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Enzyme-Linked Immunosorbent Assay for Determining Specific Immunoglobulin M in Infections Caused by *Leptospira interrogans* serovar *hardjo*

ANDREW R. MILNER,* KIMBERLY B. JACKSON, KEITH WOODRUFF, AND IAN J. SMART

Victorian Department of Agriculture, Veterinary Research Institute, Parkville, Victoria 3052, Australia

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An automated enzyme-linked immunosorbent assay detecting specific immunoglobulin M in infections with *Leptospira interrogans* serovar *hardjo* was evaluated on 69 patients. The test was sensitive and simple to perform, requiring a single dilution of test serum, with data expressed as units of antibody activity interpolated from a reference serum pool.

Confirmation of clinical diagnosis of leptospirosis often relies on laboratory testing because, in man, the clinical signs are not pathognomonic (5). Although culture of blood or cerebrospinal fluid can give a confirmatory diagnosis, the organisms grow slowly in vitro, often requiring weeks for detection and identification. Serological diagnosis is more rapid and is currently based on the demonstration of a seroconversion or rising antibody titer in sequential samples in the microscopic agglutination test (MAT). Although effective, the MAT has a number of drawbacks including the requirement for a continuous supply of live organisms for antigen, high specificity for individual serogroups, and subjectivity in the reading (12). To circumvent these problems, Adler et al. (1) and Terpstra et al. (11) reported on the use of the enzyme-linked immunosorbent assay (ELISA) as a diagnostic test for leptospirosis. Terpstra et al. (11) used a genus-specific test with no specificity for antibody class and concluded that the ELISA was sensitive and relatively easy to perform. Adler et al. (1) in a study of 15 patients, of whom 6 were infected with *Leptospira interrogans* serovar *hardjo*, indicated that immunoglobulin M (IgM) was the predominant class of antileptospiral antibody produced in acute leptospirosis and that the detection of specific IgM by ELISA appeared to be suitable as a diagnostic test. The present communication describes a large-scale comparison of an anti-IgM ELISA with the MAT for the detection of anti-*hardjo* antibodies. In southeastern Australia, serovar *hardjo* is the major cause of leptospirosis in both humans and cattle (8, 10). The ELISA technique is simple and economical to perform, requiring only one dilution of test serum with data expressed as units of antibody activity interpolated from a reference curve (7).

MATERIALS AND METHODS

Patient samples. A total of 138 serum samples from 69 individuals were tested retrospectively. Acute and convalescent samples from each patient were submitted to the Veterinary Research Institute from hospitals and medical practitioners throughout Victoria for confirmation of diagnosis of acute leptospirosis.

Agglutination test. The MAT (3) used viable *hardjo* antigen grown in Johnson and Harris-modified Ellinghausen and McCullough medium (EMJH) for 2 to 3 days at 30°C until turbid and then diluted to approximately 2×10^8 bacteria per ml in phosphate-buffered saline (PBS; pH 7.2). Sera were titrated in twofold dilutions from 1:64 to 1:8,192, with the endpoint defined as the dilution at which 50% of the leptospires present were agglutinated. Sera which did not react at a dilution of 1:64 were considered negative. The specificity of the serological reaction was assessed with *L. interrogans* serovar *pomona* antigen grown in the same way.

ELISA. ELISA antigens were grown in EMJH medium for 2 to 3 days at 30°C, washed three times in PBS, and sonicated twice for 10 s. Antigens have proved stable for at least 4 months at 4°C without loss of titer.

