituga	960
L. canicola malaya H6	<20
L. canicola portlandvere 63-69	20480

[a] Viable whole cells of each serovar were used as the antigen.

[b] Titer is expressed as the average reciprocal dilution at which 50% of the leptospires were agglutinated.

Figure 5. ELISA results using the 4DB ascites fluid to detect 12 *canicola* serovars. Heat-inactivated whole cells were diluted 1:128 and captured by the polyclonal anti-*canicola* rabbit serum. A 1:14,000 dilution of the 4DB ascites fluid was added followed by a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgM. The substrate was ABTS.

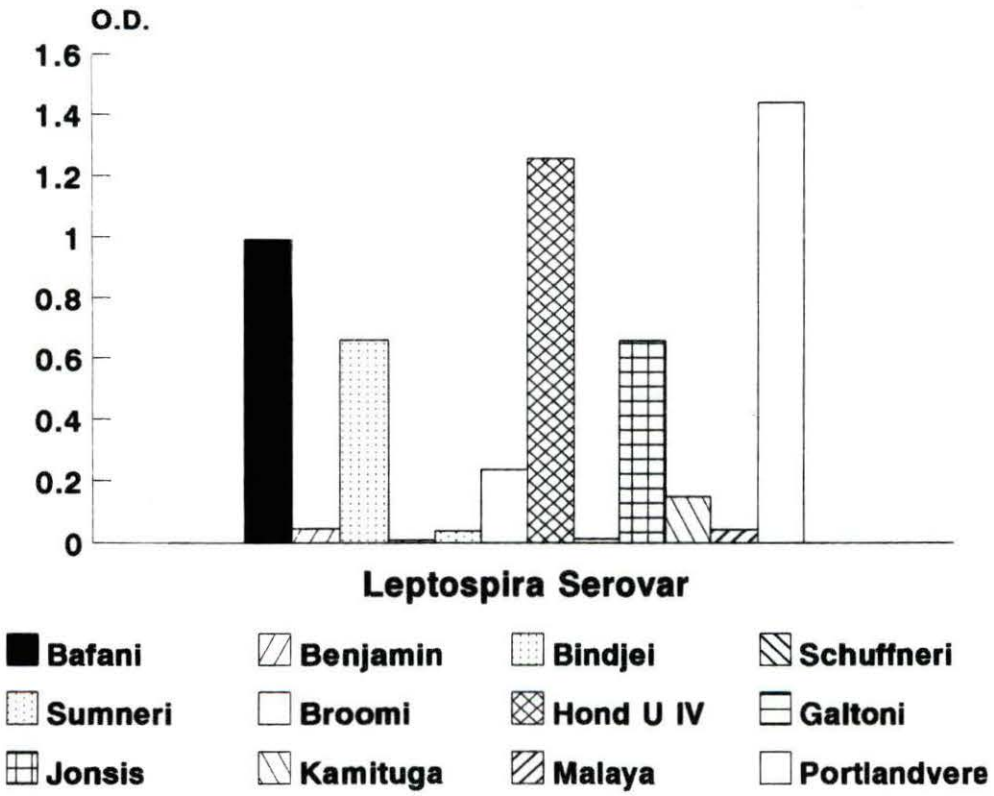
O.D.
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Leptospira Serovar
Bafani
Benjamin
Bindjei
Schuffneri
Sumneri
Broomi
Hond U IV
Galtoni
Jonsis
Kamituga
Malaya
Portlandvere

Figure 6. ELISA results using the 4DB ascites fluid to detect six inactivated whole cells of *Leptospira interrogans* serovars. Inactivated whole cells were diluted 1:128 and captured by the polyclonal anti-*canicola* rabbit serum. A 1:14,000 dilution of the 4DB ascites fluid was added followed by a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgM. The substrate was ABTS.

