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One-point method for serological diagnosis of leptospirosis: a microcapsule agglutination test

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SUMMARY

This paper describes a simple and rapid microcapsule agglutination (MCA) test. The results obtained by this new test have been compared with those obtained by the microtitre MCA and the microscopic agglutination (MA) test. The procedures required for the new test are easier and can be performed more rapidly than those necessary for the microtitre MCA test. Furthermore, the new test is more sensitive than the MA test in the early stages of leptospirosis.

This new test appears satisfactory as a screening test for the early diagnosis of leptospirosis.

INTRODUCTION

The haemagglutination test has been used for serological diagnosis of leptospirosis, however it is known that erythrocytes are unsuitable as antigen carriers (Williams, 1977). An alternative to using red blood cells as the antigen carrier, the microcapsule agglutination test (MCA-LS) which uses an artificial carrier, has been developed (Arimitsu *et al.* 1982). The microcapsule (MC) carrier is made from a synthetic polymer and is very stable, having a uniform chemical quality. No antigenic activity was detected on the surface of MC particles, and non-specific reactions caused by the carriers were reduced.

Application of the MCA-LS test to serological diagnosis of leptospirosis has been studied by Arimitsu and her colleagues (Arimitsu *et al.* 1982) and to *Treponema pallidum* (MCA-TP) by Kobayashi *et al.* (1983). Polyurea, containing red dye, was selected as the wall material of the particles to facilitate the ease of reading the agglutination patterns. Sonicated leptospires and treponema antigens are adsorbed on to the surface of MC particles which have been treated with glutaraldehyde or tannic acid.

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The MCA-LS test was carried out using microtitre methods, which included both single and mixed antigens; these were compared to the microscopic agglutination test (MAT), which has been widely used as a conventional standard method. It was shown that the MCA-LS test was more sensitive than the MA test in the early stages of leptospirosis. Using serum fractions and 2-mercaptoethanol treatment, the test was found to have high sensitivity to the IgM antibodies (Arimitsu, 1982). The MCA-TP method was compared with the TPHA (*Treponema pallidum* haemagglutination test) and the FTA-ABS (fluorescent treponemal antibody-absorption) test and other tests for serodiagnosis of syphilis, and it was concluded that the test was useful for the diagnosis of early syphilis and suitable for use as a routine diagnostic test (Kobayashi *et al.* 1983).

Early diagnosis is necessary for the prompt treatment and prevention of leptospirosis. The use of mixed antigens in the MCA-LS test has been found satisfactory for the detection of several important serovars of leptospira and is considered suitable for the purpose of the screening test.

In this report the one-point MCA test was studied. In the original microtitre MCA-LS test (Arimitsu *et al.* 1982), test sera were serially twofold diluted, and special tools such as an expensive autodiluter or manual dilutions were needed. In addition, the procedure was time-consuming. In the one-point test, only one dilution of about 300-fold was used; the procedure is therefore very simple and rapid and does not require special tools or skills.

MATERIALS AND METHODS

Leptospira strains

Six serovars were used; *Leptospira icterohaemorrhagiae* strain RGA, *L. autumnalis* strain Akiyami A, *L. hebdomadis* strain Hebdomadis, *L. australis* strain Ballico, *L. canicola* strain H. Utrecht IV and *L. pyrogenes* strain Salinem. The organisms were cultivated in Korthof medium containing 10% rabbit sera at 32 °C for about 4–7 days.

Sera.

The following sera were from patients diagnosed clinically and serologically as having leptospirosis. Sixteen paired sera which were collected during pyrexial stages of disease and during convalescence, 27 single-point sera from patients with Weil's disease, a series of sera taken from an Akiyami A disease patient who had clinical signs of icterus during the course of illness and 15 sera from leptospirosis caused by serovars other than *L. icterohaemorrhagiae*. These were obtained from the National Institute of Health of Japan. In addition, more than 300 sera from non-leptospirosis patients were obtained from various hospitals in Tokyo. These patients were not diagnosed as leptospirosis clinically.

