

## LABORATORY DIAGNOSIS OF SCRUB TYPHUS

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### Summary

Laboratory diagnosis of scrub typhus is achieved by the isolation of organisms and serological means. Since the isolation procedure is long and laborious, serological diagnosis is the most common tool. Weil-Felix reaction is most widely used, but has definite limitations. The preferred and most reliable test for diagnosing scrub typhus is the indirect immunofluorescent technique.

Diagnosis of rickettsial diseases relies principally on clinical recognition and is aided by such epidemiological considerations as residence or recent travel in endemic areas and recent exposures to relevant arthropods. Recognition of scrub typhus on the basis of clinical signs and symptoms is often difficult due to the variability of disease manifestations which in many instances mimic those of other infectious diseases present in the same area. An eschar, rash and generalized or regional lymphadenopathy are the main features of the illness. However, in many patients, one or more of these findings are not present. Symptoms as headache, fever, and conjunctival injection are not specific enough to allow differentiation of scrub typhus from other common infections such as murine typhus, leptospirosis, typhoid fever, and dengue.

Serological tests are useful to confirm an initial clinical diagnosis or to establish a retrospective diagnosis of rickettsial disease. The tests depend on showing a rise in antibody titer during the course of the infection. When the test results become available to the clinician, the patient has already recovered, is undergoing treatment, or has died. All rickettsial infections can be treated successfully with broad spectrum antibiotics, provided therapy is started early in the disease. Therefore, early clinical diagnosis and treatment are critical in preventing severe disease and death.

### ISOLATION PROCEDURES

Generally, the only specimens pertinent to laboratory diagnosis are blood samples or tissues obtained under aseptic conditions at autopsy. If isolation of the organism is attempted, the specimen should be processed

and inoculated into animals immediately. If this is not possible, the specimen should be placed in a glass or plastic container, quick-frozen and maintained at  $-65^{\circ}\text{C}$  until examined.

Smears of infected tissues may be made and examined directly for the presence of rickettsiae after staining with Giemsa stain. The organisms are usually very scarce in these smears, so passage of the tissue material in laboratory animals is highly recommended.

The specific diagnosis of scrub typhus is made by isolating *Rickettsia tsutsugamushi* from the patient's blood during the acute illness or from tissues obtained at necropsy. A suspension of ground blood clot or of tissue is inoculated intraperitoneally into white mice.<sup>1</sup> Signs of infection in mice include inactivity, ruffled fur and ascites. Those mice infected with the virulent strains may die within 10 to 24 days after inoculation. Microscopic examination of impression smears made from the spleen surface or the scrapings from the parietal peritoneum of moribund mice and stained by Giemsa method reveal both intracellular and extracellular rickettsiae. Routine isolation in mice is long and laborious, often requiring between 2 to 3 months before definitive diagnosis can be given. Strains of *R tsutsugamushi* vary greatly in their virulence for mice regardless of whether they were recovered from chiggers, small mammals or humans.

Another laboratory host used to propagate the organisms is the developing chick embryo. Established strains of *R tsutsugamushi* grow well in the yolk sacs of embryonated eggs from hens maintained on antibiotic-free feed. The yolk sacs of the 5 to 6 day old embryos are inoculated, and the eggs are incubated at  $35^{\circ}\text{C}$ . Depending on the infectious nature of the inoculum, death occurs between 6 to 12 days

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without pathognomonic lesions. The cultivation of the rickettsiae in yolk sacs has made these organisms available in great quantity and sufficient purity to provide stock suspensions and antigens for different serological tests. Although adapted strains can readily propagate, primary isolation of rickettsiae by this means is less reliable. Nevertheless, adaptation of the organisms to cultivation in embryonated eggs is necessary, if the strain of rickettsiae cannot be characterized due to the reduced virulence for laboratory mice. This procedure is very tedious and difficult, and must be performed in a sterile manner. Adaptation may occur after a few passages, but usually many months of intensive effort are required.

As described previously, the standard methods for isolating rickettsial organisms are long and laborious. Recently, the detection of scrub typhus rickettsiae in peripheral monocyte cultures derived from experimentally infected monkeys and dogs has been reported.<sup>2</sup> The technique is simple and inexpensive, and the time required for isolation is markedly reduced. The evaluation of this culture technique for the isolation of *R tsutsugamushi* from human scrub typhus patients is in progress.

#### SEROLOGICAL PROCEDURES

The disease is most often diagnosed serologically by analyzing the antibodies that appear during the course of the illness. Serum specimens should be obtained during the acute and convalescent stages of the disease and ideally tested simultaneously.

