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Short paper

Development of *in vitro* assays for measuring the relative potency of leptospiral bacterins containing serogroups *canicola*, *grippotyphosa*, *icterohaemorrhagiae*, and *pomona*

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ABSTRACT

Historically, potency testing of bacterins containing *Leptospira* involved a hamster vaccination-challenge assay. The United States Department of Agriculture (USDA) has long recognized that an *in vitro* system has several inherent advantages over the animal model. This is a review of the work performed at the USDA to replace the hamster vaccination-challenge model used to test *Leptospira* bacterins. The work covered a span of approximately 20 years and resulted in the development of USDA monoclonal antibody based enzyme-linked immunosorbent assays (ELISAs) for the quantitation of antigen in bacterins containing *Leptospira* serogroups *canicola*, *icterohaemorrhagiae*, *pomona*, and *grippotyphosa*. The monoclonal antibodies used in the assay a) recognize lipopolysaccharide-like epitopes on the surface of the whole cell, b) agglutinate the homologous leptospiral serovars but do not agglutinate heterologous leptospiral serovars or heterologous bacterial species, and c) passively protect hamsters against a homologous challenge but fail to protect hamsters against heterologous challenges. Once developed, the performance of each ELISA was evaluated at the USDA followed by industry evaluation. Serials that passed the hamster vaccination-challenge assay yielded ELISA relative potency values of 1.0 or greater. These ELISAs have been shown to be a reproducible, sensitive, specific, and inexpensive alternative to the current Codified hamster potency assay.

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1. Introduction

Leptospirosis is a global disease affecting both animals and man. *Leptospira (L.) interrogans* serogroups *canicola*, *icterohaemorrhagiae*, and *pomona*, and *Leptospira kirshneri* serogroup *grippotyphosa* cause disease in dogs, swine, and cattle in the United States (U.S.). While the manifestations of the disease vary in these animals, vaccination is the best preventive measure to control infection and, thereby, potential transmission to human beings.

The Codified potency test for leptospiral bacterins licensed by the U.S. Department of Agriculture (USDA) is a hamster potency assay in which hamsters are vaccinated with a specified dilution of the bacterin followed by inoculation with a virulent challenge approximately two weeks post-vaccination [1–4]. Additional hamsters are used as nonvaccinated controls and to perform back-titrations for verification that an appropriately virulent challenge was administered. A satisfactory bacterin must protect at least 80% of the vaccinated hamsters while at least 80% of the nonvaccinated controls must succumb to the challenge. Since one satisfactory potency test must be conducted for each serovar present in a bacterin, a minimum of 160 hamsters is often needed to test a single multivalent serial prior to release if the Codified requirements are followed.

The hamster potency assays were first Codified in 1974. However, increasing cost associated with the use of large numbers of hamsters and increasing concerns for animal welfare and human health to conduct the vaccination–challenge assay stimulated the development of *in vitro* assays during the ensuing decades. Assays measuring relative potencies of serials compared to qualified references were considered viable alternatives to the Codified requirements.

The production of sensitive and specific monoclonal antibodies (MAbs) directed against protective antigens of serovars commonly used in the Codified hamster vaccination–challenge assay was necessary for developing the *in vitro* assays. By the late 1980s and early 1990s, MAbs had already been produced by several laboratories in an attempt to characterize the leptospiral antigens that confer protection in the host [5–17]. Between 1989 and 1996, the

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USDA generated MAbs against hamster-virulent challenge organisms of serogroups *canicola*, *icterohaemorrhagiae*, *pomona*, and *grippotyphosa* using a combination of Triton X-100 detergent extracts and inactivated whole cells of the respective serogroups. Once the appropriate MAbs were generated, enzyme-linked immunosorbent assay (ELISA) parameters were established, and randomly selected licensed bacterins were evaluated with the assay. In the mid to late 1990s, veterinary biologics manufacturers were asked to participate in the ELISA validation by performing the assay in their own laboratories and to provide feedback to the USDA. The assays were fine-tuned in the early 2000s based on industry feedback and internal testing to standardize ELISA formats at the Center for Veterinary Biologics (CVB).

The assay for measuring Relative Potency (RP) in bacterins has previously been described in detail [18,19], but the work will be briefly reviewed here for clarity to the reader. Previously unpublished data on the preliminary chemical characterization of the epitopes recognized by these MAbs and evaluation of the respective ELISAs since their initial development will be presented in greater depth.

2. Materials and methods

2.1. MAb and antiserum production

MAb and antiserum development for each serovar is identical to the procedures previously described [18,19]. Briefly, for the antiserum production, challenge strains of *Leptospira* were inoculated intravenously (IV) into rabbits with 1.0, 2.0, 4.0, and 5.0 mL of the P80-BA semisolid culture. The first three inoculations were given at five-day intervals and the final inoculation was given seven days after the third inoculation. Blood was obtained by cardiac puncture on the tenth day, and the serum harvested, filtered, and stored at −70 °C in one mL aliquots.