Preparation of leptospira antigens

The leptospira antigens were prepared using the procedure previously described by Arimitsu (Arimitsu *et al.* 1982). Cells of each of the six strains were disrupted with a sonicator (Model 200 M; Kubota Medical Appliance Supply Co. Ltd) at 9 kHz for 20 min. Disrupted antigens were adjusted to the same optical density at 280 nm and two mixed antigens were prepared as follows: antigen A contained

L. autumnalis, *L. hebdomadis* and *L. australis*, and antigen B contained *L. icterohaemorrhagiae*, *L. canicola* and *L. pyrogenes*.

Preparation of polyurea MC particles

The method was almost the same as previously described (Arimitsu *et al.* 1982). A 50 ml portion of 5% maleic anhydridemethyl-vinylether copolymer (Gantrez AN-149, GAF Corporation) solution was added to a mixture of 11·8 g of diisopropylnaphthalene (Kureha Chemical Industry Co. Ltd) and the mixture was emulsified by agitation until an average drop size of *ca.* 5·5 μm was obtained. After addition of 2·5 g of urea, 0·25 g of resorcinol, 0·3 g of ammonium chloride and 6·7 ml of 37% formaldehyde solution, the emulsion was heated at 60 °C for 2 h. The polyurea MC (average particle size, *ca.* 5·5 μm; specific gravity, *ca.* 1·16) was used after washing four times with purified water.

Sensitization of the MC particles and freeze drying

Method of sensitization was the same as described previously (Arimitsu *et al.* 1982). The two kinds of mixed antigens were used to prepare MC antigens A or B, respectively. The sensitized MC particles were adjusted to 1·5% in PBS solution (pH 7·2) containing 3% of bovine serum albumin, and 0·4 ml of the suspension were distributed in 3 ml vials and freeze dried by using Model SVAC-200 special (Seiwa Shinku Corporation).

Microtitre MCA-LS test

The microtitre MCA-LS test was conducted by the same method as described previously (Arimitsu *et al.* 1982).

MA test

The test was carried out by a microtechnique of the Schüffner–Mochtar method, as stated before (Galton *et al.* 1965). Living antigens were used.

One-point MCA test

(1) The lyophilized MC was resuspended with 2·5 ml of distilled water, which is about 6·25-times the volume before lyophilization. The final concentration of the MC becomes 0·24%, which gives the desired patterns of agglutination (data not shown). (2) The reconstituted MC antigens were left at room temperature for more than 30 min and, 0·3 ml each of the A- and B-type MC antigens was transferred to a disposable polyacrylamide tube (diameter 12 mm, length 78 mm). (3) Each test serum was sampled by using disposable 1 μl loops and was then mixed into each of the two tubes containing A- or B-type MC antigens. These test sera were consequently diluted about 300-fold. Previously we have reported that the borderline between positive and negative appears to lie at about a 40- to 80-fold titre. (Arimitsu *et al.* 1982). One-point MCA test was therefore determined at about a titre of 300. (4) The tubes containing the mixture of antigen and serum were left standing vertically in a rack with a mirror, and the agglutination patterns at the bottom of the tubes were read through the mirror after 3 h, or after being kept overnight at room temperature. Antigens without serum were used as a negative control.

Table 1. *The effect of heat inactivation of patient's sera without leptospirosis*

Sera	Number of serum	Number of positive serum(%)	After inactivation
Unknown patients*	128	4 (3·1%)	All changed to (−)
Syphilis patients	128	2 (1·6%)	All changed to (−)
RA (+) patients	72	0	—
CRP (+) patients	41	1 (2·4%)	Not changed to (−)†
Total	369	7 (2·0%)	1 (0·27%)†

* These patients had no clinical symptoms of leptospirosis.
† This serum changed to negative after filtration (0·45 μm).

