Application of Serologic Diagnosis of Tsutsugamushi Disease (Scrub Typhus) in Korea Where the Disease was Recently Recognized to be Endemic

Yunsop Chong

In Korea, tsutsugamushi disease is a recently recognized infection. It has become clear that it is more prevalent than leptospirosis or hemorrhagic fever with renal syndrome. Accurate diagnosis of the disease is necessary for the selection of effective antimicrobial agents which can prevent fatalities and shorten the course. For the diagnosis, various serologic tests are used. Sensitivity and specificity of a test depend on various factors. In this report, microbiological aspects of the infection were briefly described and the Weil-Felix, indirect immunofluorescence and indirect immunopersidase tests were compared for their applicability in routine use and usefulness in the diagnosis. Their interpretations were also briefly discussed.

Key Words: Tsutsugamushi disease, scrub typhus, serologic diagnosis.

Tsutsugamushi disease (scrub typhus) is the infection with Rickettsia tsutsugamushi (Tamya 1962). In Korea, as the disease was only lately recognized (Lee et al. 1986; Yi et al. 1986), it can be assumed that accurate diagnosis was not always achieved. For the diagnosis of the disease, serological tests have been widely used. The sensitivity of a serologic test depends on the nature of the test itself, but the specificity depends on the prevalence of the disease together with the prevalence of cross reacting antibodies in a population (Brown et al. 1983). Until we accumulate more information on these matters, we need to select and apply the serologic tests cautiously. The purpose of this paper is to review, briefly, R. tsutsugamushi infection and discuss the application of the serologic tests for the diagnosis.

R. TSUTSUGAMUSHI AND THE INFECTION

R. tsutsugamushi (Weiss and Moulder 1984) is considered, by some investigators, to be a misnomer and R. orientalis is the correct name, as “tsutsugamushi” in Japanese means a mite, but not a rickettsia (Kawamura 1984). R. tsutsugamushi is stained by the method of Giemsa or Giemenez, it is slightly smaller than the other rickettsiae and has a size of of 0.3–0.5 μm × 1.2 μm. It can be cultured in the yolk sac of chicken embryos or on continuous cell lines. Various vertebrates, such as man, goat, pig, monkey, rat, mouse, rabbit, guinea pig, cotton rat, hamster and dog are susceptible. The mouse is most often used as an experimental animal. Several antigenic types are recognized and the three main types are Gilliam, Karp and Kato (Weiss and Moulder 1984). Additional types may be added in the future (Tamura et al. 1984).

Tsutsugamushi disease is transmitted by the biting of larva or chigger mites. The vectors include various species of Leptotrombidium. L. akamushi, L. pallida and L. scutellare are the vectors in Japan (Tamya 1962). In Korea, L. akamushi has not been reported, but the other two species exist throughout the country (Chung 1986). In Japan, it is known that not all mites, but only 1–3% of L. akamushi, 0.2–0.5% of L. pallida and 0.02–0.1% of L. scutellare harbour the rickettsiae (Kawamura 1984). Contrary to other rickettsia, the mites were shown to be infected naturally with R. tsutsugamushi and to transmit it transovarianly. The chigger mites which missed the chance to take a tissue juice meal may survive the cold winter and transmit the disease in the spring (Suto 1987). As the chigger mite has a small size of around 0.2 mm and as the biting, which lasts for 1–2 days, does not cause pain or irritation, the host can not be aware of the feeding.

Tsutsugamushi disease occurs in India, Pakistan,
Japan, the northern part of Australia, islands of the Pacific Ocean, the southern part of Siberia, China and Indonesia (Weiss and Moulder 1984). In Japan, it was a well known disease and was already recorded in 1810. The disease was recognized in summer in the tributaries of the Shinano river in the Niigata Prefecture. Later, it was found that the infection also occurred in other parts of Japan in spring and autumn. To distinguish the two forms, the summer type was called classical and the other, new type (Taniya 1962). In Japan, during the period of 1965 to 1974, the disease became very rare and only 3–17 cases were reported annually (Ujiie 1981; Kawamura 1984). Since 1975, the disease started to increase and in 1984 there were 970 reported cases (Suto 1986). The infection drew renewed interest because of the increased cases and occasional fatalities (Suzuki et al. 1981).

