



Laboratory Diagnosis of Scrub Typhus

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Introduction

Scrub typhus is an important cause of acute undifferentiated febrile illnesses in India. However, diagnosed with difficulty, because of its nonspecific clinical presentation, low index of suspicion and the lack of diagnostic facilities. The current review discuss the laboratory diagnosis of Scrub Typhus.

Laboratory Diagnosis: Different types of samples can be collected for laboratory investigation but it depends on diagnostic method to be used. The laboratory should be contacted in advance to decide on the types of specimen to be collected. (*Table 1*)

Serological Tests: For a test to be useful in the diagnosis of an acute rickettsial infection, the most important criteria are sensitivity and the length of delay between the onset and appearance of detectable antibody titers. Conversely, when the test is to be used for seroepidemiologic studies, it should be highly specific to prevent false-positive results due to cross-reacting antibodies. In primary infection with *O. tsutsugamushi*, a significant antibody titer is observed at the end of the first week, concomitant with the detection of IgM antibodies, whereas IgG antibodies appear at the end of the second week. In the case of reinfection with *O. tsutsugamushi*, IgG antibodies are detectable by day 6, with IgM antibody titers being variable (1).

a) Weil-Felix Test: The cheapest and most easily available serological test but is notoriously unreliable. The Weil-Felix test is based on the detection of antibodies to alkali based carbohydrate antigen which are shared by some rickettsiae and certain strains of *Proteus* species, *P. vulgaris* OX19, and OX2 and *P. mirabilis* OXK. The OX-K strain of *Proteus* mirabilis was demonstrated to agglutinate with sera from scrub typhus patients and was further used in the diagnosis of *O. tsutsugamushi*- related infections. In India the antigen can be procured from Central Research Institute (CRI), Kasauli, Himachal Pradesh. By the Weil-Felix test, agglutinating antibodies are detectable after 5 to 10 days following the onset of symptoms, with the antibodies detected being mainly of the immunoglobulin M (IgM) type. Fifty per cent of patients have a positive test result during the second week with a titre of 1:1000 to 1:1500 which declines rapidly during convalescence (2).

b) Complement Fixation (CF) Test: With the development of techniques for growing rickettsiae, the complement fixation (CF) test was adapted for the detection of antibodies

specific for rickettsiae. This test is now being replaced by a complement-fixation test. It is a serological test to detect specific antibody or specific antigen in a patient's serum. Each patient's serum is systematically tested against five *O. tsutsugamushi* serotypes. An IgM titer >1:32 and/or a four-fold increase of titers between two sera confirm a recent infection. However, due to cross-reactions among serotypes, it is difficult to identify accurately a specific serotype (3). Washed particulate rickettsial antigens are species specific for the typhus group, but cross-reacting antibodies among groups are observed (4). The CF test is strain specific for *O. tsutsugamushi*. This specificity, particularly with acute-phase sera, implies that all strains endemic to a region must be used to ensure the detection of every positive serum specimen (5). Antibody titres obtained by the CF test correlate better with IgG titers than with IgM titers obtained by immunofluorescence assay.

c) Indirect Hemagglutination Test: The indirect hemagglutination test detects antibodies to an antigenic erythrocyte-sensitizing substance (ESS) used to coat human or sheep erythrocytes that are either fresh or fixed in glutaraldehyde. The ESS is rickettsial group specific with cross-reactivity among Rocky mountain spotted fever (RMSF) and rickettsialpox (6). This test detects both IgG and IgM antibodies, but agglutination is more efficient with IgM antibodies

d) Latex Agglutination Test: In the latex agglutination test, ESS is used to coat latex beads. The reactivity is not exactly the same as that of the indirect hemagglutination test, because the ESS on latex beads probably contains more antigenic fractions than the ESS adsorbed onto erythrocytes. This test is rapid (15 min) and does not require elaborate instrumentation. Latex agglutination is reactive with IgG and IgM antibodies, but the agglutination efficiency of this test is greater when the antirickettsial IgM/IgG ratio is 1. This test allows the demonstration of antibodies within 1 week after the onset of illness. Significant antibody titers disappear after 2 months.