The appropriate concentration of antigen used in the ELISA was determined by checkerboard titration to be the amount prepared from 10^5 organisms per 50 μl. Flat-bottomed microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 50 μl of antigen per well in 0.1 M sodium carbonate buffer (pH 9.5). The wells were washed three times with PBS containing 0.1% Tween 20 (Sigma Chemical Co., St. Louis. Mo.) (PBS-Tween), and 50 μl of the appropriate serum dilution in ELISA buffer (0.1 M Tris hydrochloride [pH 8.0], 0.5 M NaCl, 2×10^{-3} M EDTA, 0.05% Tween 20, 5×10^{-5} M thimerosal, 0.2% bovine serum albumin) was added to duplicate wells. Incubation was for 60 min at room temperature. A series of six duplicate dilutions of a reference positive serum pool was also added to each plate. After the wells were washed three times in PBS-Tween, 50 μl of affinity-purified goat anti-human μ-chain conjugated to horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) was added to each well and incubated for 60 min at room temperature. The appropriate dilution of the conjugate was determined by checkerboard titration against the antigen. Generally, the conjugate was diluted 1/400 for use. After the wells were washed three times in PBS-Tween, 100 μl of 5-aminohydroxybenzoic acid (E. Merck AG, Munich, Federal Republic of Germany), purified by the method of Ellens and Gielkens (4) with hydrogen peroxide at a final concentration of 6×10^{-3}%, was added to each well. After 60 min, optical density at 492 nm was measured with an automated Titertek Multiskan spectrophotometer.

The absorbance of the highest concentration of the reference serum pool was specified as 100% bound, and the absorbance of each sample was expressed as a percentage of this value. Generally, the absorbance of the reference pool was approximately 1 absorbance U. A reference curve, plotting the percentage bound in each reference sample

* Corresponding author.

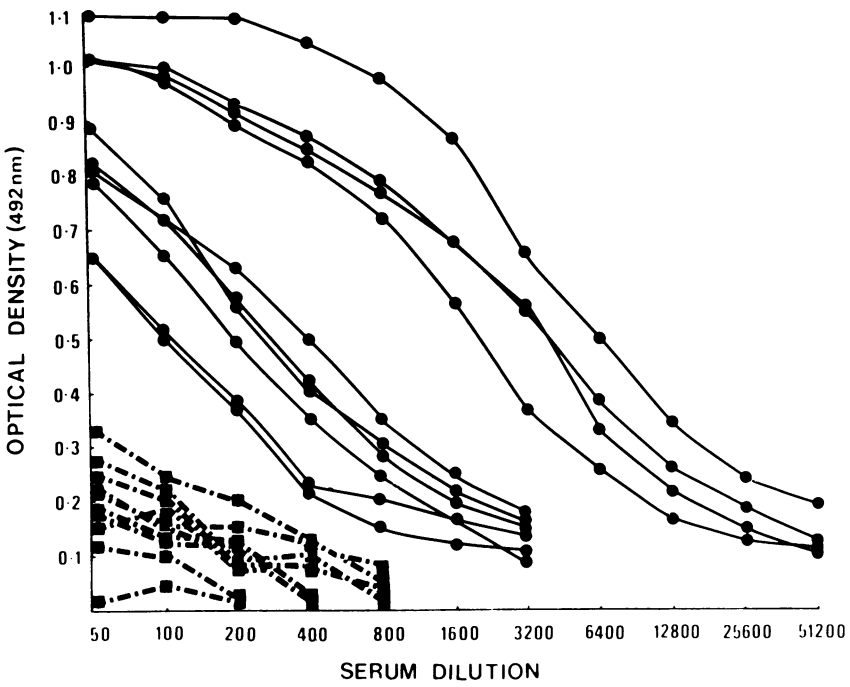
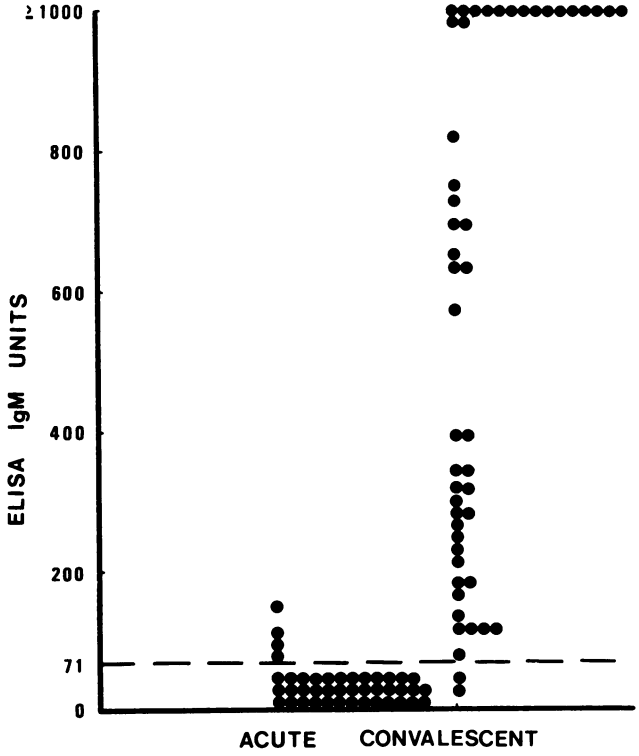