TSUTSUGAMUSHI DISEASE IN KOREA

The disease was first reported among the United Nations Forces personnel during the Korean war. Foreign investigators subsequently isolated R. tsutsugamushi from two patients and from the rodents and mites collected from the area near the demilitarized zone (Munro-Faure et al. 1951; Jackson et al. 1957). Chun et al. suggested the presence of the disease among Korean natives by demonstrating the presence of antibodies against Proteus OXK antigen in some normal subjects (Chung 1986).

However, it was not until 1986 that the serologically proven tsutsugamushi disease was first reported among native Koreans. Yi, an internal medicine practitioner in the southern port city of Chinhoe, Korea, had noted febrile patients with rashes and lymphadenopathy every autumn since around 1980 (Yi et al. 1986). In 1985, he found eschars on some of these patients and the diagnosis of tsutsugamushi disease was made by the Weil-Felix and indirect immunofluorescent antibody (IFA) tests. In the same year, Lee et al. (1986) reported tsutsugamushi disease among the patients admitted to Seoul National University Hospital, which were first suspected to be hemorrhagic fever with renal syndrome or others.

Subsequently, it became clear that in most parts of Korea, the disease exists (Chang et al. 1988b; Jang et al. 1987; Kim et al. 1987a; Kim et al. 1987b; Kim et al. 1987c; Kim et al. 1988a; Kim et al. 1988b; Lee et al. 1988; Park and Chun 1988) and it is more prevalent than hemorrhagic fever with renal syndrome or leptospirosis (Chang et al. 1988b). It can be safely assumed that in the 1970s, the disease was rare, as it was in Japan, and consequently misdiagnosis must have been rare. However, in the early 1980s when the disease was not known to be present in Korea, the patients must have been misdiagnosed as having hemorrhagic fever with renal syndrome, leptospirosis or other diseases. All of these three diseases occur in the autumn in Korea, and patients contract the diseases after exposure to scrub or cultivated farm lands. Murine typhus is also now reported to occur in Korea, complicating the diagnosis (Kim et al. 1988b).

Up to the present, the antigenic types of R. tsutsugamushi isolated from Korean patients have been Gilliam and Karp types. The Gilliam type was relatively more prevalent in the north and Karp in the south (Chang and Kang 1987; Chang et al. 1988a; Chong et al. 1989). Serological evidence showed that the disease was more prevalent in Cheju island, Chungnam and Chungpuk Provinces (Chang et al. 1988b).

LABORATORY DIAGNOSIS

In endemic areas, once tsutsugamushi disease is suspected, the clinical diagnosis is considered not difficult to make because most of the patients have one or more eschars on the skin, besides acute onset, high fever of 39–40°C, rashes and lymphadenopathy, together with a history of exposure to scrubbs (Yi et al. 1987). An eschar, a black scab formed on the site of mitebiting, is found on patients with tsutsugamushi disease and spotted fever. However, in Korea, physicians were not familiar with the disease and even if the disease was suspected, an eschar was not always found. Also, the clinical features may not be typical. Yi et al. (1986) reported that all of the patients had rashes and most of them lymphadenopathy. However, the findings differed depending on the investigators. This may be accounted for by the difference in the stages of the disease. Primary care clinicians may observe more of these findings. Bourgeois et al. (1982) reported that rashes are more frequently observed in the primary infections and lymphadenopathy more frequently in the reinfections. As not all tsutsugamushi disease can be diagnosed by clinical findings, laboratory diagnosis is required.

