Evaluation of the one-point microcapsule agglutination test for diagnosis of leptospirosis

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We have developed a one-point microcapsule agglutination test (MCAT) for the serodiagnosis of leptospirosis. The MCAT kit was evaluated for use in humans by six WHO Collaborating Centres for Reference and Research on Leptospirosis. The laboratories classified their serum samples on the basis of the microscopic agglutination test (MAT) and the following screening tests: enzyme-linked immunosorbent assay (ELISA), macroscopic (slide) agglutination test, or the complement fixation test. The MCAT may in some instances give a positive result earlier in the course of the disease than MAT or the ELISA IgM; on the other hand, it did not detect antibodies against some serovars, for example, those of the Sejroe or Australis serogroup in Slovakia. In contrast, however, the MCAT detected antibodies to serovar hardjo (the same serogroup as Sejroe) in patients from the United Kingdom and the Russian Federation.

Introduction

Leptospirosis, a zoonosis with a worldwide distribution, is an acute febrile illness caused by microorganisms of the genus Leptospira. A diagnosis of human leptospirosis should be considered for all patients who present with an unknown disease with acute fever. The first leptospiroemic phase is characterized by acute systemic infection and by the presence of leptospires in the blood and cerebrospinal fluid under darkfield microscopy. This phase lasts 4–7 days; however, it is difficult to diagnose the disease at this stage. From the eighth day onwards (seldom earlier), serovar-specific antibodies may be demonstrated in blood serum by the microscopic agglutination test (MAT) (1), which is most widely employed as the standard reference test because of its high specificity and sensitivity. Currently, over 200 pathogenic Leptospira serovars have been identified (2). MAT requires multiple serovars of live leptospira, which involves a risk of infection and maintenance of a large number of stock cultures to provide antigens. These factors limit the test’s usefulness for routine application in diagnostic laboratories.

We have developed microcapsules (MCs) of a synthetic polymer for use as an antigen carrier and reported a passive MC agglutination test (MCAT) for detecting antibodies to leptospires (3). The high specificity and sensitivity of the MCAT to IgM antibodies make it suitable for the early diagnosis of leptospirosis. Furthermore, since the test is cross-reactive to antibodies from many members of the Leptospira genus, it may have an application as a genus-specific test (4). A later development has been its use as a single-dilution (about 1:300) screening test for the early diagnosis of leptospirosis (5).

As a preliminary study to determine whether the MCAT could have an application outside Japan, we used it to demonstrate leptospiral antibodies in human serum samples from China, Republique of Korea, and Italy. The one-point MCAT gave encouraging results with the serum samples from China and Republic of Korea, but was less promising with the samples from Italy, possibly because of the presence of a greater number of serovars in Italy than in China and Republic of Korea (6, 7). Other data obtained from Italy have been more promising, however, although the one-point MCAT failed to detect antibodies to serovar bratislava (8).

Subsequently, the test has been evaluated by six of the WHO Collaborating Centres for Reference and Research on Leptospirosis, and we report the results in this article.
Materials and methods

One-point MCAT

The MCAT kit* consists of two vials of lyophilized reagents (A and B) and one vial of a diluent (1% bovine serum albumin–PBS, pH 7.2). Reagents A and B are microcapsules sensitized separately with a mixture of sonicated antigens of Leptospira australis, L. autumnalis, and L. hebdomadis (reagent A); and L. canicola, L. icterohaemorrhagiae, and L. pyrogenes (reagent B). A test-tube rack with an oblique mirror and a number of disposable test-tubes are also provided with the kit.

The test procedure was as follows. After the reagents were reconstituted with the designated amounts of diluent, 0.3 ml of each reagent was transferred to corresponding tubes marked A or B. A sample of the test serum (about 1 μl) was then added to both test-tubes and diluted about 300-fold. The tubes were mixed well and allowed to stand in the rack at room temperature for 3 hours. The reaction outcome was classified as 3+, 2+, +, ±, or − according to the settling patterns. A reaction classified “+” was considered positive.

