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Evaluation of a Commercial Enzyme-Linked Immunosorbent Assay for Detection of Immunoglobulin M Antibody in Diagnosis of Human Leptospiral Infection

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The PanBio Leptospira immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) is a commercially available screening test for the diagnosis of acute leptospiral infection. The ability of the test to diagnose early or recent *Leptospira interrogans* infection was assessed by testing sera with known microagglutination test (MAT) titers to serovars pomona, hardjo, copenhageni, and australis. The IgM ELISA detected all 41 cases of early or recent leptospiral infection (sensitivity, 100%), with a positive ELISA result seen in many cases before MAT antibody titers reached 1:50. Thirty-eight of 41 patients showed seroconversion (fourfold or greater increase in titer by MAT, 2 of 41 patients had a single sample with elevated titer, and 1 patient from whom leptospires were isolated from a blood sample failed to show MAT titers, despite a seroconversion (negative to positive result) in the ELISA. Follow-up sera obtained from 8 of 12 patients (67%) for 3 to 48 months after the acute stage of illness showed persisting IgM antibody. However, the range of levels detected in these samples (maximum ELISA ratio, 2.0) was lower than the range seen when infection was recent. Reactivity in the IgM ELISA was observed for only 1 of 59 serum samples from asymptomatic donors (specificity, 98%) and 16 of 233 serum samples from patients with Ross River virus, brucella, Epstein-Barr virus, cytomegalovirus, mycoplasma, Q-fever, toxoplasma, hepatitis A virus, *Treponema pallidum*, or *Borrelia burgdorferi* infection (specificity, 93%), with the majority of these patients showing lower levels of IgM in comparison to those in patients with leptospiral infection. We conclude that this ELISA is sufficiently sensitive for use as an initial screen for leptospiral infections, with subsequent confirmation of positive test results by MAT.

Diagnosis of human leptospiral infection relies on either isolation of the causative organism from body fluids or demonstration of a rise in specific serum antibodies. Isolation is difficult and not always successful, and the detection of leptospires in body fluids by dark-field microscopy is limited due to proteinaceous filaments (pseudoleptospires) that can be present (8). The microscopic agglutination test (MAT) is the reference test for diagnosis and detects antibodies at serovar levels (4). However, the test is time-consuming, requires a specialized operator for interpretation, can show low sensitivity and thereby necessitate the use of paired sera, involves the use of a battery of leptospires belonging to different serovars, requires the maintenance of stock cultures, and uses live organisms, creating a risk of laboratory-acquired infection (20). An immunoglobulin M (IgM) enzyme-linked immunosorbent assay (ELISA) is often used as an alternative to MAT in routine diagnostic laboratories (4, 6, 14, 20). As well as being easier to perform, ELISA can easily accommodate a large number of samples and gives a less subjective result than MAT. In this study we compared the performance of a rapid (60-min) commercially available ELISA for the detection of leptospira-specific IgM (PanBio, Brisbane, Queensland, Australia) with MAT. Our study indicates that this ELISA is a suitable screening test for the determination of leptospira IgM antibodies in the routine clinical laboratory.

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MATERIALS AND METHODS

Patients and serum samples. Most blood samples used in this study were referred to the Institute of Medical and Veterinary Science, in Adelaide, South Australia, for leptospiral serology from either general practitioners or hospitals in South Australia; those from patients 35 to 40, however, were referred to the Institute of Medical and Veterinary Science from Lautoka, Fiji. Of the patients seen in South Australia, most had presumed occupational exposures to leptospires because they were meat workers, dairy farmers, or stockmen; exposures for patients 34 and 41, however, were recreational and occurred outside South Australia.