Laboratory diagnosis includes hematological and biochemical tests of blood and microscopic examination of tissue preparations (Suto 1986). White blood cell count is generally decreased. A relative increase of neutrophils with a marked shift to the left may be observed, together with decreased platelet counts and fibrinogen levels. C-reactive protein may become positive and a moderate increase of serum aspartate aminotransferase (GOT) and alanine aminotransferase
Serologic Diagnosis of Tsutsugamushi Disease

(1) and a definite increase of lactate dehydrogenase may be observed. However, as all of these findings are nonspecific, specific tests are required for the diagnosis of tsutsugamushi disease. Microbiological and serological diagnosis of tsutsugamushi disease include the isolation of R. tsutsugamushi from blood samples of suspected patients by inoculating mice, and the detection of the antibody against R. tsutsugamushi from patients' sera. The definite determination of the etiologic agent and the antigenic type, and testing of the virulence require the isolation of R. tsutsugamushi. However, it is only possible to isolate the organism during the acute phase of the disease. It also takes at least 10 days. Virulence of R. tsutsugamushi varies depending on the strain. To increase isolation of weakly virulent strains, nude mice are often used or the mice need to be injected with cyclophosphamide along with the specimens (Kawamura 1984). The isolation requires special skills in animal inoculation and tissue culture which are not suitable for the busy clinical microbiology laboratories. Our first attempts to isolate R. tsutsugamushi from blood specimens of patients with suspected tsutsugamushi disease often failed due to premature death of the mice and bacterial contamination of L cells (Chong et al. 1989). Therefore, serologic tests are widely used for the laboratory diagnosis.

Weil-Felix Agglutination Test

There are nonspecific and specific serological tests for the diagnosis of tsutsugamushi disease. Previously, the Weil-Felix test, a nonspecific tube agglutination reaction, was widely used because of the easy availability of Proteus OXK antigen and the simple test procedure. It is possible to run the test even in a small laboratory. The problems with the test are very low sensitivity and specificity. Brown et al. (1983) reported a specificity of 0.97 when a cut-off titer of ≥320 was applied. However, the sensitivity was only 0.25 in the first week of the illness and 0.60 even after the 3rd week. In our previous studies, the patients only rarely showed a titer of ≥320 (Chong et al. 1988). In Japanese literature (Kanai and Kanai 1983), a titer of ≥40 was considered significant. This titer may be significant. I observed that sera of patients other than those with tsutsugamushi disease did not have a titer of ≥40 (Table 2). However, with the low cut-off point, a false positive test may result. Brown et al. (1983) reported false positive results in leptospirosis patients.

Complement Fixation (CF) Test

Various specific tests have been used for the diagnosis of tsutsugamushi disease. For example, the CF test has been used effectively. The test was valuable previously because of its high specificity and it can usually differentiate the type of R. tsutsugamushi infected. Lee et al. (1988) used the test in Korean patients and suggested that the infections in the central part of Korea were due to the Gilliam type, which was later proven by isolation of the rickettsia (Chang and Kang 1987; Chang et al. 1988a). However, because of its technical difficulties, the routine use of the CF test is now largely replaced by other specific tests (Kawamura 1987).

Indirect Immunofluorescent Antibody Test

The serological methods most widely used these days are the micro-indirect immunofluorescent antibody (IFA) test and micro-indirect immunoperoxidase (IIP) tests. In addition enzyme-linked immunosorbent assay (ELISA) and immune adherence hemagglutination, and an avidin-biotin complex method of the IFA test are being developed (Kawamura 1987).

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Table 1. Seropositive rate of 1,773 serum specimens from acute febrile patients against Leptospira, Rickettsia and Hantaan virus in 1987

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Positive No.</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptospira</td>
<td>219</td>
<td>(12.3)</td>
</tr>
<tr>
<td>R. tsutsugamushi</td>
<td>487</td>
<td>(27.5)</td>
</tr>
<tr>
<td>R. typhi</td>
<td>241</td>
<td>(13.6)</td>
</tr>
<tr>
<td>Hantaan virus</td>
<td>160</td>
<td>(9.0)</td>
</tr>
</tbody>
</table>

*Modified from Chang et al. (1988b).