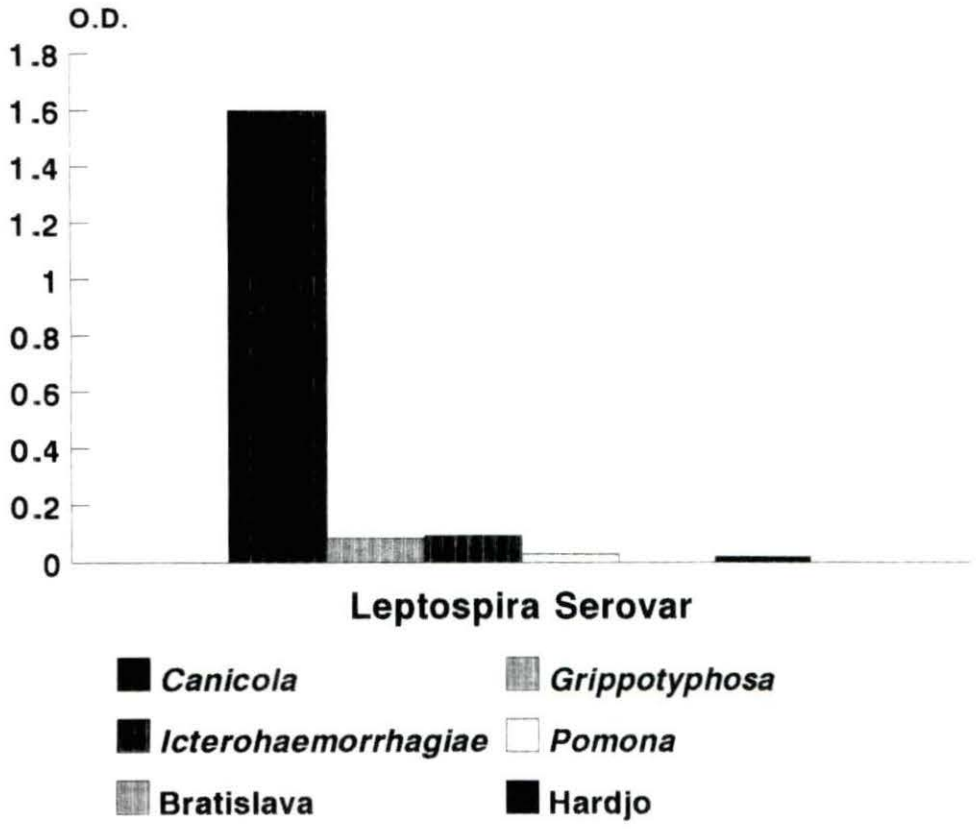
O.D.
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Leptospira Serovar
Canicola
Icterohaemorrhagiae
Bratislava
Grippotyphosa
Pomona
Hardjo

Table 3. Passive protection of hamsters with the 4DB MAb against a virulent challenge of serovars *canicola*, *icterohaemorrhagiae*, *grippotyphosa*, and *pomona*[a]

Vaccination Material	Hamsters Surviving Homologous Challenge (*L. canicola*)	Hamsters Surviving Heterologous Challenge[b]		
		(I)	(G)	(P)
4DB MAb [c]	5/5[d]	0/5	0/5	0/5
Controls (DNT MAb) [e]	0/5	0/5	0/5	0/5

[a] Control hamsters were passively immunized with an equal antibody concentration of ascites fluid prepared against the dermonecrotic toxin of *P. multocida* Type D. Hamsters were vaccinated with the 4DB MAb and challenged 24 h later with virulent organisms.

[b] Heterologous *L. interrogans* serovars *Icterohaemorrhagiae* (I), *Grippotyphosa* (G), and *Pomona* (P).

[c] The 4DB ascites fluid contained 275 mg of IgM per milliliter.

[d] Results expressed as survivors per total animals challenged.

[e] The volume of DNT ascites fluid contained 275 mg of IgG_1 per milliliter.

Table 4. Preparations used to determine the specificity of the *L. canicola* ELISA[a]

Preparation[b]	Adjuvant[c]	ELISA OD
Lc	NA	1.786
Ph	AlOH + Quil A	0.022
Ec	AlOH	0.040
LHIP	AlOH	0.011
Schol	AlOH	0.021
Cf	AlOH	0.045
App, Hpara, Pm	AlOH	0.035
App	Oil	0.002

[a] Preparations containing no *L. canicola* organisms were used as well as the ELISA test reference bacterin as a positive control. Optical density values represent a 1:2 dilution.