FIG. 1. Titrations of anti-*hardjo* IgM in sera from two groups of 10 individuals with (●) or without (■) MAT evidence of recent *hardjo* infection.

against the logarithm of its antibody content, was constructed, with the 100%-bound sample defined as containing 1,000 antibody U. The antibody activity of each test sample was read off this curve.



RESULTS

The characteristics of the ELISA for the detection of anti-*hardjo* antibody activity were determined by comparing titrations of convalescent serum samples from 10 patients who had seroconverted to serovar *hardjo* in the MAT with those of 10 patients who had no evidence of MAT reactivity (Fig. 1). The shape of the dose-response curves differed markedly between the two groups. By inspection, testing at a single dilution of 1:100 appeared to be adequate for discrimination between reactors and nonreactors. Quantitation of the amount of antibody present was obtained by comparison with dilutions of the reference serum pool which was made up of the four sera with the strongest responses (Fig. 1).

The between- and within-test reproducibility of the ELISA was evaluated by using sera with midrange antibody activity (231 U) tested daily for 8 days. The coefficient of variation, calculated by the method of Rodbard (9), was 20.9% between assays and 15.8% within assays.

The ability of the ELISA to detect recent serovar *hardjo* infection was compared with that of the MAT by using acute and convalescent serum samples from two groups of patients. The first group, 19 patients with no evidence of MAT antibody in either serum, were considered not to have leptospirosis and were thus used to obtain the level of background reactivity in the ELISA. On the basis of these results (data not shown), at a serum dilution of 1/100, 71

FIG. 2. Comparison of ELISA anti-*hardjo* IgM activity in acute and convalescent serum samples from 50 patients who seroconverted in the MAT. A titer of 71 antibody U at a serum dilution of 1/100 was used to discriminate between positive and negative sera. Eight acute sera were unavailable for ELISA testing.