Table 2. Weil-Felix OXK titer of control subjects

<table>
<thead>
<tr>
<th>Age group (yr)</th>
<th>&lt;10</th>
<th>10</th>
<th>20</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-19</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>20-29</td>
<td>7</td>
<td>5</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>30-39</td>
<td>8</td>
<td>1</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>40-49</td>
<td>7</td>
<td>2</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>50-59</td>
<td>9</td>
<td>1</td>
<td>4</td>
<td>14</td>
</tr>
<tr>
<td>60-69</td>
<td>6</td>
<td>2</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>Total</td>
<td>42</td>
<td>12</td>
<td>8</td>
<td>62</td>
</tr>
</tbody>
</table>

*Modified from Chong (1986).
The IFA test has many advantages over the CF test. It is more sensitive and the antigen preparation for the IFA test is easier. Peritoneal smears prepared from infected mice treated with or without cyclophosphamide can be used as antigen (Yamamoto and Murata 1987). Now antigens prepared from R. tsutsugamushi grown in established cell lines such as L cells are more often used with satisfactory results. Strains of Gilliam, Karp and Kato are usually used for the test. Although one type of antigen may react with antibodies against other antigenic types, it was recommended to include antigenic types of local prevalence to have an optimal titer. In this regard, we should include both Gilliam and Karp, as Korea is now known to have those infections. The IFA test can determine both IgG and IgM antibodies when the appropriate antiglobulin is used. It was reported that it is sometimes difficult to determine IgM antibody titer due to the competition with IgG antibody. In such cases pretreatment of test sera to remove interfering IgG antibodies may result in an accurate IgM antibody determination (Yamamoto and Murata 1987). The fluorescein labeled conjugate is very stable, and can be used for some time after making a working dilution, if kept in a refrigerator. The test is rapid and not very complicated, which makes it suitable for routine diagnostic testing. One major disadvantage is the need for a fluorescent microscope.

**Indirect Immunoperoxidase Test**

The indirect immunoperoxidase (IIP) test (Suto 1985) has the major advantage of not requiring a fluorescent microscope. The slide is read with an ordinary light microscope. Moreover the stained slide can be stored for an extended period of time and can be used for quality control or interlaboratory comparison. The titer is reported to be 4–8 times higher than the titer of the IFA test. The author compared IFA and IIP tests (Chong et al. 1988) and it was found that the two showed comparable titers (Table 3). The titer may depend on the quality of the conjugate used. Suto (1986) insists on the use of a special brand. I used a different one (Accurate Chemical and Scientific Corp., Westbury, N.Y.) for the comparison. A higher titer is an advantage because of the possibility of early detection of antibody. Suto (1985) reported that with the IIP test, IgM antibody can be detected a few days after the onset of the disease and IgG antibody several days afterwards. In the author's study, many patients showed high titer of IgG in the first week of illness (Fig. 1). A very high antibody titer can be a disadvantage in the laboratory work.

<table>
<thead>
<tr>
<th>IFA test titer</th>
<th>No. of specimens with IIP titer:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;20</td>
</tr>
<tr>
<td>≤20</td>
<td>6</td>
</tr>
<tr>
<td>40</td>
<td>4</td>
</tr>
<tr>
<td>80</td>
<td></td>
</tr>
<tr>
<td>160</td>
<td>1</td>
</tr>
<tr>
<td>320</td>
<td></td>
</tr>
<tr>
<td>640</td>
<td></td>
</tr>
<tr>
<td>≥1280</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>10</td>
</tr>
</tbody>
</table>

*Modified from Chong (1986).