[b] Commercial licensed bacterins containing no *L. canicola* organisms. Lc = ELISA test reference, Leptospira Canicola Bacterin; Ph = Pasteurella Haemolytica Bacterin; Ec = Escherichia Coli Bacterin; LHIP = Leptospira Hardjo-Icterohaemorrhagaie-Pomona Bacterin; Schol = Salmonella Choleraesuis Bacterin; Cf = Campylobacter Fetus Bacterin; APP, Hpara, Pm = Actinobacillus Pleuropneumoniae-Haemophilus Parasuis-Pasteurella Multocida Bacterin-Toxoid; App = Actinobacillus Pleuropneumoniae Bacterin.

[c] NA = non-adjuvanted; AlOH = aluminum hydroxide.

Table 5. Inactivated whole cells used to determine the specificity of the *L. canicola* ELISA[a]

Organism[b]	ELISA OD
Lc	1.883
Lg	0.010
Li	0.031
Lp	0.001
Bb	0.062
Pm	0.031
Schol	0.022

[a] Organisms evaluated included various *L. interrogans* serovars and heterologous bacteria. The ELISA test reference bacterin served as a positive control. Optical density values represent a 1:2 dilution.

[b] Lc = *L. canicola* ELISA test reference bacterin; Lg = *Leptospira grippotyphosa*; Li = *Leptospira icterohaemorrhagiae*; Lp = *Leptospria pomona*; Bb = *Bordetella bronchiseptica*; Pm = *Pasteurella multocida* type D; Schol = *Salmonella choleraesuis*.

Figure 7. Results using the 4DB MAb in the ELISA to detect antigen in a 5-ml dose aluminum hydroxide-adjuvanted product. Two antigen elution methods were evaluated as well as evaluation of the bacterin without prior treatment. After antigen in the samples were captured, a 1:14,000 dilution of the 4DB ascites fluid was added followed by a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgM. The substrate was ABTS. TR: test reference, NT: not treated, Cit: sodium citrate, KH_2PO_4: potassium phosphate buffer.

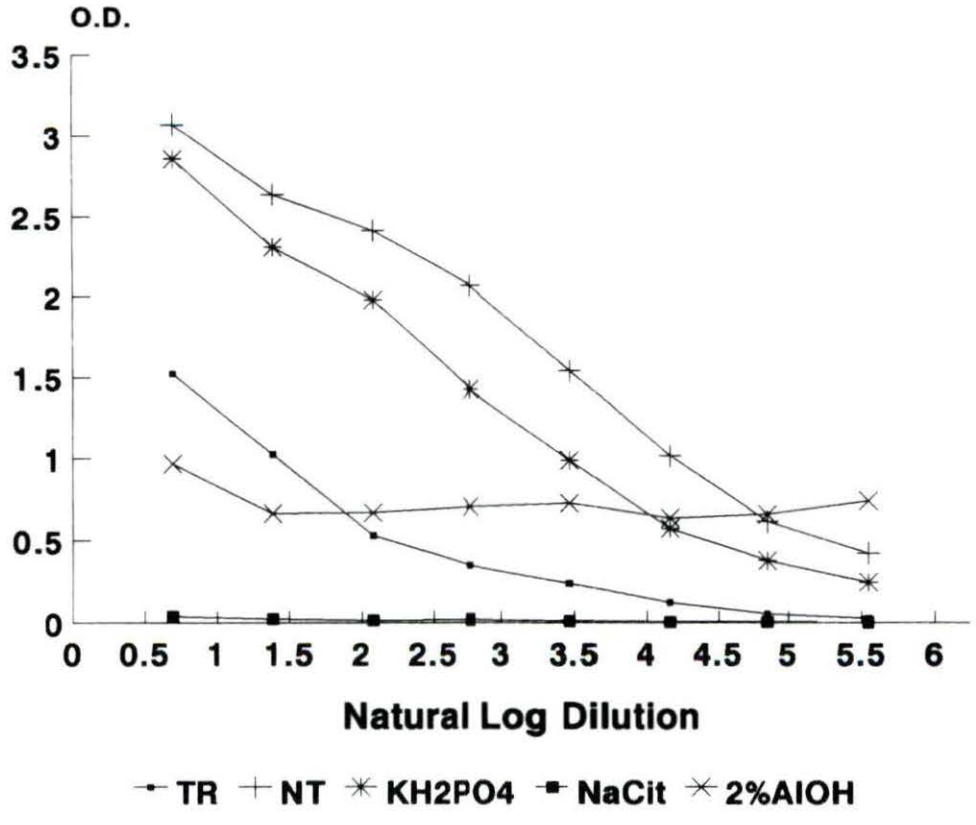
O.D.
3.5
3
2.5
2
1.5
1
0.5
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5 6
Natural Log Dilution
TR NT KH2PO4 NaCit 2%AlOH

