

A card test for the serodiagnosis of human leptospirosis

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SYNOPSIS A macroscopic agglutination test for detecting leptospiral antibodies in human sera is described. The test utilizes a stained preparation of non-pathogenic leptospire and is performed on the Brewer diagnostic cards used for bovine brucellosis screening. The agglutination of the stained antigen is more easily observed than the current macroscopic slide test using unstained leptospiral antigens. The non-pathogenic serotype *patoc* is agglutinated by sera from humans infected with serotypes *pomona*, *hardjo*, *ballum*, and *copenhageni* with a sensitivity of 94% in comparison with the microscopic agglutination test.

The laboratory diagnosis of recent leptospiral infections in man may be established either by isolating the infecting organism or by demonstrating a rise in the specific antibody titre. The isolation of the organism has been shown to be feasible, at least for the New Zealand serotypes (Christmas *et al.*, 1974), and is of undoubted epidemiological importance. However, the time taken to culture and identify the organism allows confirmation of the diagnosis in retrospect only. For the detection of specific antibodies the microscopic agglutination test (MAT) (World Health Organization, 1967) is still the standard reference test for leptospiral diagnosis. However, the disadvantages of maintaining a battery of live cultures for the performance of the MAT are well known.

A large number of preliminary tests using genus specific antigens or pools of antigens have been developed. Those involving agglutination of the non-pathogenic serotype *patoc* (Combiescu *et al.*, 1958), haemagglutination (Palit and Gulasekharan, 1973; Sulzer and Jones, 1973), haemagglutination-lysis (Cox *et al.*, 1957), complement fixation (Elian and Nicaña, 1964; Turner, 1968), and immunofluorescence (Torten *et al.*, 1966) all require specialized equipment, and some involve the growth of large volumes of leptospire for the extraction of antigen.

A simple macroscopic slide test reported by Galton *et al.* (1958) is the basis of the commercial screening

antigens² which are used by some local laboratories in New Zealand. This report describes an improvement of this test and the use of a single non-pathogenic (serotype *patoc*) antigen as a screening test for human leptospirosis.

Material and Methods

ORGANISMS

Leptospira interrogans, serotypes *pomona*, *hardjo*, and *ballum*, were isolated from human cases of leptospirosis in New Zealand. Serotype *copenhageni* was a New Zealand bovine isolate obtained from D. R. Ris, Wallaceville Animal Research Centre. *Leptospira biflexa* serotype *patoc* was obtained from the World Health Organization, Leptospira Reference Laboratory, Brisbane, Australia, where all the strains were serotyped. The leptospire were grown and maintained in synthetic Tween 80-albumin medium (EMJH)³.

RABBIT IMMUNIZATION

All leptospire for immunization of rabbits were grown in modified Vervoort's medium containing 10% rabbit serum and supplemented with vitamin B₁₂ (Wolff, 1954). Immune rabbit sera were prepared in New Zealand White rabbits by giving an intramuscular injection of the leptospire centrifuged from approximately 20 ml of culture, and emulsified in 2 ml of a mixture of equal parts of saline and incomplete Freund's adjuvant. When necessary, booster injections of 5 ml of culture were given intraperitoneally to obtain homologous MAT titres of at least 1:6400.

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²Difco Bacto-Leptospira antigens

³Difco Bacto Leptospira EMJH Code 0794, 0795

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HUMAN SERA

Human sera were obtained from culturally proven cases of leptospirosis either in New Zealand, or in Denmark in the case of *copenhageni*, or were selected at random from specimens received for leptospiral serology at the National Health Institute.

ANTIGEN PREPARATION

Leptospire were grown in 50 ml volumes of medium. Well-grown cultures were killed by the addition of 1:10 000 thiomersal (Merthiolate, Lilly), centrifuged, washed twice in normal saline, and resuspended in 1% (w/v) Ponceau S in 0.01M phosphate buffered saline, pH 7.3 (PBS). After standing overnight at 5°C leptospire were washed in PBS until the supernatant was colourless. The staining procedure was repeated when necessary to produce a deep red-brown stained antigen. Stained leptospire were resuspended in PBS to a concentration of 5% (v/v) for the test. For the preparation of antigen pools equal volumes of the 5% suspensions of the different serotypes were mixed. Three preparations were made: (a) a pool of the four New Zealand isolates, serotypes *pomona*, *hardjo*, *ballum*, and *copenhageni*; (b) a pool of the four New Zealand isolates plus serotype *patoc*; (c) serotype *patoc* alone.

Thiomersal 1:10 000 was added to all final preparations. The antigens have been stored at 5°C for up to six months with no variation in activity.

To prepare formalinized antigens the leptospire were resuspended in 0.25% formol saline for 3 hours, washed, and then stained with 1% Ponceau S in PBS.

