Diffusion in gel-enzyme-linked immunosorbent assay—a new serological test for leptospirosis

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SUMMARY A new serological test, diffusion in gel-enzyme-linked immunosorbent assay (DIG-ELISA) was developed and compared with the microscopic agglutination test (MAT) for the serological diagnosis of leptospirosis. The results suggest that DIG-ELISA is a viable alternative to the MAT because of its simplicity, sensitivity, versatility and potential for standardisation.

Diagnosis of leptospirosis in humans is based on clinical, cultural or serological parameters. However diagnosis based on clinical grounds alone is considered unreliable and thus should be confirmed by either cultural isolation and/or the demonstration of a rise in specific antibody titre between acute and convalescent sera.^{1,2} Cultural isolation may be attempted from the blood or occasionally cerebrospinal fluid (CSF) during the first week of the disease. After the first week of the disease blood cultures are rarely successful and so culture must be attempted from alkaline urine.¹⁻³ However not infrequently, because of prior antibiotic treatment, the length of time needed for culture and the suitability of specimens for culture in relation to the course of the infection, serology remains the principle method of diagnosis.

Of all current serological tests the microscopic agglutination test (MAT) is regarded as the reference test.³ The test is considered elaborate, expensive, time-consuming, thus remaining a specialised test of reference laboratories only.³ Furthermore the use of either living or formalised antigens and the subjectiveness in assessing the degree of agglutination can lead to considerable variation in antibody titres between laboratories using this test. As a result of variabilities in MAT titres between different laboratories becoming apparent in a survey of leptospirosis in New Zealand, the diffusion in gel-enzyme-linked immunoassay (DIG-ELISA) was investigated as an alternative test.^{4,5}

Material and methods

LEPTOSPIRAL SEROVARS

Leptospira interrogans serovars *hardjo* and *pomona* (the most prevalent serovars causing human disease in New Zealand)⁶ were obtained from the National Health Institute, Wellington, NZ. Strains of these serovars were cultured at 30°C in the dark in bovine serum albumin Tween-80 medium (Difco laboratories). Thiomersal-preserved strains of the same serovars were also kindly donated by Wellcome, NZ Ltd.

RABBIT ANTISERUM

Bivalent anti-*hardjo* and anti-*pomona* serum was produced in an 8 month New Zealand White female rabbit. The rabbit was bled for pre-immune serum and then sensitised with an intramuscular injection containing 10⁹ of thrice washed leptospiral cells of each serovar, emulsified in a 50:50 mixture of Freund's complete adjuvant (Difco laboratories). The rabbit was then rested for 21 days, and the immunity boosted with the same concentration of leptospires but this time emulsified in Freund's incomplete adjuvant. Thereafter the rabbit was immunised with 2 weekly 0.5 ml intravenous injections containing 5 × 10⁸ leptospires and then bled.

Monospecific anti-*hardjo* and anti-*pomona* sera were kindly donated by Mr S Flint, ICI-Tasman Ltd, Wellington, New Zealand. These were raised in New Zealand White rabbits by repeated intravenous injections at 5-7 day intervals using increasing

volumes (1 ml, 2 ml, 4 ml, 6 ml) from 4-6 day old cultures.

HUMAN SERA

These were obtained from patients displaying clinical symptoms of leptospirosis submitted by their medical practitioner.

MICROSCOPIC AGGLUTINATION TEST (MAT)

This was performed in microtitre trays as outlined in the Manual of Clinical Immunology.⁷

PEROXIDASE CONJUGATES

Antirabbit immunoglobulins (lot 040) and anti-human-IgM (lot 120) and -IgG (lot 080A) conjugated to horseradish peroxidase were purchased from Dako, Copenhagen, Denmark. The titre used for DIG-ELISA using antirabbit immunoglobulins was 1/1000 and for antihuman immunoglobulins 1/500.

DIFFUSION IN GEL-ENZYME-LINKED IMMUNOASSAY (DIG-ELISA)

This was performed according to Elwing and Nygren^{4,5} but with the following modifications. Standard bacteriological polystyrene Petri dishes (8.5 cm diameter) were coated with 20.0 ml of Wellcome antigen (consisting of 2.5×10^9 leptospires suspended in 0.85% NaCl) overnight at room temperature. The dishes were rinsed twice with distilled water and then coated with 20.0 ml of 0.5% bovine serum albumin (BSA, Sigma fraction V) for 30 min at 37°C. The dishes were washed twice with pH 7.2 phosphate-buffered saline (PBS) followed by coating with 20.0 ml of PBS containing 1% wt/vol Noble Agar (Difco laboratories) and 0.1% wt/vol BSA. When the agar had set, wells (3 mm diameter) were punched into the agar using a number 1 cork borer. The agar plug was removed by vacuum and 20 µl of test sera (diluted if necessary in PBS containing 0.5% wt/vol BSA and 0.05% vol/vol Tween 20 (Sigma)) added.