Figure 8. Results using the 4DB ascites fluid in the ELISA to detect antigen in a 5-ml dose oil-adjuvanted product. Two antigen elution methods were evaluated as well as evaluation of the bacterin without prior treatment. After antigen in the samples were captured, a 1:14,000 dilution of the 4DB ascites fluid was added followed by a 1:2,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgM. The color substrate was ABTS. TR: test reference, NT: not treated, DOC: desoxycholate, SDS: sodium dodecyl sulfate.

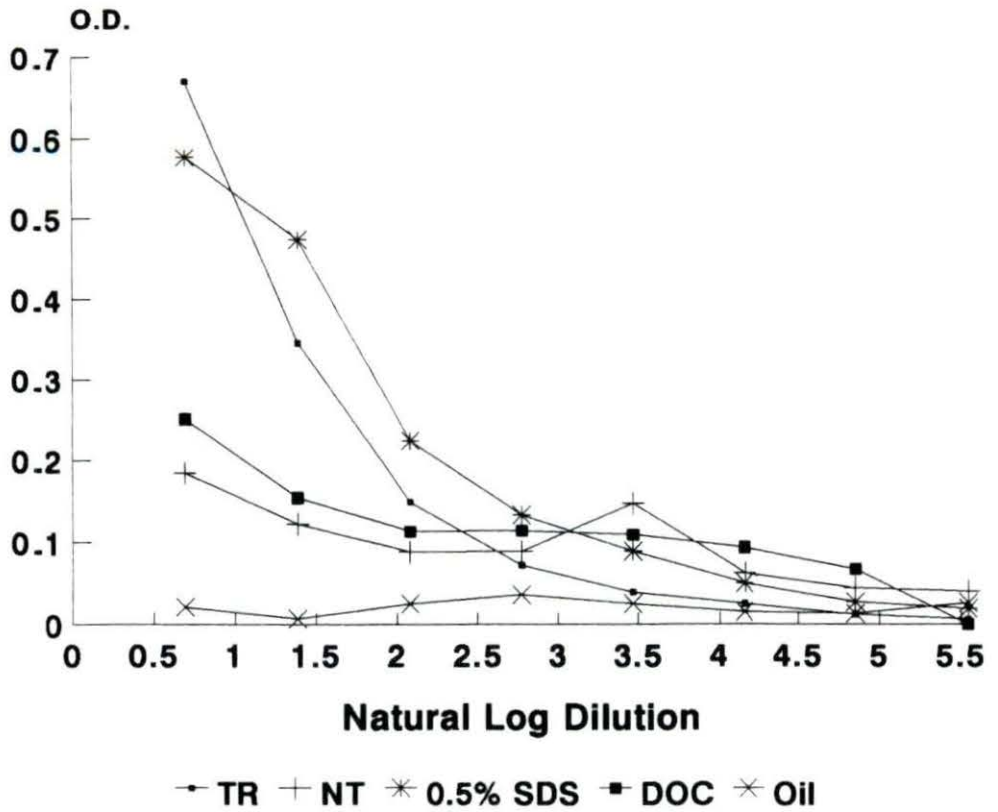
O.D.
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0 0.5 1 1.5 2 2.5 3 3.5 4 4.5 5 5.5
Natural Log Dilution
TR NT 0.5% SDS DOC Oil

Table 6. Bacterins evaluated for relative potency by the *L. canicola* ELISA

Total No. Products	Host Animal Dose	No. Firms	Adjuvant in Product				
			AlOH	Oil	Combination[a]	Other[b]	None
9	1-ml dose	2	-	-		5	4
71	2-ml dose	8	50			7	14
17	3-ml dose	1			17		
86	5-ml dose	6	65	13	8	-	-
2	10-ml dose	1			2	-	-

[a] Products containing a combination of adjuvants.