e) Enzyme-linked Immunosorbent Assay (ELISA): ELISA was first introduced for detection of antibodies against *Rickettsia typhi* and *Rickettsia prowazekii*. The use of this technique is highly sensitive and reproducible, allowing the differentiation of IgG and IgM antibodies. This technique

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was later adapted to the diagnosis of Rocky Mountain Spotted fever (RMSF) and scrub typhus. A "paper ELISA," was proposed for the detection of anti-O. tsutsugamushi antibodies. Its first steps are similar to those used for the IFA, but an anti-human IgG peroxidase conjugate and substrate-saturated filter paper, on which the reaction is visualized, are used (7).

f) Indirect Immunofluorescence Antibody (IFA): IFA is the gold standard and is used as a reference technique in most laboratories. For scrub typhus, the sensitivity of IFA is low if high specificity is required. Detection of rickettsiae by using immunofluorescence allows the confirmation of infection in patients prior to their seroconversion. Samples can be tested fresh or after formalin fixation and paraffin embedment. Biopsy specimens of the skin with a rash around the lesion, preferably petechial lesions are the most common samples used. In animals or patients with fatal cases of infection, bacteria are detectable at autopsy in the tissues of numerous organs such as liver, spleen, kidney, heart, meningeal membranes, or skin (8,9).

g) Indirect Immunoperoxidase (IIP): IIP is a modification of the standard IFA method that can be used with a light microscope, and the results of these tests are comparable to those from IFA. An immunoperoxidase assay has been developed as an alternative to IFA for the diagnosis of scrub typhus. The procedure is the same as IFA, but fluorescein is replaced by peroxidase. The advantage of the immunoperoxidase assay is that the results can be read with an ordinary light microscope. In addition, it provides a permanent slide record (10-12).

h) Microimmunofluorescence: The rickettsial IFA adapted to a micromethod format is the test of choice for the serodiagnosis of rickettsial diseases (13). The micro-IFA has the advantage that it can simultaneously detect antibodies to a number of rickettsial antigens (up to nine antigens) with the same drop of serum in a single well containing multiple rickettsial antigen dots. IFA allows the detection of IgG and IgM antibodies or both. The identification by IFA of specific IgM antibodies to the various species of rickettsiae provides strong evidence of recent active infection, although the diagnosis may be obscured by a prozone phenomenon. This technique is, furthermore, affected by RF, thus requiring the use of a RF absorbent before IgM determination.

i) Western Immunoblot: Western immunoblot assay with sodium dodecyl sulfate-gelelectrophoresed and electroblotted antigens is a powerful serodiagnostic tool for seroepidemiology and confirmation of serologic diagnoses obtained by conventional tests. It is especially useful in differentiating true-positive from false-positive results created by cross-reacting antibodies.

j) Line blot Assay: The line blot assay allows the testing of more than 45 antigens simultaneously. It is a useful test for large-scale screening of sera when quantitative titers

are not needed or when tests against a large number of agents are required that might be considered for patients with nonspecific or atypical clinical presentations (14).

Isolation of Rickettsiae: In the past, only research laboratories that had biosafety level 3 containment and personnel with extensive experience in cultivating rickettsiae were able to isolate rickettsiae from clinical specimens but results are not available in time to guide clinical management. During recent years, the development of cell culture systems for viral isolation has led to an increase in the number of laboratories suitably equipped to isolate rickettsiae. Since different rickettsial diseases may have indistinguishable clinical manifestations, the isolation of new isolates followed by their molecular characterization is critical for the discovery of new rickettsial diseases. The isolation of rickettsiae may be attempted with several samples: buffy coat of heparinized blood, defibrinated whole blood, triturated clot, plasma, necropsy tissue, skin biopsy, and arthropod samples.