PROCEDURE

The test was performed on Brewer diagnostic cards¹ used for the brucellosis card test. However, similar results were obtained using glass slides viewed against a white background. 0.025 ml each of antigen and of serum were mixed, and tilted to and fro either manually or on an automatic card rocker for 4 minutes at room temperature. The test was read immediately and the positive results were graded from 1+ to 4+, depending upon the amount of agglutination. A negative control serum was included in each test, and care was taken not to mistake drying around the edge of the drop for agglutination.

Results

RABBIT ANTISERA

The stained antigen preparations reacted well with their homologous rabbit antisera. There were also

considerable cross-reactions among the serotypes tested. The individual results are shown in table I. Rabbit antisera also reacted with the pool of the four pathogens to the same extent as with their homologous antigens. The agglutination of the stained antigens was more easily observed than the agglutination of the unstained commercial antigens (Difco). Formalinizing the leptospire before staining had no adverse effect.

Antiserum	Antigen				
	<i>pomona</i>	<i>hardjo</i>	<i>ballum</i>	<i>copenhageni</i>	<i>patoc</i>
<i>pomona</i>	4+	4+	1+	4+	1+
<i>hardjo</i>	4+	4+	4+	4+	4+
<i>ballum</i>	4+	3+	4+	4+	3+
<i>copenhageni</i>	4+	3+	3+	4+	4+
<i>patoc</i>	1+	1+	1+	3+	1+

Table I Reactions of leptospiral stained antigens with rabbit antisera

Note 1+ to 4+ denotes degree of agglutination

HUMAN SERA

Fourteen sera representing serial specimens from four patients with culturally proven leptospirosis were tested with the three stained antigen preparations. The results in table II show that the inclusion of *patoc* produced agglutination to higher titres than the pool of pathogens alone, and that when the sera were tested against *patoc* alone an even greater degree of agglutination resulted.

To evaluate the suitability of the *patoc* antigen card test as a single preliminary test for the serodiagnosis of leptospirosis, a further 70 sera from 23 culturally proven cases of human leptospirosis (10 *pomona*, 9 *hardjo*, 4 *copenhageni*) were tested. In all 23 cases there was a rise in the MAT titre after an initial negative (less than 1:100) or low titred serum taken at the same time as the blood culture. Unfortunately, five of the initial sera were unavailable for card tests. Two of the second serial specimens failed to react in the card test; however, in both cases, the third serial specimen showed a rise in the MAT titre and agglutinated the *patoc* card test antigen. The results of the reactions of the previous 14 sera are included with these results in table III.

One hundred and eighty human sera were then selected at random from the specimens received at the National Health Institute for leptospiral serology. Seventy-eight of these were positive in the MAT and 102 were negative (less than 1:100). The results of card tests using the *patoc* antigen are recorded in table IV. All of the five 'false negative' sera in the card test belonged to series of sera taken from five different patients.

¹Hynson, Westcott, and Dunning, Inc, Baltimore, USA

Patient	Serotype Isolated	Days from Onset	Serum Dilution					Homologous Reaction	
			0	1:2	1:4	1:8	1:16	Card Test	MAT Titre ²
1	ballum	30	a/b/c ³	a/b/c	a/b/c	a/b/c	a/b/c	3	1 600
		80	1/1/1	1/2/2	2/2/2	1/1/2	1/1/2	3	3 200
2	copenhageni	6	1/1/2	1/1/2	1/1/2	0/0/2	0/0/1	1	0
		11	1/1/3	1/2/3	1/2/4	1/2/4	1/2/3	2	300
		27	1/1/2	1/2/3	2/2/3	2/1/3	2/1/3	2	10 000
3	hardjo	2	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0	0
		13	0/2/3	1/2/3	1/2/3	1/2/2	0/2/2	1	1 600
		27	1/2/3	1/2/3	1/2/3	1/1/2	0/1/2	2	1 600
		128	0/1/2	0/1/2	0/0/1	0/0/0	0/0/0	1	200
4	pomona	13	0/0/0	0/0/0	0/0/0	0/0/0	0/0/0	0	0
		22	1/1/2	1/1/2	1/1/1	0/0/0	0/0/0	1	800
		28	1/1/2	1/1/2	0/1/1	0/0/1	0/0/0	1	400
		50	1/1/1	1/1/1	1/0/1	0/0/0	0/0/0	1	400
		130	1/0/1	1/0/1	1/0/0	0/0/0	0/0/0	1	100

Table II Reactions of human sera with three stained antigen preparations

¹Results are recorded as follows: a = reaction with pool of pathogens
b = reaction with pool including *patoc*
c = reaction with *patoc* alone.