[b] Products containing adjuvants other than aluminum hydroxide or oil.

Table 7. Representative ELISA results for phases I and II of the *L. canicola* ELISA using random leptospiral bacterins[a]

Host Animal Dose	Relative Potency Range Phase I	Phase II
1-ml dose	4.55-5.48	NA[b]
2-ml dose	1.14-9.18	3.06-4.17
3-ml dose	0.27-0.38	2.36-2.46[c]
5-ml dose	2.34-4.21	4.90-38.20
10-ml dose	2.04-2.08	NA

[a] Results are expressed as the range of highest relative potency calculation as determined by the NVSL RelPot computer program.

[b] There were no products that required testing by phase II. All products of this host animal dose that were tested had passed in phase I. NA = not applicable.

[c] These are products that failed in phase I and were re-tested in phase II.

Table 8. Comparison of hamster protection and the ability of the *L. canicola* ELISA to detect an unsatisfactory bacterin[a]

Bacterin Dilution	No. Organisms Injected	No. Survivors[b]	No. Organisms Represented[c]	ELISA Results[d] I	II
Undiluted	4.375×10^7	5/5 (100%)	1.051×10^7	7.01	
			2.102×10^8		12.31
1:3	1.458×10^7	5/5 (100%)	3.505×10^6	3.81	
			7.009×10^7		5.11
1:6	7.292×10^6	5/5 (100%)	1.752×10^6	NVC	
			3.504×10^7		3.31
1:12	3.646×10^6	5/5 (100%)	8.761×10^5	NVC	
			1.752×10^7		2.69
1:24	1.823×10^6	4/5 (80%)	4.381×10^5	NVC	
			8.762×10^6		1.94
1:48	9.115×10^5	2/5 (40%)	2.190×10^5	NVC	
			4.380×10^6		1.08
1:96	4.557×10^5	1/5 (20%)	1.095×10^5	NVC	
			2.190×10^6		0.61
1:192	2.279×10^5	2/5 (40%)	5.476×10^4	NVC	
			1.095×10^6		NVC
1:384	1.139×10^5	0/5 (0%)	2.738×10^4	NVC	
			5.474×10^5		NVC
1:768	5.697×10^4	0/5 (0%)	1.369×10^4	NVC	
			2.737×10^5		NVC

[a] The NVSL reference *L. canicola* bacterin was diluted to 1/80th of a 2-ml dose and used to vaccinate hamsters followed by a virulent *L. canicola* challenge two weeks later. The same bacterin was evaluated against the ELISA test reference bacterin according to the ELISA protocol without diluting to 1/80th of a 2-ml dose

[b] Number of hamsters surviving a virulent *L. canicola* challenge. Results are expressed as the number of survivors per total number of animals challenged.

[c] Number of organisms used in the ELISA at a 1:2 dilution.

[d] Results are expressed as the highest RP values as determined by the RelPot computer program. RP values for phase I are not adjusted for the 1:20 dilution. The bacterins were evaluated in both phases of the ELISA protocol. NVC, no valid combinations.

DISCUSSION

Because of the increasing concern for reducing animal costs, improving animal welfare and human health risks, the development of an in vitro assay for measuring the relative potency of leptospiral bacterins containing serovar *canicola* was developed. This ELISA system was developed to address these concerns.

A monoclonal antibody directed against surface antigens of the Moulton strain of serovar *canicola* was developed using a Triton X-100 extract of this serovar. The selected hybridoma, 4DB, was shown to secrete IgM-type antibody that was shown to be protective, agglutinating, and serovar-specific. The 4DB MAb appears to react with an epitope present on LPS-like moieties of whole cells of the Moulton strain of serovar *canicola* (Figures 1-4).