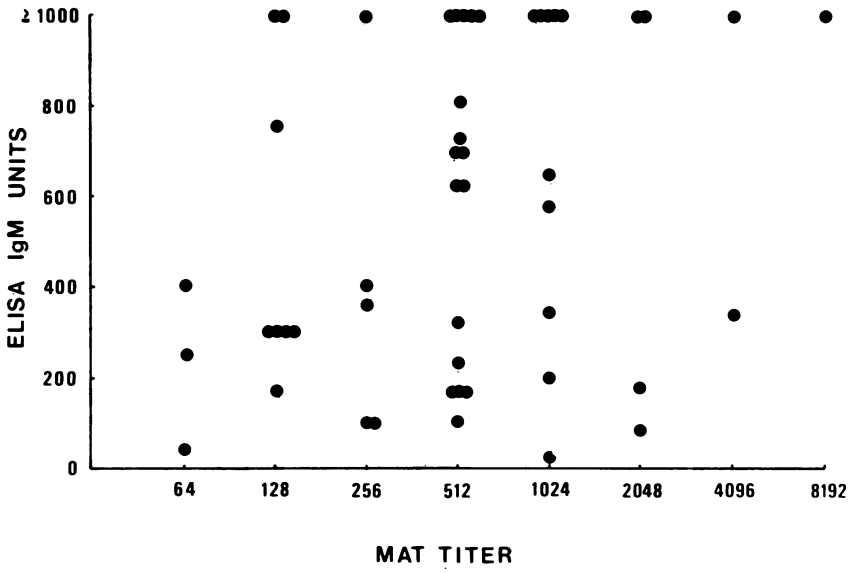


FIG. 3. Comparison of anti-*hardjo* antibody activity in the ELISA and MAT performed on convalescent sera from 50 patients with serological evidence of recent *hardjo* infection.

antibody U was chosen as a discrimination value between infected and noninfected patients. This discrimination value is not expected to be exceeded in 92% of all noninfected individuals, as determined by one-sided, distribution-free tolerance limits set at a 95% tolerance probability (6).

The second group, 50 patients, all had serological evidence of active serovar *hardjo* infection, having seroconverted to *hardjo* in the MAT. Only 42 acute sera were available for ELISA testing. Of these, 38 (90%) contained fewer than 71 antibody U of anti-*hardjo* IgM (Fig. 2). Convalescent sera from all 50 patients were tested, of which 48 (96%) had more than 71 antibody U. A total of 95% of all comparisons agreed. No significant correlation at the 5% level was observed between ELISA IgM antibody units and the MAT titer (Spearman's rank coefficient of correlation, $r = 0.18$) (Fig. 3). When the 50 convalescent sera were tested against serovar *pomona* antigen, all were negative in the MAT, but 4 had ELISA titers >71 U (99, 175, 213, and 541 U). All of these four sera had titers >1,000 U when tested against *hardjo* antigen.

DISCUSSION

In this study, the diagnostic potential of the ELISA was compared with that of the MAT. Seroconversions, or fourfold changes in the MAT, which are indicative of recent infection (1, 8, 10), were almost invariably (96%) associated with elevations in antibody activity against serovar *hardjo* as measured by the ELISA. Furthermore, there was close agreement between these tests on the classification of sera as positive or negative. It appears, however, that the two tests were influenced by different antibody populations (Fig. 3), presumably because the antigens responsible for the agglutination reaction may be only a small subset of the total detected by the ELISA (1). This is consistent with the observation that the ELISA was capable of detecting antibody activity in 10% of acute-phase sera which were negative by the MAT.

A problem associated with the interpretation of serological diagnostic tests for leptospirosis is the persistence of the antibody response in convalescent patients. With the MAT, Blackmore et al. (2) detected antibody up to 20 years after infection, with a marked variation occurring in the maximum responses between individuals. They indicated that it is therefore not usually possible from a single MAT result to estimate retrospectively the time at which infection occurred. With the ELISA, we also found that antibody activity persists in serum usually at levels of <200 U for at least 9 to 12 months after seroconversion (data not shown). Nevertheless, the observation that 34% of patients who seroconverted to serovar *hardjo* had ELISA titers of ≥1,000 U (Fig. 3) indicates a strong initial antibody response to infection and suggests that this magnitude of response in a single serum sample may be indicative of recent infection.

In conclusion, this ELISA test for the detection of anti-*hardjo* IgM proved sensitive, reproducible, and easy to perform. The use of a single test serum dilution to discriminate between the presence and absence of an antibody response enabled the testing of 36 duplicate test samples, a 6-point reference curve, and known positive and negative standards on each microplate. Results can be read visually or automatically and can easily be analyzed by computer. The ELISA is especially suitable for laboratories that do not have facilities for continuous culture of leptospires, because the antigen is stable for at least 4 months at 4°C.

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