Serological Diagnosis of Scrub Typhus by Indirect Immunofluorescence. (28107)

F. Marilyn Bozeman and Bennett L. Elisberg (Introduced by E. B. Jackson)
Department of Rickettsial Diseases, Walter Reed Army Institute of Research, Washington, D.C.

Three different laboratory approaches are currently available for detection of *Rickettsia tsutsugamushi* infections in man. The simplest of these is the demonstration of agglutinins for *Proteus mirabilis* OXK in the patient's serum. This procedure, known as the Weil-Felix Reaction (WFR), has 2 serious limitations: (a) not all scrub typhus patients develop OXK antibody(1,2), a positive WFR being demonstrable at times in no more than 50% of the cases(3); and (b) these antibodies may appear in the sera of patients infected with louse-borne relapsing fever(4) or leptospirosis(3,5) in the absence of concurrent scrub typhus. The complement fixation reaction (CFR) is unsatisfactory owing to the high degree of strain specific reactivity of "soluble" antigens prepared from *R. tsutsugamushi* and the marked antigenic heterogeneity that exists among various strains of this rickettsia causing human disease(6,7). Although the most reliable evidence of infection is recovery of the specific rickettsiae in mice inoculated with the febrile patient's blood, strains have been encountered which cause disease in man, but are virtually avirulent for mice(3). In such instances, infection of test mice can be demonstrated only by their ability to survive challenge with a known pathogenic strain—a lengthy procedure which often takes 5-6 weeks. From consideration of the serious deficiencies in currently available methods for laboratory diagnosis of scrub typhus, the need for a rapid reliable diagnostic test is obvious.

An immunofluorescent reaction which can be used for specific diagnosis of scrub typhus infection in man is described here. It is patterned after the system used by Goldwasser and Shepard(8) in their studies of epidemic and murine typhus infections.

Materials and methods. Preparation of antigen smears. The Karp and Gilliam strains of *R. tsutsugamushi, R. mooseri* (Wilmington strain), *R. prowazekii* (Breinl strain), *R. rickettsii* (Bitterroot strain), *R. burnetii* (Henzerling strain), *Proteus mirabilis* OXK, and the OX19 and OX2 strains of *Proteus vulgaris* were used to prepare smears for fluorescent staining.

The rickettsiae were grown in the yolk sacs of embryonated eggs. Heavily infected yolk sacs, as judged by examination of Macchiavello or Giemsa stained smears were triturated individually in McIlvaine's buffered saline pH 7.6 to make a 20% suspension. This suspension was centrifuged at 1000 rpm for 2 minutes, and the mid-zone portion removed. In order not to have too many organisms in the smears, this material was diluted further with buffered saline; a 1:5 to 1:20 dilution was made depending upon the concentration of rickettsiae in the original yolk sac. Smears were made by placing 0.005 ml of the diluted infected yolk sac suspension on a slide and allowing it to spread. Four smears, each about 5-6 mm in diameter, were placed on one slide. Several hundred slides were prepared at one time. These preparations were air-dried, fixed in acetone for 10 minutes at room temperature, and stored in microscope slide boxes at −20°C until used. No deleterious effect was noted after storage for 6 months.

Smears of the *Proteus* strains were made from a 1:10 dilution of standard Weil-Felix antigens(9) prepared at Walter Reed Army Institute of Research; they were heat-fixed and stored at −20°C.

Scrub typhus sera. Sera collected in Malaya during the illness and convalescence of 15 scrub typhus patients were tested. Diagnosis was established in each case by isolation of a strain of *R. tsutsugamushi.* The majority of the cases were sporadic and occurred in different areas of the country. Six of the patients had nondescript, mild illnesses, and 9 were moderately severe cases with typical clinical features. Eleven of the patients were treated with chloramphenicol; 4 had no