Blood was collected by venipuncture and was allowed to clot. Samples were centrifuged, and the serum was collected, aliquoted, and stored at −20°C until it was assayed. Serial serum samples ($n = 117$) collected for up to 1,460 days after the onset of infection from 41 patients with *Leptospira interrogans* infection (15 patients infected with serovar hardjo, 18 infected with serovar pomona, 7 infected with serovar copenhageni, and 1 infected with serovar australis), diagnosed on the basis of the MAT and/or culture, were tested by the ELISA. In addition, 59 serum samples donated by healthy individuals and 233 serum samples taken from patients in the acute phase of Ross River virus (RRV), brucella, Epstein-Barr virus (EBV), cytomegalovirus (CMV), chlamydia, mycoplasma, Q-fever, toxoplasma, hepatitis A virus, *Treponema pallidum*, or *Borrelia burgdorferi* infection were also tested by the ELISA to evaluate the specificity of the test.

ELISA. In the Leptospira IgM ELISA (PanBio) serum was diluted 1/100 in the diluent provided with the ELISA kit before transfer to leptospira antigen-coated microwell strips for 20 min at room temperature (100 μl/well). After washing with phosphate-buffered saline containing 0.05% Tween 20, bound IgM was detected via a 20-min incubation with anti-human IgM peroxidase (100 μl/well) and, after washing again, a 10-min incubation with tetramethylbenzidine substrate (100 μl/well). The reaction was stopped by the addition of 100 μl of 1 M phosphoric acid per well, and the strips were read at 450 nm with a microtiter plate reader. Positivity was determined by comparison to a reference serum sample (cutoff calibrator). A positive sample was defined as having a sample: calibrator absorbance ratio of ≥1.0, and a negative sample was defined as having a sample:calibrator absorbance ratio of <1.0.

MAT. MAT was performed as described previously (5, 7), with the following serovars of *L. interrogans* used as antigen: hardjo, pomona, tarassovi, and copenhageni. Serial twofold dilutions of the sera were made in 0.01 M phosphate-buffered saline (pH 7.2) starting from 1:50. MAT was performed by incubating

TABLE 1. Comparison of MAT and ELISA for patients with serovar hardjo infections

Patient no.	Post-onset interval (days)[a]	ELISA ratio	MAT titer
1	9	3.6	400
	16	5.7	1,600
	450	1.4	<50
2	7	1.4	<50
	21	4.5	1,600
	52	3.2	800
	90	2.3	400
	240	1.0	<50
	630	0.7	50
3	3	0.1	<50
	14	4.4	6,400
	1,186	1.0	200
4	3	0.3	<50
	6	1.2	<50
	14	7.7	1,600
	22	8.6	1,600
	510	1.5	100
5	12	0.6	<50
	15	2.6	100
	28	3.0	400
6	5	0.1	<50
	18	6.7	400
7	3	1.6	100
	10	2.9	1,600
8	1	0.1	<50
	14	5.1	400
9	0	NT[b]	<50
	11	4.0	800
10	0	0.3	<50
	14	4.0	800
	68	2.9	800
11	0	NT	<50
	14	NT	800
	210	2.4	100
	222	2.3	100
12	7	4.4	800
	15	6.2	3,200
13	4	0.1	<50
	15	3.0	3,200
14	4	0.2	<50
	18	4.2	3,200
15	2	0.7	<50
	17	4.9	3,200

[a] Post onset interval represents days after the onset of illness.
[b] NT, not tested.

the sera at 37°C for 90 min with suspensions of live leptospires (1×10^8 to 2×10^8 per ml) of each strain. The titer was the highest dilution which gave 50% agglutination. Leptospira infection was diagnosed by a fourfold increase in MAT titer and/or isolation of the organism except in two patients (patients 23 and 29), for whom diagnosis was based on clinical presentation and elevation of the MAT titer (titer, 400) in one serum sample.