Leptospiral serovars used in the MAT

In the Russian Federation, strains responding to 13 serogroups were used in the MAT; the serum samples were positive to the following serovars: canicola, grippotyphosa, copenhageni, pomona, sejroe, and wolffi. In France 19 serovars were used: australis, autumnalis, castellonis, bataviae, canicola, cynopteri, grippotyphosa, hebdomadis, copenhageni, icterohaemorrhagiae, javanica, panama, pomona, pyrogenes, hardjo, sejroe, wolffi, tarassovi, and patoc. In the United Kingdom the following 11 serovars were used: australis, autumnalis, ballum, bataviae, canicola, celledoni, icterohaemorrhagiae, javanica, pyrogenes, hardjo, and saxkoebing. In Slovakia the following 14 representative indigenous serovars were used: bratislava, jalna, grippotyphosa, copenhageni, icterohaemorrhagiae, mozdok, pomona, balcanica, hardjo, istrica, polonica, saxkoebing, sejroe, and patoc. In the MAT used in Australia, the following 14 serovars were employed: australis, bulgarica, canicola, celledoni, grippotyphosa, kremastos, szwajzak, copenhageni, pomona, robinsoni, zanoni, hardjo, medanensis, and tarassovi. Finally, in the Netherlands, the following 18 serovars were used: australis, ballum, bataviae, canicola, celledoni, cynopteri, grippotyphosa, hebdomadis, copenhageni, icterohaemorrhagiae, poi, mini, pomona, hardjo, saxkoebing, tarassovi, andamana, and patoc.

Serum specimens

A total of 327 serum samples, including 43 paired sera, 13 series of sera, and 29 negatives as controls were collected by six WHO Collaborating Centres for Reference and Research in Leptospirosis. The distribution of serogroups was as follows: Australis (19); Autumnalis (9); Ballum (6); Bataviae (10); Canicola (13); Celledoni (3); Cynopteri (8); Grippotyphosa (37); Hebdomadis (8); Icterohaemorrhagiae (63); Javanica (1); Panama (10); Pomona (21); Pyrogenes (6); Sejroe (69); and Tarassovi (1). The serogroup of 14 serum samples could not be determined because of cross-reactions between Australis, Autumnalis, Icterohaemorrhagiae and Sejroe. As controls, samples of sera from 29 nonleptospiral patients were used; for example, those with Lyme disease, renal syndrome, hepatitis, jaundice of unknown origin, and acute respiratory viral infection.

Tests used

All the serum specimens were tested by MAT, the basis of serological diagnosis of leptospirosis and the most widely used procedure. As screening tests, the complement fixation test (CF) (9), the macroscopic (slide) agglutination test (10), and enzyme-linked immunosorbent assay (ELISA) IgM and IgG (11) were used.

Results

Sensitivity of the one-point MCAT in the early stages of illness

We investigated the suitability of the one-point MCAT for diagnosing the early stages of leptospirosis, compared with MAT and ELISA IgM. A total of 54 serum samples were collected from patients 3–10 days after onset of the disease (Table 1). The one-point MCAT detected antibodies in 35 (64.8%) serum samples compared with the 20 (37%) detected by MAT, and 21 (38.9%) by ELISA IgM. The sensitivity of the one-point MCAT to detect IgM antibodies therefore appears to be greater than that of MAT or the ELISA IgM.

Sensitivity of the one-point MCAT to serogroups

A total of 137 MAT-positive serum samples taken from patients between 11 days and 180 days after onset of illness were tested using the one-point

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* Supplied by Japan Lyophilization Laboratory, Kiyose City, Tokyo, Japan.
MCAT. Not all the serum samples from France and Australia were included in the analysis because it was not known when after the onset of illness they were collected. However, such serum samples were included if they had high antibody titres. Table 2 shows that the one-point MCAT failed to detect antibodies against Pomona, Icterohaemorrhagiae, Grippotyphosa, Sejroe, Australis, and Hebdomadis serogroups, as shown by the magnitude of the positive ratios; also, although the one-point MCAT kit contains the serovars <i>australis</i> and <i>hebdomadis</i>, it failed to detect antibodies to Australis (45.5% of tests) or Hebdomadis (40%) serogroups. There were only five samples in the Hebdomadis serogroup. The one-point MCAT could not detect the <i>bratislava</i> and <i>jalna</i> serovars in the Australis serogroup; also, it was not able to detect antibodies to the Grippotyphosa (72%) or Sejroe (46.2%) serogroup, although the kit did not contain serovars from these serogroups. Antibody to the <i>ISTRICA</i> serovar of the Sejroe serogroup was not detected at all by the one-point MCAT.

**Results obtained by each participating laboratory**

In Australia, of the 30 serum specimens tested, 13 were negative by MAT, with no false-positives in the one-point MCAT. The remaining 17 of the MAT-positive serum specimens were tested using CF and one-point MCAT; seven were positive in both CF and the one-point MCAT (41.2%). The one-point MCAT detected antibodies against two of three <i>hardjo</i> serovars. No information on the number of days after onset of disease was provided for any of the serum samples collected. The test results were read against the criteria supplied with the kit.