The MAT and ELISA results show that the 4DB MAb is specific for Serogroup canicola. Agglutination occured with only the Serogroup canicola and failed to agglutinate the remaining Serogroup representatives (Table 1). Similar results were seen when tested by ELISA (Figure 5). In contrast, results obtained with different Serogroups show differences when 12 serovars of the Serogroup canicola were evaluated by the MAT and the ELISA. *Leptospira canicola* serovars that had high agglutination titers did not always have the highest optical densities by ELISA (Table 2 & Figure 6). Similarly, those serovars yeilding the highest optical density values by ELISA did not always have the lowest MAT titers. These differences may be inherant in methodology of the test employed. The MAT is used to obtain an end-point in antibody titer (15). Titers of $\leq$ 1:200 are considered to be non-specific (15). The MAT involves the use of viable antigens that are in suspension and are freely accessible to the antibody. Scoring of the percent agglutination is subjective, and there is a wide lab-to-lab variation in MAT results (15). In the ELISA, antigen is captured by

polyclonal antisera prepared against serovar *canicola*. Assume that a particular organism contains a low number of the 4DB epitope and that the polyclonal antisera contains antibody against this epitope. Once the organism (antigen) is bound by the polyclonal antisera, potential 4DB binding sites are eliminated. This results in a low ELISA OD value but may result in a high MAT titer. Alternatively, at a 1:100 dilution, the polyclonal capture serum could contain high affinity antibodies specific for antigens of other *canicola* serovars and could explain the higher OD values with serovars yielding low MAT titers (*bindjei* (bindjei), and *jonsis* (jones)). It is noteworthy that the most commonly used serovars in production of leptospira bacterins in the United States are serovars *portlandvere* as well as the Moulton and Hond U IV strains of serovar *canicola*.

A total of 185 licensed leptospiral bacterins were evaluated with this ELISA system. All bacterins tested had an RP of $\geq$ 1.0. Most bacterins passed the ELISA test when diluted 1:20 (phase I). Those that failed to pass in phase I, did pass when assayed without prior dilution (phase II). Possible explanations for these results include: a) a wide antigenic concentration of serovar *canicola* in leptospiral bacterins, b) interference with detection of *canicola* antigen by other viral and bacterial components, c) differences in adjuvants used in leptospiral bacterins, and d) the efficiency of antigen elution. The latter is probably the most plausible explanation for these results.

This ELISA-based detection system approaches the sensitivity of the hamster potency assay (Table 8) and indicates that the ELISA test reference bacterin represents an immunogenic sample. Results of the correlation study show that hamsters were protected with 41.6% less organisms as comapred to the number of organisms required for an ELISA RP of 1.0 or greater. This difference is represented by a single 2-fold dilution.

Because the currently encoded potency assay is an in vivo model, if this experiment were repeated several times, the dilution that provided hamster protection may equal the dilution that would result in an RP of 1.0.

With regard to correlation of the ELISA data with in vivo data, four facts must be realized: a) the hamster is not a natural reservior for leptospirosis, b) in addition to antibody-mediated processes, there are also cell-mediated processes involved in the response to *Leptospira* infection, c) the hamster potency assay required the bacterin to be diluted to 1/80th of the host animal dose, and d) the ELISA protocol does not involve diluting the bacterins to 1/80th of the host animal dose. For these reasons, any ELISA developed for measuring the realtive potency of bacterins will never parallel the in vivo model exactly.

The use of this ELISA provides a reproducible alternate method for testing the *L. canicola* component of leptospira bacterins. In addition, this test system could be used for other purposes, such as screening of master seed cultures used in the manufacture of leptospiral bacterins and for screening cultures for cross-contamination by other *L. interrogans* serovars. This ELISA could be modified to be used for diagnostic purposes, such as detection of leptospires in biological samples, histopathology, etc. Other assays currently being developed in this laboratory to measure anitgen(s) of serovars *grippotyphosa* and *icterohaemorrhagiae* will provide additional in vitro testing methods for the evaluation of leptospiral bacterins and reduce even further the need for in vivo testing of these products.

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GENERAL SUMMARY

Leptospira interrogans serovars *pomona* and *canicola* are the cause of leptospirosis in dogs, cattle, swine, and horses. Virulence attributes of *L. interrogans* include motility, a hemolysin, proposed cytolysin production, and evasion of the host immune response (26-33). Vaccination has been shown to be an effective preventative measure (25,38,41,43,50,51). After an active infection, immunity is life-long and is serovar-specific (25).