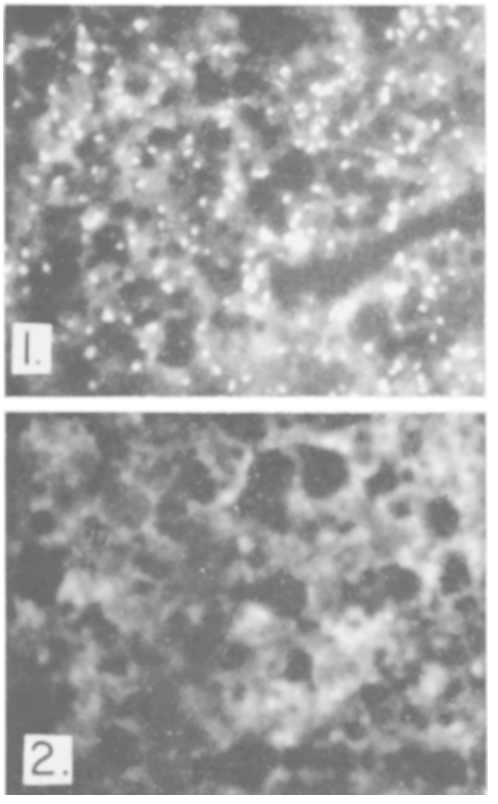

FIG. 1. Photomicrographs of *R. tsutsugamushi* (Gilliam strain) in smears of yolk sac material stained by indirect immunofluorescence. (1) Positive reaction with 1:40 dilution of 22-day serum from scrub typhus patient ST-14. Bright spots in photomicrograph represent fluorescing rickettsiae; they appeared bright yellow-green against a dull green background on direct examination. (2) No rickettsiae were visible when 1:10 dilution of normal human serum was added to smear. (420×)

smears were air-dried and mounted in buffered glycerin pH 7.2 for examination.

Examination of preparations. Slides were examined the same day they were prepared. The intensity of fluorescence was recorded from 0 (no fluorescence) to 4+ for maximum fluorescence. Degree of fluorescence was recorded for each serum dilution. The final fluorescent antibody titer of a serum was designated as the highest dilution which gave a 1+ fluorescence. Fig. 1 (top) shows positive fluorescence of *R. tsutsugamushi* when the smear was treated with scrub typhus convalescent serum. The rickettsiae were not stained when normal human serum was added to the smears (lower picture).

Results. The results of the immunofluorescent tests on the sera from the 15 scrub typhus patients in Malaya are presented in Table II. Increases in antibody against both Karp and Gilliam rickettsiae were demonstrated in all the cases. The final fluorescent antibody titers, however, varied slightly, depending on the strain employed. In 9 of the cases, there was a 16-fold or greater increase in antibody titer between the initial and convalescent serum specimens with both the Karp and Gilliam antigens. Three other patients had at least a 16-fold rise with one of the antigens, but the increment was less with the other strain. The last three patients displayed a 4-fold increase with both antigens.

With this group of sera, Gilliam was somewhat the better or more sensitive antigen, *i.e.*, in 10 of the patients, consistently higher titers (4-fold or greater) were obtained with Gilliam than with Karp strain rickettsiae, while Karp yielded higher titers in 2 instances. The sera from the other 3 patients titered the same with both antigens. Irrespective of final titers, a serological diagnosis of scrub typhus could have been made on all of the patients employing either antigen, with the exception of ST-12. This particular patient's second serum, drawn on the 12th day, titered only 1:10 with the Karp strain, but 1:640 with Gilliam.

In the present study, the number of cases from whom specimens were collected at optimal times are too few to permit a meaningful analysis of time of appearance, rate of development, and persistence of antibodies. However, the following comments are warranted. Antibody was demonstrated regularly in sera collected after the 7th day of illness, and in several instances low levels of antibody (1:10 and 1:40) were found in sera taken before the 7th day. Significant titers were present during the latter part of the second week and increased thereafter, reaching a maximum sometime during the 3rd and 4th weeks. Antibody was still present in a specimen obtained 6 months after the onset of illness.

Only 6 of the 15 scrub typhus patients exhibited significant rises in *Proteus* OXK agglutinins in the WFR. Tests in which fluorescent staining of *Proteus* OXK bacilli was

TABLE I. Sources of Sera Used to Evaluate the Specificity of the Scrub Typhus Immunofluorescent Reaction.