In France, a total of 63 serum samples from 46 patients were classified into four groups based on the MAT results, as follows: not leptospirosis; could be leptospirosis; probable recent acute leptospirosis; and confirmed leptospirosis. The number of days after the onset of illness when they were collected was not known for all the serum specimens. The one-point MCAT results were compared with those obtained using the macro-agglutination test (TR), ELISA IgM, and MAT. The positive titre of ELISA IgM was ≥200. In the first group, 10 serum samples were from nine individuals who did not have leptospirosis. All these samples were negative in the MCAT; two were false-positives in the ELISA IgM; and one was a false-negative in the TR. In the second group, 24 serum samples from 22 patients were classified as “could be leptospirosis” (either recent or old); eight samples (33.3%) were positive in the MCAT; and three (12.5%) were positive in both TR and ELISA IgM. In the third group, 15 serum samples from 14 patients were probably recent leptospirosis cases with negative ELISA IgM; and five (33.3%) were positive in both the TR and MCAT. In the last group, 12 serum samples were from 10 patients with confirmed leptospirosis; ten (83.3%) were positive in the one-point MCAT; and nine (75%) were positive in the ELISA IgM, whereas five (41.7%) were positive in the TR.

In the Netherlands, 46 serum samples were collected from patients suffering from or who had suffered from leptospirosis caused by different serovars. The positive ELISA IgM titre was ≥160. Of the 46 serum specimens, 40 (87%) were positive in the MAT, 27 (58.7%) were positive in the ELISA IgM,

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### Table 1: Comparison of the suitability of the one-point microcapsule agglutination test (MCAT), microscopic agglutination test (MAT), and enzyme-linked immunosorbent assay (ELISA) IgM for the early diagnosis of leptospirosis

<table>
<thead>
<tr>
<th>MAT</th>
<th>One-point MCAT&lt;sup&gt;a&lt;/sup&gt;</th>
<th>ELISA IgM&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>Serum samples&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20</td>
<td>34</td>
</tr>
<tr>
<td>Percentage</td>
<td>37</td>
<td>63</td>
</tr>
</tbody>
</table>

<sup>a</sup> One-point MCAT versus MAT; χ² test = 8.34 (significant at 1%).

<sup>b</sup> One-point MCAT versus ELISA IgM; χ² test = 7.27 (significant at 1%).

<sup>c</sup> Serum samples were taken 3–10 days after the onset of illness.

### Table 2: Positive rates by serogroup of antibody, as detected by the one-point microcapsule agglutination test (MCAT)

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>No. of positives/No. tested&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Australis</td>
<td>5/11 (45.5)%</td>
</tr>
<tr>
<td>Autumnalis</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td>Ballum</td>
<td>3/3 (100)</td>
</tr>
<tr>
<td>Bataviae</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>Canicola</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>Celledoni</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>Cynopteri</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>Grippotyphosa</td>
<td>18/25 (72)</td>
</tr>
<tr>
<td>Hebdomadis</td>
<td>2/5 (40)</td>
</tr>
<tr>
<td>Icterohaemorrhagiae</td>
<td>31/34 (91.2)</td>
</tr>
<tr>
<td>Javanica</td>
<td>1/1 (100)</td>
</tr>
<tr>
<td>Pomona</td>
<td>11/12 (91.7)</td>
</tr>
<tr>
<td>Pyrogenes</td>
<td>2/2 (100)</td>
</tr>
<tr>
<td>Sejroe</td>
<td>12/26 (46.3)</td>
</tr>
<tr>
<td>Total</td>
<td>103/137 (75.2)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Serum samples were collected from 11 days to 180 days after the onset of illness.

<sup>b</sup> Figures in parentheses are the percentage of samples that were positive.
and 34 (74%) were positive in the one-point MCAT. These serum samples were tested using the slide agglutination test. As antigens, 16 serovars were used and divided into the following eight groups:

- I: icterohaemorrhagiae and copenhageni;
- II: canicola and ballum;
- III: grippotyphosa and pomona;
- IV: australis and hebdomadis;
- V: mini and saxkoebing;
- VI: poi, tarassovi and bataviae;
- VII: patoc;
- VIII: hardjo and hardjobovis.

Of the 46 serum specimens, 37 (80.4%) were positive in the slide test.

In the Russian Federation, a total of 45 serum samples, including two paired and two series of serum samples from patients with acute and convalescent leptospirosis, as well as six control serum samples from patients with cholestatic hepatitis, Lyme borreliosis, viral hepatitis, jaundice of unknown origin or acute respiratory viral infection, were investigated using MAT, the slide agglutination test, and the one-point MCAT. The number of days after onset of disease was identified for all but one of the serum samples. The positive titre in the slide agglutination test was taken to be ≥8. Of the 45 samples, 41 (91.1%) were positive in the MAT, 28 (62.2%) were positive in the slide agglutination test, and 32 (71.1%) were positive in the one-point MCAT. Only one serum sample, taken 6 days after onset of the disease, was negative in the MAT, but strongly positive in the one-point MCAT. Six other control patients were all negative in the three tests.