Reading of the MC agglutination pattern

The degree of the MC agglutination was expressed by the following scores; 3+, when the MC pattern had slipped; 2+, when the MC pattern covered the entire bottom of the tube; 1+, when the MC pattern was apparently larger than the negative control; ±, if slight MC agglutinations were noticed; and −, if no agglutination was observed.

RESULTS AND DISCUSSION

Non-specific reactions

In order to check non-specific reactions, various sera from syphilis patients, RA(+) (rheumatoid arthritis test) and CRP(+) (C-reactive protein test) patients and patients without a definite diagnosis were tested (Table 1). Seven of 369 sera gave positive readings: the positive sera contained lipid-like floating and other substances probably due to contamination by bacteria. Haemolysis did not cause non-specific reactions, because some specimens with intense haemolysis showed no positive reaction.

In order to determine the effect of heat treatment on the non-specific reactions, the seven positive sera were inactivated at 56 °C for 30 min; six reverted to a negative result. Only one serum from a CRP(+) patient remained positive after inactivation, but changed to negative after millipore filtration (pore size, 0·45 μm: Millex-HA, Millipore Corporation). The unsensitized MC particles did not show any reaction with the seven sera; therefore non-specific reactions are not caused by the MC particles themselves.

Comparison between one-point and microtitre MCA tests

Twenty-seven sera from Weil's disease patients were examined by the one-point and the microtitre MCA tests. The correlation between the results of both tests is shown in Fig. 1, although the result of the one-point test is only semi-quantitative.

Using these results, the positive limit of the one-point test was considered to be equal to a titre of 160 by the microtitre test. Since the sera were used at a tenfold dilution, MAT was considered positive at > 10. Specimens which were negative by the one-point test had titres lower than 80 by the microtitre test and titres below 10 by the MA test; specimens which were positive by the one-point test had titres of more than 10 by the MA test.

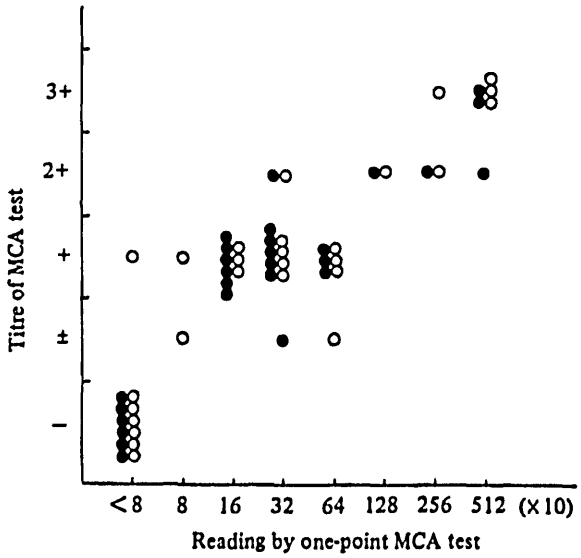

Fig. 1. Correlation of one-point MCA test and microtitre MCA test with 27 sera from patients with Weil's disease.

Table 2. *Comparison between one-point MCA and MA tests of 16 paired sera from Weil's disease patients*

	One-point MCA test				MA test	
	A*	B*	A	B		
Patient Stage. . .	Acute		Convalescence		Acute	Convalescence
1	−	−	+	+	<10	160
2	+	+	+	+	<10	160
3	+	+	+	+	10	20
4	2+	2+	+	+	10	20
5	+	+	+	+	10	20
6	+	+	2+	2+	10	40
7	+	+	+	+	10	40
8	2+	2+	2+	2+	10	80
9	2+	2+	2+	2+	10	80
10	2+	2+	2+	2+	20	40
11	+	+	+	+	20	40
12	+	+	+	+	20	160
13	3+	3+	2+	2+	80	160
14	+	+	−	−	40	80
15	2+	2+	2+	2+	160	320
16	+	+	+	+	160	320

* Type of MC.