The current potency test for licensing leptospiral bacterins involves a hamster assay in which 80% of vaccinated hamsters must survive a virulent challenge (1). This assay is very expensive, time consuming, and involves exposure of personnel to live organisms that are pathogenic for humans. One of the policies of the National Veterinary Services Laboratories (NVSL) is to reduce the use of animals for testing when possible. An in vitro assay for measuring the relative potency of leptospiral bacterins is in line with this policy. This thesis describes the development of in vitro assays for measuring the relative potency of leptospiral bacterins containing serovars *pomona* and *canicola*.

Monoclonal antibodies directed against surface antigens of serovars *L. pomona* and *L. canicola* were developed using Triton X-100 extracts of these serovars. The *L. pomona* 2D7 hybridoma clone secreted IgA-type antibody. The *L. canicola* 4DB hybridoma clone secreted IgM-type antibody. Both MAbs were shown to be agglutinating by MAT and protective in hamsters. Specificity of the two MAbs was demonstrated with no cross reactivity between heterologous serovars.

There are many antigenic leptospiral proteins that have been described. Some of the major cross-reactive antigens are part of the axial filaments (5,10,22,23,24). Two proteins of the axial filament with approximate molecular mass of 30 kDa and 40 kDa are the most evident in

SDS-PAGE electrophoresis (5-7,41,50,59). It is known that the axial filmanet has some immunogenic properties (9,58,31). There are several reports of MAbs directed against leptospiral LPS (54,55,60,61-63). Both the 2D7 (*L. pomona*) and 4DB (*L. canicola*) MAbs reacted with antigens on the intact cell of the respective serovars. Since leptospiral bacterins are composed of inactivated whole cells of *L. interrogans* serovars, these cell surface antigens represent an excellent parameter for relative potency assessment.

The *L. pomona* 2D7 clone was shown to react with an epitope present on an LPS-like moiety of 14-16 kDa in the Triton X-100 extract and a single LPS-like moiety of 18 kDa in the whole cell preparation. There are two possible explanations for the difference in the sizes detected by the 2D7 MAb. First, the 18 kDa moiety may represent the 14-16 kDa moiety in association with other surface membrane components on the intact cell. The extraction process may have removed these associated components yielding the 14-16 kDa antigen. Secondly, the 14-16 kDa moiety may be present in a three-dimensional conformation that is different when inserted in the membrane as compared to the conformation of the extracted antigen.

The 4DB MAb was shown to react with an epitope on three different moieties (i.e. bands) present in the *L. canicola* Triton X-100 extract and whole cell preparation. Based on the results of proteinase digestion and LPS-silver stain studies, it can only be speculated that the 4DB MAb reacts with an epitope present on three LPS-like moieties present in Triton X-100 extract and whole cell preparations of serovar *canicola*. They do not prove that these moieties are LPS, but they do indicate that an LPS-like substance is associated with the epitope recognized by the 4DB MAb. This is be consistent with literature that leptospiral LPS differs from classical LPS (5,11-15). A third explanation for the difference in sizes of moieties recognized by the 4DB MAb may be due to post-translational

molecules being produced during heat-inactivation. By raising the temperature of the growth medium, the organisms begin synthesizing heat-shock proteins and other molecules related to the pathogenesis of the organism. At 56° C proteins that may have been secreted from the cell in response to the initial rise in temperature, may denature (coagulate) and aggregate on the cell surface.

The LPS silver stain used to stain LPS in the *pomona* and *canicola* preparations probably stained some protein in addition to the LPS. This is likely due to: a) fluctuations in room temperature, and/or b) incorrect concentration of ammonium hydroxide used in the color development solution. The oxidation of the LPS-like moieties may have been affected by changes in room temperature. On the other hand, if too much ammonium hydroxide was present, proteins may have been stained.

The two MAbs were used to develop in vitro assays for measuring the relative potency of bacterins containing *L. pomona* (2D7) and *L. canicola* (4DB). Both in vitro assays involve coating microtitration plates with polyclonal rabbit antisera to capture antigen in bacterins. Antigen is detected by adding a specified dilution of ascites fluid containing monoclonal antibodies against the appropriate serovar. After incubation with anti-mouse horseradish peroxidase antibody conjugate, the antigen-antibody complex is visualized with either TMB (*L. pomona*) or ABTS (*L. canicola*). The blank subtracted optical density (OD) values for each test sample are then used to calculate the relative potency using the NVSL RelPot program (version 3.0).