A) Embryonated chicken egg yolk sacs: have been widely used in the past, but they are now being replaced by cell culture systems. The mouse is the species of choice for the isolation of *R. akari*, *Rickettsia australis*, and especially *O. tsutsugamushi*. Blood clot ground in skimmed milk or any suitable medium is inoculated intraperitoneally. The animals have to be observed for 3-4 weeks. Smears from peritoneum, tunica and spleen of animal, stained by Giemsa or Gimenez methods demonstrates rickettsiae.

B) Cell cultures: Cell culture, described for more than 60 years, is now the most widely used method for isolating rickettsiae from clinical samples. Vero cells, MRC 5 cells are being used frequently but L929 mouse fibroblast cell monolayer in tube culture is best suited for the isolation of *R. rickettsii* and *O. tsutsugamushi* from blood (15).

C) Shell Vial Assay: More recently, the shell vial assay, detection of the microorganism being possible in 48 to 72 h in most cases (16). Inoculation should be made onto two types of cells. Vero or L929 cells have been shown to allow better and faster isolation of rickettsiae, especially from heavily infected samples, than HEL or MRC5 cells (17). Nevertheless, HEL or MRC5 cells have the advantage that once a monolayer is established, contact inhibition prevents further division and the cells can then be used for prolonged incubation. For an optimal yield, blood should be collected on heparin anticoagulant, avoiding EDTA or sodium citrate, which leads to detachment of the cell monolayer from coverslips. Erythrocytes should not be inoculated onto shell vials because they lead to high background levels at the time of examination with a UV microscope. The centrifugation step after inoculation of the shell vial is critical for the sensitivity of the technique, because it enhances rickettsial attachment to and penetration of cells (17). Delay between the time of sample collection and inoculation onto shell vials also appeared to be critical. Presumptive

Table 1. Specimen Collection and Transport

| Procedure | Specimen | Shipment |
|-----------------------|---|---|
| Culture | Skin or l. node biopsy, Heparinised blood (Buffy coat), Defibrinated whole blood Triturated clot,Triturated clot,Plasma Necropsy tissue & arthropod samples | Over dry ice at -80°C |
| PCR | Skin rash, lymph node biopsies or EDTA Blood | Ship at room Temp EDTA blood : Conserve at +4°C and then ship at room temp for PCR. |
| Serology | Serum: Collect two serum specimens 10 days apart | Conserve at +4°C, then ship at room Temp |
| Immuno histochemistry | Skin rash biopsies lymph node biopsies | Formalin or paraffin-embedded shiped at room temp |

identification of a rickettsial isolate may be achieved by microscopic examination after staining. Rickettsiae appear as short rods, not stained by staining with the Gram stain but which are visible after Giemsa or Gimenez staining.

Molecular Biology-Based Identification: The first proposed molecular biology-based identification method was based on PCR-restriction fragment length polymorphism (RFLP) analysis of the gene encoding the OmpA protein. Molecular detection using polymerase chain reaction (PCR) is possible from skin rash biopsies, lymph node biopsies or ethylenediaminetetraacetic acid (EDTA) blood. O. tsutsugamushi can be demonstrated by standard and by nested PCR. Realtime PCR assays are as sensitive as standard PCR but are more rapid and can give quantitative