For the MAb production, Triton X-100 supernatants containing outer envelope membranes of hamster virulent challenge strains for each of the respective serogroups were harvested via centrifugation after overnight incubation in tris-tricine, 5 mM ethylenediaminetetraacetic acid solution (pH 8.6) containing 0.01% Triton X-100. BALB/c mice were immunized three or more times intraperitoneally (IP) every 7–10 days for approximately four weeks with the respective supernatants, inactivated whole cells of each serogroup, or antigen extracts for heterologous bacterial organisms. A final injection was always administered IV. In some instances, mice were immunized with both Triton-X-100 extracts and heat inactivated whole cells at various stages of the growth curve.

The myeloma cells from sensitized mice were fused with murine spleen cells to form hybridomas, and positive clones were selected based on (a) high ELISA optical density values with the homologous antigen and no cross reactivity with heterologous antigens, and (b) western blot analysis. The selected clones were used to prepare ascites fluids, which were screened by ELISA, western blot analysis, and passive protection studies in hamsters.

2.2. Electrophoresis and western blotting

Triton X-100 extracted antigens and inactivated whole cells of the respective leptospiral serogroups were separated in homogenous gels with a 14% [wt/vol] polyacrylamide gel containing 0.1% SDS. In specific experiments, samples were digested with either proteinase K (100 μg/mL) for 20 h at 60 °C or 200 mM sodium *m*-periodate for 48 h at 4 °C prior to electrophoresis. Gels were either stained for lipopolysaccharide (LPS) with a modified silver nitrate stain [20], for total protein using Coomassie Brilliant Blue, or subjected to western blotting. Ascites containing each respective MAb was diluted to approximately 1:100 in phosphate buffered saline containing 0.05% Tween (PBST) prior to immunoblotting. Peroxidase labeled goat anti-mouse immunoglobin specific for the isotype of each MAb was used to detect bound immunoglobulin after addition of the 4-chloronapthol substrate.

2.3. Passive protection

For each passive protection study, ten hamsters were injected IP with 0.5 mL of undiluted ascites fluid for the respective serogroup. Ten hamsters (control group) received an equal volume of sterile ascites hybridoma fluid or ascites fluid prepared against the dermonecrotic toxin of *Pasteurella multocida* Type D. Hamsters were challenged 24 h after vaccination with 0.25 mL of infected hamster liver homogenate (10–10,000 LD_{50}). A separate group of hamsters served to calculate the lethal dose for 50% of the hamsters (LD_{50}). The number of survivors was determined for all groups.

2.4. MAT

Microscopic agglutination tests (MATs) were performed according to standard protocols [21]. Briefly, serial twofold dilutions of the respective ascites fluids and homologous serovar specific antisera were prepared and incubated with viable cultures (with nephelometer readings of 20–30) of representative serovars. Viable whole cells of each serovar were used as the antigen, and the titer was expressed as the reciprocal dilution at which 50% of the leptospires were agglutinated. Heterologous bacterial species tested included *Escherichia coli, Salmonellae, Campylobacter, Bordetella, Erysipelothrix, Pasturella, Staphylococcus, Streptococcus*, and *Rhodococcus* species (Tables 1–4). Ascites and serovar specific antisera were considered positive if the titer was 1:1000 or greater.

2.5. ELISA

The optimal dilution of each MAb and associated antisera for use in their respective ELISA was determined through a checkerboard titration. Regarding the recognition of antigen in bacterins, initial protocols for serogroups *pomona*, *canicola*, *grippotyphosa*, and *icterohaemorrhagiae* ELISA included an antigen elution (removal of antigen from adjuvant) step prior to assaying products since a large number of licensed products did not perform well in the ELISAs without this step. Most licensed products did not require an antigen elution step for the *pomona* ELISA. Current protocol details are provided in Supplemental Assay Methods (SAMs) 624–627 supplied by CVB [22–25]. In brief, the capture antisera were diluted in 0.05 M carbonate buffer, pH 9.6 prior to overnight incubation at 4 °C. After washing, antigen diluted in PBST pH 7.2 was added to the plate, and then detected with the respective MAb diluted in PBST pH 7.2 supplemented with 0.1% polyvinyl alcohol (PVA) and 1% normal rabbit serum. Horseradish peroxidase-conjugated goat anti-mouse (alpha) conjugate and 2,2′-azino-bis(3-ethylbenzothia zoline sulfonic acid) were used as the antibody conjugate and substrate, respectively.

Bacterins used in the ELISAs were licensed commercially available, adjuvanted bacterins (monovalent and multivalent) from the USDA's repository. Reference bacterins were produced by commercial laboratories prior to 2000 for the USDA. These reference bacterins (RBs) were used in the development and initial evaluation of the ELISAs in the 1990s. The protective dose of the RBs was titrated by limiting dilution in hamsters for the initial assay evaluation.

Four additional reference bacterins (one per serogroup) were produced by veterinary biological manufacturers between 2004 and 2006 to be used as National Reference Standards (NRSs) and