Differences were encountered in regard to treatment of the bacterin prior to use in the ELISA protocols. As opposed to the 2D7 MAb, the 4DB MAb bound to aluminum hydroxide adjuvant. When a commercial 2% aluminum hydroxide gel solution was diluted to correspond to the final concentration in an accompanying treated bacterin, the 4DB MAb was shown to be bound to

the gel. This may be due to the chemical nature of the IgM-type antibody as compared to the IgA-type antibody. An IgM is pentameric and contains more disulfide bonds and carbohydrate groups in the hinge region than does an IgA molecule. Because of possible differences in the avidity of these two MAbs, the *L. canicola* ELISA protocol required that bacterins adjuvanted with aluminum hydroxide be treated to elute the antigen from the adjuvant prior to evaluation in the ELISA. Although the 4DB MAb did not bind to an oil preparation, the oil appeared to hinder detection of all epitopes present in the bacterin. For this reason, those bacterins containing oil as the sole adjuvant required treatment to elute the antigen. There were several bacterins that contained a combination of adjuvants as well as some products that contained adjuvants that did not interfere with the detection of the antigen.

All products evaluated by either ELISA assay met the minimum relative potency requirement of 1.0 or greater. The presence of adjuvants in bacterins does not appear to hinder detection of epitopes by the *pomona* 2D7 MAb. Although the *canicola* 4DB MAb did not bind to an oil preparation, the oil appeared to hinder detection of all epitopes present in the bacterin. For this reason, those bacterins containing oil as an adjuvant require treatment to elute the antigen.

Both ELISA's approximated the sensitivity of the hamster potency assay for the respective serovars. Whenever developing an in vitro assay as an alternative to an in vivo model, it is important to realize that several obstacles must be overcome. These ELISA's measure a single protective immunogen (epitope) that is present in multiple copies on the surface of the organism. It would be naive to think that this one immunogen (epitope) is the only one of importance. On the contrary, one must think of more than one immunogen that is involved in the host immune response to infection. This is particularly true with bacterial

infections. Another obstacle to developing the "perfect" ELISA system is the extreme intercellular interplay involved in response to most infections. Most infections involve both arms of the immune system and include the cellular responses to a wide variety of cytokines. These interactions cannot be reproduced in an ELISA-based detection system. Another important variable to be considered is the adjuvant(s) that is used as an immunostimulant. Some of these not only affect humoral responses, but also cell-mediated responses. For some of these adjuvants, antigen may be adequately eluted. For others, there is no known method of eluting the antigen. For those products containing combinations of adjuvants, the problem is compounded by the fact that either a) the adjuvants may interfere with each other, or b) antigen may not be able to be eluted efficiently for application to an ELISA system. There is also the potential for new adjuvants to be developed that require less antigenic mass to produce protective immunity. In this case, these ELISA assays would have to be modified to reflect these adjuvants. In addition, the physiological state of the host must be considered when evaluating correlation data. Is the animal immunosuppressed (i.e., viral infection, genetically pre-disposed to suppression, currently under steroid therapy, stress, malnutrition, etc)? What is the hormonal state of the animal (i.e., estrus, pregnancy, parity, etc)? Given the various environmental and genetic interactions related to immune function, to expect exact parallelism with in vivo data would be ludicrous.

The development of alternate methods for testing of veterinary biologicals is a great advance over in vivo assays. In vitro assays are safer, more precise, and quicker to perform as compared to in vivo assays in laboratory animal models. However, the correlation of in vitro data with in vivo data is not always exact. The multitude of cellular interactions and the presence of cytokines that are present with infectious

diseases in the intact animal is an obstacle which may never be overcome with an in vitro test system. In addition, there is the lack of assessment of the effect of the presence of adjuvants in most leptospiral bacterins. The more immunostimulatory adjuvant will require less antigen to be present in the product to elicit an adequate protective humoral response. Since no elution treatment will ever release 100% of the bound antigen, this is a variance that may require more attention. This thesis describes the development of an alternative method for measuring potency of leptospiral bacterins containing serovars *pomona* and *canicola*. Work is planned for developing similar assays for serovars *icterohaemorrhagiae*, *grippotyphosa*, *bratislava*, and *hardjo*.

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