Disease	Country	No. of patients
Rickettsial infections		
Murine typhus	Malaya	4
	U.S.	1
Tick typhus	Malaya	3
Spotted fever	U.S.	3
Q fever	Malaya	5
Bacterial infections		
Relapsing fever (louse-borne)	Ethiopia	7*
(tick-borne)	U.S.	1†
Leptospirosis	Puerto Rico	4‡
	U.S.	4‡
Proteus (urinary tract)	U.S.	3

* Received from Dr. E. S. Murray, Harvard School of Public Health.

† Received from Dr. John D. Keye, Shasta Co. Dept. of Public Health, Calif.

‡ Received from Dr. A. D. Alexander, Division of Veterinary Med., Walter Reed Army Inst. of Research.

antibiotic therapy. Only 6 patients developed significant increases in *Proteus* OXK agglutinins. None of these scrub typhus patients' sera fixed complement at an initial dilution of 1:10 in the presence of 16 units of soluble antigen(10) prepared from yolk sacs infected with either Karp or Gilliam strains.

The strains of *R. tsutsugamushi* recovered from these patients showed considerable variation in the severity of the disease produced in adult white mice following intraperitoneal inoculation. Three of the strains maintained in mice consistently killed all the mice inoculated with a 20% spleen suspension; 2 were mildly virulent, causing occasional deaths during the third week following inoculation, while 10 strains infected mice, but did not kill them.

Control sera. Table I summarizes pertinent information concerning the sources and numbers of control sera used to evaluate the specificity of the fluorescent antibody test for diagnosis of scrub typhus. Sera from persons infected with 2 biologically different classes of agents were selected for testing. These were from patients with (a) murine typhus, tick typhus, and Q fever which are the other rickettsioses known to be endemic in Malaya, and (b) from certain non-rickettsial infections which may evoke a *Proteus* OXK agglutinin response, *i.e.*, louse- and tick-borne relapsing fevers, leptospirosis, and *Proteus* urinary tract infections. All of the patients in the group of bacterial infections had *Proteus* OXK agglutinins except the tick-borne relapsing fever case.

Normal yolk sac diluent. Yolk sacs harvested from uninoculated 11-12 day old hen's eggs were homogenized with phosphate buffered saline pH 6.0 to make a 50% suspension. The suspension was extracted for 1-2 hours in a separatory funnel with 2 volumes of ether-alcohol mixture (9 parts absolute ether—1 part 95% ethyl alcohol). The aqueous phase was reextracted with 1 volume of alcoholic ether. After removing the excess ether under vacuum, the suspension was centrifuged at 1500 rpm for 10 minutes. The supernatant fluid was removed, adjusted to pH 7.2 with NaOH and stored at −20°C.

Staining of preparations. Prior to use, each antigen smear was ringed with a vaseline-ether-sudan black solution to prevent serum placed on one smear from running into the serum on an adjacent smear. Four-fold serial dilutions of serum, beginning at 1:10, were prepared in normal yolk sac diluent. Unless the sera were diluted at least 1:10, non-specific staining of the background made estimation of intensity of fluorescence of the rickettsiae difficult. A 0.01 ml aliquot of the diluted serum was layered on smears of each of the different rickettsial and bacterial antigens. The slides, kept horizontally, were placed in a plastic slide box kept humid with wet filter paper, and incubated at 35°C for 30 minutes. The sera were removed by rinsing the slides for a total of 10 minutes in 2 changes of buffered saline pH 7.2 and the slides were allowed to dry in the air at room temperature. Fluorescein isothiocyanate labelled anti-human horse globulin (Roboz Surgical Instrument Co., Washington, D.C.) was diluted with an equal volume of normal yolk sac diluent and 0.01 ml of the diluted conjugated globulin was added to each smear. The slides were incubated in a moist chamber, as before, for 1½ hour. The excess conjugate was washed off by twice immersing the slides in buffered saline for 5 minutes, followed by a final rinse in distilled water. The

TABLE II. Diagnosis of Scrub Typhus by Immunofluorescence.