The first serological procedure developed for a rickettsial infection was the Weil-Felix reaction,<sup>3</sup> and despite its limitations, it is still widely used. This is probably due to the easy accessibility of the antigens and the simplicity of performance. This reaction is based on the fact that the antibodies produced in certain rickettsial diseases react with the polysaccharide O antigens of the X strains of *Proteus* species. Epidemic and murine typhus infections elicit OX19 agglutinins, while spotted fever group infections, except rickettsialpox, develop agglutinins for OX19 or OX2 or both. The agglutinins to OXK appear usually 10 to 14 days after onset of scrub typhus and decline rapidly over the next several months. However, only 40 to 60% of the scrub typhus patients develop the OXK agglutinins.<sup>4</sup> Because of the

ubiquity of the *Proteus* species and the occurrence of low-titered antibodies to them in a high percentage of the population, serum titers are not considered significant unless they are 1:160 or greater. Frequently, titers do not rise to 1:160 and only a tentative diagnosis can be made on 4-fold rise in titer, eg from 1:20 to 1:80, during the course of the disease. False positive reactions have been seen with sera from patients with *Proteus* urinary tract infections, leptospirosis<sup>5</sup> and relapsing fever.<sup>6</sup> A negative Weil-Felix reaction does not exclude scrub typhus infection. Therefore, this provides only a presumptive diagnosis. Additional tests using specific rickettsial antigens are required.

The most common serological technique used for the diagnosis of rickettsial diseases is the complement fixation test. However, this test is not widely used in scrub typhus diagnosis, because of the marked diversity of antigenic strains of *R tsutsugamushi*. It becomes impractical to produce strain-specific complement fixation antigens for all existing strains.

Currently, the preferred serological test for scrub typhus is the indirect fluorescent antibody technique.<sup>7</sup> The antigen-antibody reaction, if present, is identified by staining the complex with a fluorescein-labeled anti-human globulin and noting the intensity of fluorescence of the rickettsiae. This technique is less strain-specific than the complement fixation reaction. The use of multiple prototype antigens is recommended to detect a significant diagnostic rise in antibody titer. If the acute serum is collected relatively late in the course of illness, the antibody level with one antigen might be of such magnitude that a diagnostic rise can only be demonstrated with other antigens. Presently, in our laboratory 8 strains of *R tsutsugamushi* are used in the test system. This test provides a means of establishing a serological diagnosis of the first and successive illnesses caused by scrub typhus organisms. It is the most reliable test available for serological diagnosis of scrub typhus. It must be mentioned that this technique is neither simple nor inexpensive. Its use in other than the reference laboratories depends upon the availability of fresh, infected yolk sacs for making the smears, since commercial preparations are unavailable.

The direct fluorescent antibody technique

can be used to identify *R tsutsugamushi* organisms in cells or tissues of humans and animals.<sup>8</sup> Recently, the technique has been applied to the identification of rickettsiae in naturally-infected chiggers.<sup>9</sup> In this technique, the globulin of the strain-specific antibody is conjugated with the fluorescein isothiocyanate, and the resulting conjugate is applied directly to the antigen. Since this reaction is strain-specific, new antigenic strains, not in the test system, may go undetected.

Recent advances in technology have prompted the application of various new tests for the detection of antibody, and the work on enzyme-linked immunoabsorbent assay (ELISA) with scrub typhus antigen appears to be most promising. In this test, the enzyme, which is used to label the anti-globulin, degrades the added substrate, when the labeled conjugate is attached to the specific antigen-antibody complex. If this test is successful, it should provide a means for assaying scrub typhus antibody with high degree of specificity and sensitivity.

One word of caution is necessary. That is, patients treated with antibiotics early in the disease may have delayed antibody responses, sometimes as late as 4 to 6 weeks after onset of symptoms. One should be aware of this when evaluating the results of serological tests, especially the complement fixation and agglutination reactions.

Evidence of early involvement of sensitized lymphocytes during immune response in animals experimentally infected with scrub typhus<sup>10, 11</sup> as well as the urgent need of an early diagnostic test have led research workers to evaluate in-vitro assays for cell-mediated immunity as diagnostic tools. Some of the tests which assess lymphocyte function measure their ability to proliferate (lymphocyte transformation) and their ability to produce mediators (migration inhibitory factor; leucocyte inhibitory factor; among others). These methods are still far from being incorporated into the pool of routine diagnostic tests.

In conclusion, the usefulness of serological data for diagnosis is highly dependent on the nature of the specimens submitted. For most infectious diseases, meaningful results must demonstrate a minimum of 4-fold rise in antibody titers between the acute and the con-

valescent sera. One cannot overemphasize the significance of obtaining an early serum specimen prior to an antibody rise and a suitable convalescent serum. It is also extremely important to provide sufficient clinical information so that the laboratory personnel can select the most appropriate tests. Optimal utilization of the diagnostic laboratory requires effective communication with the clinicians who require the test results.

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