Comparison between one-point MCA and MA tests on 16 paired sera from Weil's disease patients

Sixteen paired sera which showed an increase in the MA titres during the course of the illness were available. When examined by the one-point test (Table 2) the results from the acute stages were almost the same as those at the convalescence

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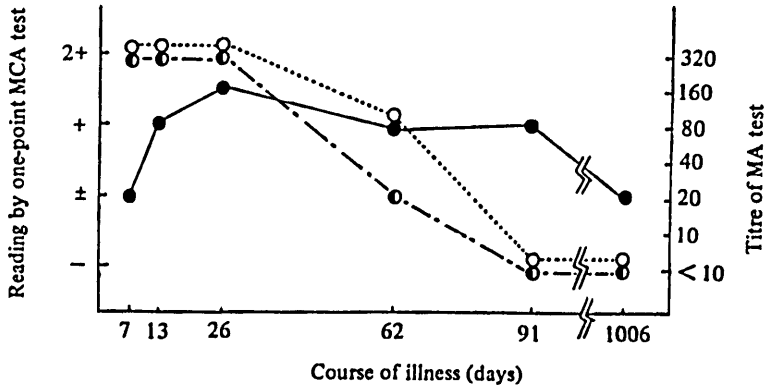

Fig. 2. Serial results with sera from a patient with leptospiral infection.

Table 3. *Comparison between one-point MCA and MA tests of different serovars*

Serovar	MA test	One-point MCA test A*	B*
L. hebdomadis	320	3+	3+
	80	+	+
	160	2+	2+
	160	+	+
	160	3+	3+
	80	3+	3+
	40	+	+
	40	+	+
L. autumnalis	160	+	+
	80	3+	3+
	160	2+	2+
L. australis	80	3+	3+
	10	−	−
L. pyrogenes	80	2+	2+
L. canicola	160	+	+

* Type of MC.

stage. In patient 2 the MA test was negative, but the one-point test was positive at the acute stage. On the other hand, in patients 4, 13 and 14, the reaction was diminished by the one-point test at the convalescence stage but the MA titre increased. It may be that the MCA test is more highly sensitive to IgM antibodies than the MA test, since the one-point test has a higher sensitivity to the acute sera than the MA test.

Results of serial serum specimens obtained from a patient with leptospirosis

Serial specimens were examined by the one-point and the MA tests (Fig. 2). The one-point test showed a definite reaction earlier than the MA test, showing a peak on day 7, while the MA test showed a peak around day 26. The results of the one-point test became negative around day 91, while the MA test still gave a high titre. The difference might be due to the higher sensitivity of the MA test to earlier antibodies and corresponding lower sensitivity to the later antibodies.

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Results of sera from patients with serovars other than L. icterohaemorrhagiae

Serum specimens from patients infected with serovars other than *L. icterohaemorrhagiae* were tested with the A- and B-type MC antigens. Table 3 shows that the MC antigens reacted with sera derived from different serovars as well as *L. icterohaemorrhagiae*. Although the components of A- and B-type MC antigens are different, similar results were obtained by the one-point method; this fact may suggest that the test is genus-specific. Only one type of mixed antigen might be sufficient for the screening test, but more tests should be carried out to confirm this point on many other serovars.

The one-point MCA test is more rapid and simpler than the microtitre method. Its use would be as a primary screening test which could be used in busy routine clinical laboratories that do not have reference facilities. The risk of infection from wild and domestic animals is very high, especially in developing countries. In these countries, tests which need special tools and techniques may be difficult to carry out for economic and technical reasons.

Although there are several screening methods available the one-point MCA seemed to be more useful and practical because: (1) the MC antigens are very stable, since they are made from stable synthetic polymers and are lyophilized; (2) the results are easily read, because the MC antigens include a red dye and the pattern is clear; (3) the MC antigens have high sensitivity to the early antibodies, they are genus-specific and can therefore be used as a screening test.

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