Table 2. Characteristics of Different Diagnostic Test

| Test | Indication | Advantage | Drawbacks | Remarks |
|-------------------------|---|---|---|--|
| Shell vial technique | Isolation from blood, tissue & arthropods | Characterization of etiologic agent, +ve result 3 days after sampling, +ve result before antibody titer rise | Limited to laboratories with biohazard facilities (BSL-3)Vials need to be inoculated immediately -ve for patients with prior antibiotic therapy | Identification of new pathogens, Allows early diagnosis before seroconversion |
| Molecular Detection PCR | Isolation from blood, tissue & arthropods | Not limited to BSL-3 labs. Results after 24 hrs after sampling May be +ve in patients on antibiotics | Facilities for molecular based tests are required | For early diagnosis before seroconversion Useful for screening arthropods |
| Immuno-detection | Isolation from blood, tissue & arthropods | Available in advanced pathology labs, +ve result 2 days after sampling May be +ve for patients on antibiotics | Requires experienced personnel | For early diagnosis before seroconversion, especially in patients with inoculation eschar |
| Weil-Felix test | Serodiagnosis | Inexpensive test | Lacks both sensitivity & specificity | Should be used only in very poor countries for diagnosis of acute cases |
| CF test | Serodiagnosis | High Specificity | Lacks both sensitivity early in disease | Should be used only for seroepidemiologic studies |
| Indirect HA | Serodiagnosis | Both Specificity & Sensitivity Early detectable antibodies | Low antibody titers in late convalescent-Phase sera | Should be used only for seroepidemiologic studies |
| Latex agglutination | Serodiagnosis | Simple, no expensive material required, commercially available | Expensive kit | Should be used in nonequipped laboratory |
| ELISA | Serodiagnosis | Both specific and sensitive | Expensive kit | Useful for both diagnosis of acute cases & seroepidemiology |
| Microo-IF | Serodiagnosis | Both specific and sensitive, commercially available | Requires fluorescence Microscope | Reference technique in most laboratories, useful diagnosis of acute cases & seroepidemiology |
| Immuno peroxidase | Serodiagnosis | Both specific and sensitive, does not require fluorescence microscope | Except for scrub typhus, cannot be used for largescale evaluation | Alternative technique to IFA. Allows permanent slide records. |
| Line blot | Serodiagnosis | Both specific and sensitive. Large number of antigens tested simultaneously. | No quantitative titers available | Large-scale screening for seroepidemiologic studies |
| Western immunoblot | Serodiagnosis | Most specific and sensitive serologic test, earliest detectable antibodies | Time-consuming | Probably best serologic tool for seroepidemiologic studies |

results(18).PCR-based detection in published reports has been based on amplification of the gene encoding the 56-kDa antigen for O.tsutsugamushi (19,20).

When and which Diagnostic Tests: The test that is most appropriate for use during the acute phase is that which detects rickettsiae in endothelial cells, followed by specific gene amplification by PCR, immunodetection with tissue biopsy specimens, and the shell vial assay (*Table-2*) (21). Because the most important considerations in the choice of a serologic assay in this situation are its sensitivity and the length of delay between the onset and appearance of detectable antibody titers, laboratories so equipped should use IFA (especially tests specific for IgM). For laboratories without a UV microscope, the indirect immunoperoxidase and latex agglutination test is a good alternative for screening sera in a laboratory not equipped with a UV microscope, but it remains too expensive for use in very poor countries, in which case the Weil-Felix test is probably the best alternative. In the case of acute infections, a case should be confirmed if testing reveals an IFA titer greater than or equal to the cutoff (which should be defined for each rickettsial disease and each area) or a fourfold rise in titer by the CF test, IFA, the microagglutination test, the latex agglutination test, or the hemagglutination assay. Doubtful cases should be investigated by Western immunoblot assay.

IFA should be considered a technique for seroepidemiology only in areas where the seroprevalence of rickettsial disease has already been established. The line blot assay should be considered as a seroepidemiologic tool since it allows the large-scale screening of sera on numerous agents in the same assay. The Western immunoblot assay is probably the most specific tool for determining the real prevalence of rickettsial diseases. In areas where a rickettsial disease is endemic. Western immunoblot assay is a useful tool to explore a possible cross-reaction with the endemic strain. Recovery of rickettsiae from resident arthropods by the shell vial assay must also be attempted in order to identify potential pathogens for humans. In all cases, the isolation and characterization of the causative pathogen from clinical samples is the definitive test. This test can be augmented with specific PCR amplifications with skin biopsy specimens. (*Table-2*) (21)

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