TABLE 2. Comparison of MAT and ELISA for patients with serovar pomona infections

Patient no.	Postonset interval (days)[a]	ELISA ratio	MAT titer
16	10	0.7	<50
	11	6.4	6,400
	25	4.7	3,200
17[b]	5	0.5	<50
	11	6.2	100
	17	7.1	800
18	9	7.1	<50
	20	6.2	800
	35	6.0	800
	210	0.9	100
19	5	2.2	<50
	17	5.2	12,800
	1,186	1.2	1,600
	1,278	1.3	1,600
20	1	0.2	<50
	8	0.2	<50
	11	6.4	3,200
	1,460	0.9	50
21	8	2.7	<50
	12	4.5	800
	24	2.9	800
	90	0.5	200
22	5	5.0	<50
	7	4.9	<50
	15	6.6	800
23	7	5.7	400
24	NS[c]	0.2	<50
	+20	2.0	3,200
	+210	1.1	100
25	NS	3.1	<50
	+16	3.4	400
	+29	2.7	400
26	5	0.8	<50
	22	3.8	3,200
27	8	NT[d]	200
	16	5.0	1,600
28[b]	2	0.1	<50
	9	3.4	<50
	100	1.0	<50
	189	0.8	<50
29	30	5.1	400
30	5	1.2	<50
	15	5.1	6,400
31[b]	15	3.1	200
	31	2.9	800
32	6	0.5	<50
	19	2.6	1,600
33	9	3.9	100
	19	3.1	800

[a] Postonset interval represents days after the onset of illness except for patients 24 and 25, for whom the interval represents the days since the first serum sample was collected (designated with the plus signs).
[b] Leptospires were isolated from the blood of patients 17, 28, and 31.
[c] NS, not specified.
[d] NT, not tested.

TABLE 3. Comparison of MAT and ELISA for patients with serovar copenhageni or australis infections

Patient no.[a]	Postonset interval (days)[b]	ELISA ratio	MAT titer
34[c]	9	NT[d]	<50
	10	NT	<50
	13	3.6	800
	16	3.5	800
	19	3.3	800
	20	3.2	800
	22	2.8	800
	25	2.8	800
	26	2.9	800
	28	2.2	800
	30	1.8	800
	32	1.6	800
35	NS[e]	3.3	400
	+10	4.8	6,400
36	NS	3.0	400
	+20	3.6	1,600
37	NS	4.4	50
	+20	4.7	400
38	NS	5.3	<50
	+6	6.9	1,600
39	NS	4.0	200
	+10	3.7	1,600
40	NS	6.0	<50
	+4	6.5	400
41	9	2.0	100
	15	2.7	3,200
	27	3.2	6,400

[a] Patients 34 to 40 were infected with serovar copenhageni; patient 41 was infected with serovar australis.
[b] Postonset interval represents days after the onset of illness except for patients 35 to 40, for whom the interval represents the days since the first serum sample was collected (designated with plus signs).
[c] Leptospires were isolated from the blood of patient 34.
[d] NT, not tested.
[e] NS, not specified.

Leptospira isolation. Leptospires were isolated from whole-blood samples collected asceptically by venipuncture. Briefly, one to five drops (25 μl/drop) were inoculated into each of five tubes containing 5 ml of Ellinghausen-McCullough-Johnson-Harris liquid medium (Difco Laboratories, Detroit Mich.). The tubes were incubated at 30°C for up to 6 weeks. They were examined for spirochetal growth once per week by placing 25 μl on a slide, placing a coverslip on the slide, and then reading the slide by dark-field illumination microscopy at ×400 magnification. If growth was detected, cultures were sent to the World Health Organization (WHO)/Food and Agriculture Organization (FAO) Collaborating Centre for Reference and Research on Leptospirosis, Brisbane, Queensland, Australia, for identification.

Data analysis. Clinical data were correlated with serum antibody levels. The proportion of patients with levels above the designated cutoff for ELISA and MAT were determined. Analysis of variance (ANOVA) and the Tukey-Kramer multiple comparison test were used to compare the mean ELISA ratios for different MAT titers and different times after the onset of infection. Statistics were performed by using Instat (Graphpad Software Inc., San Diego, Calif.).

RESULTS

Comparison of MAT and ELISA. The ELISA was compared with MAT by using serial serum specimens collected from patients with confirmed *L. interrogans* infection caused by serovars hardjo (Table 1), pomona (Table 2), and copenhageni or australis (Table 3). Elevation of the IgM titer was observed in all 41 patients by ELISA (sensitivity, 100%), with positive ELISA ratios (≥1.0) detected as early as 3 days after the clinical onset of infection. Increases in MAT titer were observed in all but one patient, from whom serovar pomona was isolated. Despite a trend toward an increasing ELISA ratio with increasing MAT titer (Fig. 1), a positive ELISA ratio was detected in 12 patients before MAT antibody titers were detected.