Patient No.	D.D.†	Weil-Felix OXK	Intensity of fluorescence* of *Rickettsia tsutsugamushi* — Karp strain, Serum dilutions 10	40	160	640	2560	10240	Titer	Gilliam strain, Serum dilutions 10	40	160	640	2560	10240	Titer
ST- 1	1	<20	0	0	0	0			<10	1	0	0	0			10
	31	<20	4	3	2	1	0		640	4	3	2	1	0		640
ST- 2	3	<20	1	±	0	0			10	1	±	0	0			10
	15	40	3	2	1	0	0	0	160	4	3	2	1	±	0	640
ST- 3	3	40	0	0	0	0			<10	0	0	0	0			<10
	13	2560	3	2	1	0	0		160	4	3	2	1	0	0	640
ST- 4	3	<20	0	0	0	0			<10	0	0	0	0			<10
	26	<20	4	2	1	0	0		160	4	4	2	1	0	0	640
ST- 5	5	<20	0	0	0	0			<10	±	0	0	0			<10
	15	<20	3	2	1	±	0	0	160	4	3	2	1	±	0	640
	20	<20	4	3	2	1	0	0	640	4	3	2	1	0	0	640
	70	<20	4	2	1	0	0	0	160	4	3	2	1	0	0	640
ST- 6	5	20	2	1	0	0			40	3	2	±	0	0		40
	12	80	4	3	2	1	0	0	640	4	3	3	2	1	0	2560
	62	160	3	2	1	0	0	0	160	4	3	2	1	0	0	640
ST- 7	5	<20	2	1	±	0			40	±	0	0	0			<10
	13	160	4	3	3	2	2	1	10240	4	3	2	2	1	±	2560
	40	<20	4	4	3	2	1	0	2560	4	3	2	1	±	0	640
ST- 8	6	<20	0	0	0	0			<10	0	0	0	0			<10
	13	<20	3	2	1	±	0	0	160	3	2	1	0	0	0	160
	21	320	4	3	2	1	0	0	640	4	3	2	1	0	0	640
ST- 9	7	<20	2	±	0	0			10	2	1	0	0			40
	26	<20	4	3	2	1	0	0	640	4	4	3	2	±	0	640
ST-10	4	<20	0	0	0	0			<10	0	0	0	0			<10
	78	<20	3	2	±	0	0	0	40	3	2	1	0	0	0	160
	187	<20	1	±	0	0	0	0	10	2	1	0	0	0	0	40
ST-11	5	<20	0	0	0	0			<10	0	0	0	0			<10
	11	<20	3	2	±	0	0	0	40	3	2	1	±	0	0	160
ST-12	6	20	0	0	0	0			<10	0	0	0	0			<10
	12	40	1	0	0	0	0	0	10	4	2	1	1	±	0	640
ST-13	4	<20	3	2	1	0			160	2	1	0	0			40
	26	<20	4	3	2	1	±	0	640	3	2	1	±	0	0	160
ST-14	9	80	3	3	2	1	0	0	640	4	3	2	1	1	0	2560
	22	160	4	3	2	2	1	0	2560	4	4	3	2	1	1	10240
ST-15	9	<20	0	±	0	0			<10	1	2	±	±	0		40
	17	640	2	1	±	0	0	0	40	4	3	2	±	0	0	160

* Intensity of fluorescence recorded 0 to 4. ± Indicates organisms were barely visible.
† Day of illness serum collected.

employed yielded comparable results, *i.e.*, only those sera which gave positive results in the agglutination tests reacted in the fluorescent antibody tests; moreover, the titers were essentially the same when determined by this method. None of the sera reacted significantly with *Proteus* OX19 and OX2 organisms. There was no correlation between the presence or absence of OXK antibody and the fluorescent antibody titers with the Karp and Gilliam antigens.

All of the sera from the cases of scrub typhus were tested by indirect fluorescence for antibodies against Q fever, spotted fever, epidemic and murine typhus. None were positive for Q fever antibodies. One patient (ST-15), who had spent several years in the jungles of Malaya, had a sustained titer of 1:10 against the Bitterroot strain of spotted fever. The low, persistent titers with murine typhus antigen (1:10 or 1:40) in 8 of the group probably can be attributed to previous immunization with epidemic typhus vaccine.

None of the control sera reacted with *R. tsutsugamushi* in the fluorescent antibody tests, with a single exception. A 1:10 dilution of a convalescent serum from a patient with leptospirosis acquired in Puerto Rico gave a 1+ fluorescence with Gilliam, but was negative with the Karp strain.