![Fig. 1. Indirect immunoperoxidase test titer in paired sera from tsutsugamushi disease patients. (Modified from Chong et al. 1988).](image-url)
Serologic Diagnosis of Tsutsugamushi Disease

Table 4. Stability of conjugate for indirect immunoperoxidase test

<table>
<thead>
<tr>
<th>Refrigeration (days)</th>
<th>Reading 1:20</th>
<th>Reading 1:40</th>
<th>Reading 1:80</th>
<th>Reading 1:160</th>
<th>Reading 1:320</th>
<th>Reading 1:640</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>++*</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>IgG</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Modified from Chong et al. (1988).
** ++: Strong positive; +: Weak positive; -: negative.

demonstrate an endpoint titer of over 5120, it requires more serum dilution steps and more antigen slides. The IIP test is reported to be highly sensitive and specific for antibody detection and differentiation of the IgG and IgM class antibodies. However, the IIP test may be occasionally difficult to read. A serum dilution of 1:20 or 1:40 may show nonspecific staining. To avoid false positive results, it is recommended that if L cell components are stained brown, the result should not be read as positive even though there are definitely stained brownish rickettsia particles.

Compared to the IFA test, there are more variable factors in the IIP test procedure. Only two reaction steps, i.e., a reaction of antigen and antibody, then of antigen-antibody complex with conjugate are required in the IFA test, while an additional reaction of antigen-antibody-conjugate complex with substrate is required in the IIP test. Contrary to the conjugate for the IFA test, the conjugate for the IIP test, once diluted to working dilution, is unstable even in a refrigerator (Table 4). The substrate is known to be stable for at least 3 weeks at room temperature (Suto 1985). However, it was not as stable at room temperatures of around 30°C. In tsutsugamushi disease, the IIP antibody was reported to remain for many years after recovery. Thus, the detection of IgM antibody may be more meaningful. The author tested some patients with a past history of tsutsugamushi disease. Even after 5 years, IgG titers of 160 to 640 were observed. Therefore, this level of IgG titer may occasionally not be significant for the diagnosis of present illness.

Interpretation of the serologic tests

Because both the IFA and the IIP tests readings are done subjectively, sometimes it may be difficult to obtain reproducible titers even in the same laboratory, not to mention comparable titers in different laboratories. As with other serological tests, use of positive control serum with known titer and a negative control serum are required to obtain reproducible results. Use of good quality reagents from the same source may also help to obtain reliable results.

Interpretation of the serologic test depends on the purpose of the test and the prevalence of tsutsugamushi disease together with cross-reacting infections in a particular area (Brown et al. 1983). For determination of the prevalence of tsutsugamushi disease, IFA and IIP tests may be very useful, because the IgG antibody may be detected even long after recovery. Titers of 20 to 40 of IFA and IIP tests are usually considered to be due to antigenic stimulation of R. tsutsugamushi.

For the diagnosis of a present illness, however, the interpretation of the serological tests result is not always so simple. As in other serological tests, demonstration of a 4 fold rise of the titer in convalescent sera is the most significant evidence of tsutsugamushi disease. However, it is not always possible to collect the convalescent sera and moreover, it is not useful for the diagnosis of suspected patients, except for retrospective confirmation of the diagnosis.

Despite its simple procedure, the Weil-Felix test is not recommended for the diagnosis of the disease because of its low sensitivity. A positive result only is significant. As to the cut-off point, a titer of ≥320 may be too high. The normal population does not have such high levels of antibody. In areas where tsutsugamushi disease is prevalent and cross-reacting diseases are rare, a titer of ≥40 may be due to the disease.

The IFA and IIP tests detect specific antibodies to R. tsutsugamushi. Although it was reported that cross reaction between R. tsutsugamushi and other rickettsiae does not exist, we may need more experience. Considering the persistence of the titer after recovery,
a single IgG antibody titer of ≥ 320 may be significant for the diagnosis of present illness. Determination of IgM antibody may resolve some problems with IgG antibody determination. However, until we have more information on the antibody titer of the normal population, we need careful interpretation of the tests. It may be useful to follow Suto's (1986) scheme in the interpretation of the IIP test. He recommended interpretation of the result based on both IgG and IgM levels together with the stage of the disease and presence of an eschar.

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