After overnight incubation at 37°C, the agar was removed, the dishes washed thrice with PBS and then flooded with 10.0 ml of PBS-BSA-Tween 20 diluted peroxidase conjugated antiserum. The dishes were then incubated at 37°C for 60 min, rinsed twice with distilled water and then coated with 15.0 ml of 1% wt/vol agarose (BDH) in pH 7.0 phosphate buffer (0.1M) containing 0.1% wt/vol of the substrate *p*-phenylenediamine dihydrochloride (BDH) and 0.01% vol/vol hydrogen peroxide (AJAX) and further incubated at 37°C for 15 min, after which the dark brown reaction zones were measured using engineering calipers (Mitutoyo, Japan).

Results

COMPARISON OF DIG-ELISA TITRES WITH MAT TITRES USING ANTIRABBIT SERA

Using a chequerboard titration of antigen versus conjugate concentration it was found that a final concentration of 2.5×10^9 leptospires and a 1/1000 dilution of peroxidase conjugate antirabbit immunoglobulins produced consistent well defined zones of reaction. A positive reaction was signified by the appearance of a dark brown zone, the diameter of which varied in size according to the antibody concentration of the serum. Those sera with high MAT titres had correspondingly larger zones of reaction as compared to sera with low MAT titres.

Table 1 compares the DIG-ELISA and MAT antibody titres for the bivalent and monovalent sera using threefold dilutions for DIG-ELISA and twofold dilutions for MAT. No direct relation between the titres of these two techniques was observed and this appeared to be related to the immunisation methods used in the production of the antisera. The bivalent antiserum exhibited a higher DIG-ELISA titre and vice versa for the monovalent sera. However the monovalent sera exhibited no serological cross-reactivity when using either homologous or heterologous antigens in the MAT. On the other hand the monovalent anti-*pomona* did cross react with both serovars at lower antibody titres when using DIG-ELISA. Preimmune rabbit serum was negative for both tests.

Table 1 Comparison of rabbit antileptospiral sera for differentiating *hardjo* and *pomona*

Antisera to serovars <i>hardjo</i> and <i>pomona</i>	Antigens (serovars)			
	<i>hardjo</i>		<i>pomona</i>	
	MAT	DIG-ELISA	MAT	DIG-ELISA
Bivalent anti- <i>hardjo</i> and anti- <i>pomona</i>	800	32 400	1600	32 400
Anti- <i>hardjo</i>	1600	3600	—	—
Anti- <i>pomona</i>	—	400	3200	1200

Sera were diluted threefold for DIG-ELISA and twofold for MAT from 1/400 to 1/32 400. The antibody titre for MAT is expressed as the reciprocal of the highest serum dilution that resulted in 50% agglutination of leptospires. The DIG-ELISA reaction zones varied from 13.2 mm (for the 1/400 dilution) to 4.0 mm for the 1/32 400 dilution.

MAT = microscopic agglutination test.

DIG-ELISA = diffusion in gel-enzyme-linked immunosorbent assay.

COMPARISON OF DIG-ELISA TITRES WITH MAT TITRES USING PATIENT SERA

The antibody titres of patients' serum measured by both MAT and DIG-ELISA are shown in Tables 2, 3 and 4. In all serum samples examined the DIG-

Table 2 Comparison of antibody titres in human sera as determined by MAT and DIG-ELISA tests with serovars *hardjo* and *pomona*

Human sera	Days between specimens	Serological titres					
		MAT		DIG-ELISA			
		<i>hardjo</i>	<i>pomona</i>	<i>hardjo</i>		<i>pomona</i>	
				IgM	IgG	IgM	IgG
A ₁	0	100	—	400	100	—	100
A ₂	14	200	—	1600	400	—	400
B ₁	0	800	—	6400	400	1600	400
B ₂	8	200	—	6400	1600	—	400
C ₁	0	200	—	1600	—	400	100
C ₂	19	100	—	1600	100	400	100
D ₁	0	400	—	6400	—	—	—
D ₂	11	200	—	1600	—	—	—
D ₃	20	200	—	1600	—	—	—
E ₁	0	400	—	6400	400	400	100
E ₂	18	400	—	6400	400	400	400
F ₁	0	—	—	100	—	—	—
F ₂	7	200	—	6400	400	400	—
G ₁	0	—	400	1600	100	6400	1600
G ₂	21	—	800	400	—	6400	1600
H ₁	0	—	400	—	—	6400	100
H ₂	18	—	200	400	—	6400	400
*I ₁	0	—	—	400	—	1600	—
I ₂	18	—	50	400	—	1600	—
I ₃	38	—	50	—	—	1600	400
†J ₁	0	—	—	—	—	—	—
J ₂	21	—	—	—	—	—	—

Sera were diluted fourfold for DIG-ELISA and twofold for MAT.