It was of interest that follow-up sera obtained from 8 of 12 (67%) patients for 2 to 48 months after the acute phase of

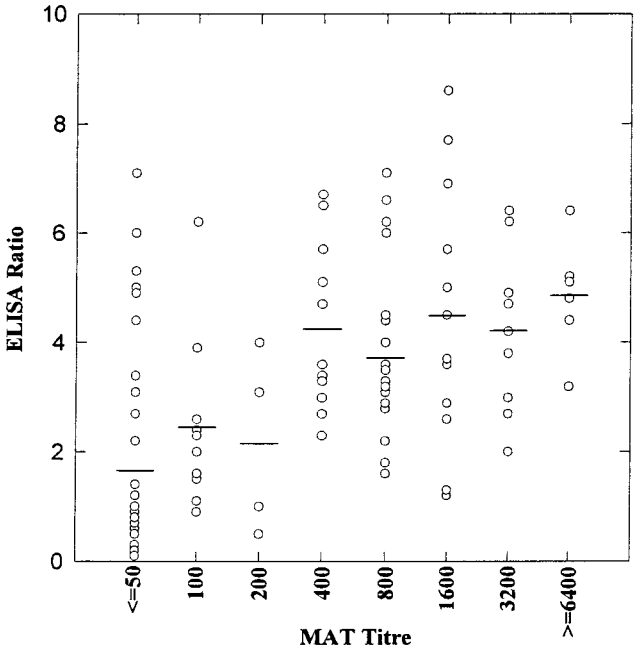

FIG. 1. Comparison of ELISA ratio and MAT titer. The mean ELISA ratio is represented by the bold line. There was a significant variation among the mean ELISA ratios with the MAT titer (one-way ANOVA, $P < 0.0001$). In addition, the Tukey-Kramer multiple comparison test revealed significant differences between the mean ELISA ratio corresponding to a MAT titer of ≤50 and those corresponding to MAT titers greater than or equal to 400 ($P < 0.01$).

TABLE 4. Relationship between ELISA reactivity and days after onset of infection

Time interval (days)	No. of patients	ELISA ratio[a]		No. of samples with ratio ≥1.0/total no. of sample (%)
		Mean	SD	
0–4	10	0.4	0.5	1/10 (10)
5–8	14	2.2	2.0	9/14 (64)
9–12	13	4.0	2.1	11/13 (85)
13–16	15	4.6	1.5	15/15 (100)
17–20	10	4.7	1.6	10/10 (100)
21–27	9	4.0	1.9	9/9 (100)
28–35	7	3.2	1.7	7/7 (100)
36–100	5	2.0	1.2	4/5 (80)
101–450	6	1.5	0.7	4/6 (67)
451–1,460	6	1.1	0.3	4/6 (67)

[a] There was a significant variation between mean ELISA ratios (one-way ANOVA, $P < 0.0001$). In addition, the Tukey-Kramer multiple comparison test revealed significant differences between the mean ELISA ratio at 1 to 4 days compared with those at 9 to 12 days ($P < 0.0001$), 13 to 16 days ($P < 0.0001$), 17 to 20 days ($P < 0.0001$), 21 to 27 days ($P < 0.0001$), and 28 to 35 days ($P < 0.05$), but no difference between the mean ELISA ratio at 1 to 4 days and those at 5 to 8 days or intervals greater than 36 days ($P > 0.05$).

TABLE 5. Reactivity of leptospira IgM ELISA with sera from patients with other diseases and asymptomatic donors