1 to 4 denotes degree of agglutination.

²MAT titres expressed as reciprocals of highest dilution giving 50% agglutination.

Time after Onset of Illness	Number of Sera	Microscopic Agglutination Test (MAT)		Card Test	
		Positive ($\geq 1:100$)	Negative ($< 1:100$)	Reactive	Non-reactive
0- 7 days	22 ¹	3	19	10	12
8-21 days	26	25	1	24	2
22-49 days	20	20	0	20	0
3- 6 months	16	13	3	16	0
Totals	84	61	23	70	14

Table III Reactions of 84 human sera from 27 culturally proven cases of leptospirosis

¹5 sera unavailable

Reciprocal of Maximum MAT Titre	Card Test		Total	Card Test	Microscopic Agglutination Test (MAT)	
	Reactive	Non-reactive			Positive ($\geq 1:100$)	Negative ($< 1:100$)
< 100	48	54	102	Reactive	134	55
100	1	2	3	Non-reactive	8	67
200	9	1	10	Total	142	122
400	10	2	12			
800	18		18			
1600	22		22			
≥ 3200	13		13			
Total	121	59	180			

Table IV Reactions of human sera with *patoc* card test antigen

In the two cases where the MAT titre was 400 the card test was positive for the next serial specimen taken three to four weeks later. In the other three cases, subsequent sera taken four to five weeks later were negative in the MAT, indicating that the sera giving the anomalous results were taken in the later stages of the infection.

If all these results are combined, 264 sera were tested with the *patoc* card test antigen. The results in table V show that there is a type I error (false

Table V Reactions of human sera with *patoc* card test antigen

Type I error (false positive) = $55/122 = 45\%$
Type II error (false negative) = $8/142 = 5.6\%$

positive) of 45% and a type II error (false negative) of 5.6%. The sensitivity, therefore, is 94%, and if the three cases where the sera were taken in the later stages of the infection can be discounted, the sensitivity is better than 96%. The specificity is 55%.

Discussion

The species of animal being studied influences the suitability of any particular leptospiral serological test, especially for establishing the infecting serotype. The broad cross reactivity of rabbit immune sera

has been demonstrated in complement fixation tests (Palit and Sharma, 1971; Robertson and Boulanger, 1963), haemagglutination tests (Palit and Sharma, 1971), haemagglutination-lysis tests (Cox, 1955), and in the macroscopic slide test (Galton *et al.*, 1958), while bovine sera show very little cross-reactivity. Cross reactions have been observed in most leptospiral serological tests when human sera have been examined, the frequent reaction with serotype *patoc* leading to the development of many genus-specific antigens (Combiescu *et al.*, 1958; Cox, 1957; Elian and Nicoaşa, 1964; Palit and Gulasekharan, 1973).

Our results show that in the card test human sera agglutinate the *patoc* antigen more strongly than they agglutinate a pool of antigens containing the infecting strain. The cross-reactions of the human sera were the same as those of the rabbit immune sera but with a much lower degree of agglutination. The card test is therefore of little value for determining the infecting serotype and there is no advantage in using either pools of pathogenic leptospire or individual antigens. Since the treatment for leptospirosis is not in general influenced by the infecting serotype, a rapid diagnosis of leptospirosis using a genus-specific antigen is adequate.

The *patoc* card test compares favourably in sensitivity to the MAT. Provided the serum was taken at least 8-10 days after the onset of illness a negative card test can reasonably be taken to preclude a diagnosis of leptospirosis. While the specificity is rather low this is not a disadvantage in a preliminary test as all reactive sera should be referred for MAT titres. The false positives could be either sera taken early in the infection before the MAT has risen to 1:100 or a reflection of natural antibody (Faine and Carter, 1968). A preliminary study of 'normal' human sera indicates a high incidence of low levels of antibody to *patoc*; however, these reactions are eliminated by diluting the serum 8-10 fold before testing in the card test. It is suggested, therefore, that all sera are tested undiluted, and that those that are reactive are re-tested in serial dilutions. As with the MAT, it is still necessary to demonstrate a rise in antibody titre in two serial specimens in order to confirm a diagnosis of leptospirosis.

The *patoc* antigen has the advantage of being non-pathogenic and more easily and cheaply grown than pathogenic strains, and it may detect antibodies to previously unsuspected strains. The test performed on cards with the stained antigen is more easily read than the macroscopic slide test; it is simple and reproducible and could be performed by unskilled personnel provided adequate controls were always included. It is suitable for field investigations where laboratory facilities are not available and where a

rapid screening test is required. Card tests for bovine brucellosis (Joint FAO/WHO, 1971; Pilet *et al.*, 1972) and for syphilis (World Health Organization, 1970) are well established and have been used satisfactorily for many years.

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