— = no detectable antibody.

*Positive culture obtained.

†J₁ and J₂ were negative control sera collected from a 9 month child.

The MAT titre is expressed as the reciprocal of the highest serum dilution that resulted in 50% agglutination of leptospirae. The DIG-ELISA titre is expressed as the reciprocal of the highest serum dilution that exhibited a zone of reaction greater than 4.0 mm.

Table 3 Comparison of the results of MAT and IgM-DIG-ELISA tests using undiluted human sera positive to serovar *hardjo*

MAT titre	No of sera	Mean zone diameter (mm) tested against serovars	
		<i>hardjo</i>	<i>pomona</i>
<25	10	4.6	4.1
25	2	10.1	9.5
50	4	11.4	10.8
100	6	12.3	12.1
200	7	13.1	11.8
400	4	13.5	11.5
800	2	14.0	11.2

Table 4 Comparison of the results of MAT and IgM-DIG-ELISA tests using undiluted human sera positive to serovar *pomona*

MAT titre	No of sera	Mean zone diameter (mm) tested against serovars	
		<i>hardjo</i>	<i>pomona</i>
<25	10	4.6	4.1
25	3	9.6	10.2
50	5	11.0	11.8
100	3	12.1	12.5
200	3	11.4	13.1
400	2	11.8	13.5
800	1	11.5	14.2

ELISA results reflected the MAT results with regard to the respective serovar titres. The DIG-ELISA titres were consistently greater than MAT titres, and this sensitivity allowed the early detection of two cases of leptospirosis (samples F₁ and I₁) which were initially negative by MAT. However this sensitivity was accompanied by extreme serological cross-reactivity at lower titres which only disappeared when titred out. Antibodies detected by DIG-ELISA were shown to belong predominantly to the IgM class. This observation was also confirmed by IgM indirect immunofluorescence and

raised IgM titres were observed to exist for as long as 38 days in sample I₃. IgG antibody titres were lower and generally not observed until later in the course of the disease.

Discussion

The quantification of antibodies by DIG-ELISA is based on the ability of antibodies to diffuse from wells in gel forming a diffusion gradient over an antigen coated polystyrene surface. The antigen-antibody complex is then made visual by means of a

colour reaction which is formed by an enzyme-conjugated anti-immunoglobulin reacting with its substrate-containing gel which is finally poured over the polystyrene surface.⁵ This technique was shown to detect and differentiate both *hardjo* and *pomona* antibodies in rabbit and human antisera. Whereas MAT detects antibodies to surface agglutinins only, DIG-ELISA detects antibodies to both surface and cytoplasmic antigens. Thus the different sensitivities of these two serological tests appeared to reflect the predominant class of immunoglobulin produced by whichever method of immunisation was used. It has been demonstrated previously that rabbit antiserum produced solely by intravenous inoculations of whole leptospires produced antibodies of mainly the IgM class (those that are efficient agglutinators), whereas antiserum produced by both intramuscular and intravenous inoculations, with or without oil-adjuvant, produced antibodies of both IgM and IgG classes.⁷ It is thought that this could explain the differences in antibody titres of the monovalent and bivalent rabbit antisera detected by the MAT and DIG-ELISA techniques.

Extensive serological cross-reactivity between serovars *hardjo* and *pomona* was evident at lower titres when using DIG-ELISA. This serological cross-reactivity due to common antigens has prompted an investigation into the possibility of using strains of the saprophytic species *Leptospira biflexa* to screen patients' sera by this technique, as is done with other genus-specific serological tests such as the complement fixation test and haemagglutination.

The results also suggest that DIG-ELISA has several advantages over the traditional MAT:

(i) it is a simpler test to read requiring only the measurement of the diameter of the reaction zone, which is directly proportional to the concentration of antibodies in the serum being tested so that the serum need not be serially diluted.

(ii) it appears to be more sensitive than the MAT and can differentiate specific antibodies of the two main immunoglobulin classes.

(iii) its versatility in that antigen-coated plates can

be made in batches and either stored at -20°C indefinitely or coated in agar and stored at 4°C for up to 1 month. The system has been applied to toxoplasma and cytomegalovirus serology both (unpublished observations) and found to be satisfactory.

(iv) its ease of standardisation using commercially available antigens and serum of known titre, since all zones of reaction greater than a negative serum are regarded as positive.

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