Disease	No. of samples with elevated ratios/total no. of samples (%)	ELISA ratios in positive samples
RRV	3/41 (7)	1.62, 2.75, 4.10
Brucella	3/14 (21)	1.15, 1.29, 1.35
EBV	2/32 (6)	1.07, 1.13
Q-fever	3/34 (9)	1.10, 1.23, 1.26
CMV	5/35 (14)	1.10, 1.26, 1.43, 2.90, 5.30
Chlamydia	0/5 (0)	
Mycoplasma	0/9 (0)	
Toxoplasma	0/12 (0)	
Hepatitis A virus	0/15 (0)	
Syphilis	0/16 (0)	
Lyme borrelioses	0/20 (0)	
Asymptomatic donors	1/59 (2)	1.99
Total	17/292 (6)	

illness showed persisting IgM antibody (Tables 1 to 3). However, the range of levels detected in these samples (maximum ELISA ratio, 2.0) was lower than the range seen when infections were recent (Table 4). Despite the low elevation of IgM titer from 36 days after the onset of infection (mean ratio, 2.0), there was no difference between this level and that found in the period just after infection (mean ratio, 2.2) (Table 4). Use of a higher ELISA cutoff ratio of 1.5 did not lead to a decrease in sensitivity, while the incidence of persistent IgM fell from 67 to 17%.

Specificity of ELISA. Positive ratios were observed for only 16 of 233 serum samples from patients with active RRV, brucella, EBV, CMV, chlamydia, mycoplasma, Q-fever, toxoplasma, hepatitis A virus, *T. pallidum*, or *B. burgdorferi* infection (specificity, 93%) (Table 5). Patients with brucella and CMV infection showed the highest levels of reactivity (21 and 14%, respectively). However, many of these patients showed lower levels of IgM in comparison to those in patients with confirmed recent leptospiral infections, and the use of a higher cutoff ratio of 1.5 increased the specificity from 93 to 98%, while the sensitivity was unchanged. Only 1 of 59 asymptomatic donors showed an elevation in the IgM titer by the ELISA (specificity, 98%).

DISCUSSION

A rapid, accurate method for the diagnosis of leptospirosis is important to both the clinician and the patient. The commercially available ELISA described in this report (the PanBio Leptospira IgM ELISA) is a suitable test for the detection of leptospiral IgM antibodies in a routine clinical laboratory. The ELISA showed excellent sensitivity (100%) and high specificity (93%) in the diagnosis of acute leptospiral infection, as reported in studies with a non-commercially available IgM ELISA (6, 15). Furthermore, the ELISA had a better sensitivity than the MAT and detected a number of cases of infection (29%) before MAT titers were detected. IgM was first detected as early as 3 days after the clinical onset of infection, and similar observations have been made in studies with other ELISAs (2, 13, 15, 18, 19). Cross-reactivity occurred mainly with sera from patients with CMV and brucella infections, while no sera from patients with nonleptospiral spirochetal infections (syphilis or Lyme borreliosis) showed cross-reactivity in the ELISA. From these studies it cannot be determined if the leptospiral IgM antibody detected in patients with nonleptospiral infections is cross-reacting antibody or persisting antibody from a previous leptospiral infection.

IgM antibodies persisted in the majority of patients (67%) from whom longer-term serum samples (≥3 months postinfection) were obtained. This persistence of IgM antibodies in patients with leptospirosis has been reported in other studies (1, 3, 11, 12, 15, 17), although the cause of the phenomenon is unknown. IgM levels were still elevated after 12 months in half the patients tested in one of those studies (15), and as observed in our study, IgM levels fell but remained slightly higher than the cutoff value long after infection (17). It was of interest that the use of a higher cutoff ratio of 1.5 did not lead to a decrease in sensitivity from 100%, while the incidence of persistent IgM fell from 67 to 17%, and the specificity with sera from patients with nonleptospiral infections increased from 93 to 98%.

In this study the IgM ELISA detected all cases of infection caused by *L. interrogans* serovars hardjo, pomona, copenhageni, and australis. The reactivity with serovar australis is of particular relevance, because this is an emerging serogroup in many parts of the world. In addition, the commercially available ELISA used in this study has also been shown to detect IgM antibodies to serovars madanensis, kremastos, nokolaevo, celledoni, canicola, grippotyphosa, szwajizak, djasiman, and tarassovi (9, 10, 16). Consequently, the commercially available ELISA described in this report will be a particularly valuable screening test in routine diagnostic laboratories that do not have the facilities or expertise to perform MAT, although confirmation of a diagnosis by MAT at a specialized reference laboratory would be recommended.

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