The one-point MCAT failed to detect antibodies against antigens of the Sejroe serogroup in all five samples; against antigens of the Grippotyphosa serogroup in three out of five samples; against those of the Australis serogroup in four out of six samples (one sample was taken from a patient who had been ill for 1 year); in only one of 12 samples of the Pomona serogroup; and in two of 12 samples from patients with Weil disease. Three cases of Weil disease and four cases with the Pomona serogroup who had a typical paradoxical reaction (12) (an early stage of infection) were also positive in the MCAT in Slovakia. These results indicate that there was good correlation between the MAT and the one-point MCAT for infections caused by the serovars included in the kit or the Pomona serogroup. However, the low correlation between MAT and MCAT for the Australis and Sejroe serogroups was caused by quite different antigens; for example, serovars bratislava or jalna. Also, as discussed above, the one-point MCAT did not include antigens from the Sejroe serogroup.

In the United Kingdom, a total of 97 serum specimens from 52 cases, including 28 paired sera and six series of sera, were tested using MAT, ELISA IgM, and the one-point MCAT. For all the serum samples unambiguous information was available on the number of days after onset of the disease when they were collected, except for a series of serum samples from patients whose disease onset was recent. According to the criterion that a positive ELISA IgM titre was ≥160, 65 (67%) serum samples gave positive reactions in the ELISA IgM. A total of 75 (77.3%) serum samples had positive reactions in the one-point MCAT, whereas 68 (70.1%) were positive in the MAT. All of the eight early serum samples were negative in the MAT. Of these samples, eight (collected on days 4, 5, 6, and 9 after onset of infection and two samples that were collected an unknown time after onset of illness) were positive in the one-point MCAT, while three samples (collected 5 days and an unknown time after onset) were positive in the ELISA IgM. Four series of serum samples were of the serovar saxkoebing; the one-point MCAT detected antibodies in only half of these specimens.

Discussion

The evaluation of the one-point MCAT varied according to the laboratory. The consensus view was that it was a useful test, detecting some cases of leptospirosis at an earlier stage than ELISA or MAT. The results for leptospirosis infections caused by serovars included in the kit’s antigens or related to them indicated a good correlation between the MAT and the one-point MCAT. The lower correlation in the Australis serogroup probably arose because of the greater antigenic differences between the serovars australis and bratislava, and jalna. The one-point MCAT would not be expected to detect the Grippotyphosa or Sejroe serogroups, since no antigens from these serogroups were included in the kit. When the test is to be used in a particular geographical area, reagents A and B should include a range of antigens appropriate to the region concerned. For example, in Central Europe reagents A and B should include antigens representing the Sejroe serogroup and the Jalna subserogroup of Australis. Thus, it is not possible for the one-point MCAT to cover all the leptospira serovars (over 200) in the world; serovar diagnosis is, however, essential for epidemiological purposes. Therefore, the MAT should be used to complete the diagnosis in such cases. As controls, 29 serum samples collected from patients with a variety
of infectious disease were used for the one-point MCAT. Not all the serum samples gave false-positive reactions.

There is no simple, perfect screening test for diagnosing leptospirosis. The strength of the one-point MCAT is its sensitivity during the early stages of the illness; however, this must be balanced against its reduced sensitivity during the later stages. If a key serovar is missing from the kit, the one-point MCAT may not detect antibodies in sera collected more than 1–2 months after onset of the disease.

Other advantages of the one-point MCAT kit are that it is simple and can be performed by relatively unskilled personnel with minimum laboratory facilities; it is also very stable and can be kept for long periods without critical storage requirements.

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Résumé
Evaluation d’un test d’agglutination sur microcapsules à dilution unique pour le diagnostic de la leptospirose
Nous avons mis au point un test d’agglutination sur microcapsules (MCAT) à dilution unique pour le sérodiagnostic de la leptospirose. Six centres collaborateurs de l’OMS ont évalué le MCAT en vue de son utilisation pour le diagnostic de la maladie chez l’homme. Les laboratoires ont classé leurs échantillons de sérum en fonction des résultats du test d’agglutination microscopique (MAT) et des tests de dépistage suivants: essai immuno-enzymatique (ELISA), agglutination macroscopique sur lame ou fixation du complément. Dans certaines circonstances, le MCAT peut donner un résultat positif à un stade plus précoce de la maladie que le MAT ou l’ELISA IgM; par contre, il n’a pas détecté les anticorps dirigés contre cer-
tains sérovars, comme ceux du sérogroupe Sejroe ou Australis en Slovaquie. Toutefois, le MCAT a détecte des anticorps dirigés contre le sérovar hardjo (sérogroupe Sejroe) chez des patients du Royaume-Uni et de la Fédération de Russie.

References