Discussion. Numerous immunologic technics have been used to study the antigenic characteristics of strains of *R. tsutsugamushi.* Except for mouse cross-immunity tests which demonstrate a close intra-group relationship among the strains(11), other procedures, *viz.,* serum protection(12), complement fixation(6,7), toxin neutralization(13), and vaccination-challenge(14), reveal the antigenic heterogeneity which exists within the species, and these latter tests do not lend themselves to the demonstration of broad immunologic responses. The inability of these procedures to detect antibody induced by a wide variety of antigenically dissimilar strains of *R. tsutsugamushi* has been the basis of the problems encountered in the serological diagnosis of scrub typhus.

The results of the indirect fluorescent antibody tests on the 15 scrub typhus patients suggest that by means of immunofluorescence, a group-specific serological reaction, which is specific for *R. tsutsugamushi,* is demonstrable. Although an antigenic comparison of the 15 strains of scrub typhus isolated from the patients has not been made, it is not unreasonable to assume appreciable antigenc variability could be demonstrated among members of the group. Previous workers have shown that antigenically different strains exist in Malaya; indeed, strains recovered from men infected in a single field differed among themselves(15). The failure to demonstrate complement-fixing antibodies in any of the convalescent sera in tests with soluble antigens prepared from yolk sacs infected with either Karp or Gilliam rickettsiae suggests that the strains causing disease in the patients probably were not closely related antigenically to either of the test organisms. Furthermore, definite differences were observed in the titers of certain of the scrub typhus sera when they were examined in parallel indirect immunofluorescent tests with both Karp and Gilliam antigens. These differences in strain reactivity, however, were not of sufficient magnitude to prevent the use of either strain for diagnosis.

Although sera of some of the 15 patients gave positive reactions when tested by the immunofluorescent technic with antigens prepared from *R. rickettsii* and *R. mooseri,* in no instance did the titers change during the period of observation. This provides further evidence of the specificity of the procedure for scrub typhus when *R. tsutsugamushi* antigens are employed.

Only one of the control sera containing OXK antibody of nonrickettsial origin stained scrub typhus rickettsiae. Minimal fluorescence of the Gilliam antigen, but not the Karp, was observed with a 1:10 dilution of serum collected on the 14th day of illness from a patient who had a severe *Leptospira icterohaemorrhagiae* infection in Puerto Rico. The significance of this finding is not clear since sera from 2 other patients infected with the same sero-type of *Leptospira,* which had higher OXK antibody titers, did not react with the scrub typhus antigens. The result, however, was reproducible just as were the 1+ reactions obtained with the acute phase sera from the first 2 scrub typhus patients shown in Table II. Perhaps interpretation of this minor degree of fluorescence by serum at the lowest dilution, *i.e.,* 1:10, should be held in abeyance.

The application of indirect immunofluorescence to the serological diagnosis of scrub typhus holds promise of providing a method for specific recognition of human disease with facility and accuracy not available heretofore. However, it will be necessary to show that the same degree of group reactivity found in the sera of patients infected in Malaya can be demonstrated in cases occurring in other parts of the world where different strains of *R. tsutsugamushi* exist.

Summary. The indirect fluorescent antibody test, in which dilutions of sera from patients with scrub typhus were tested on smears of *Rickettsia tsutsugamushi,* gave results which were serologically diagnostic of the disease in each instance. Non-specific reactions did not occur when the scrub typhus sera were exposed to antigens of other species of *Rickettsia.* Likewise, there was no reaction between *R. tsutsugamushi* smears and sera from other rickettsial infections, or with sera from patients with *Proteus* OXK agglutinins of nonrickettsial origin with one exception, *i.e.,* a case of leptospirosis from Puerto Rico.

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Survival of CR^{51} Labeled Erythrocytes in Swine.* (28108)

RICHARD B. TALBOT AND MELVIN J. SWENSON

Department of Physiology and Pharmacology, College of Veterinary Medicine, Iowa State University, Ames

Survival study of transfused erythrocytes is of general use both as a diagnostic aid and as a research tool in man and several domestic animals. Several technics have been used for detecting erythrocyte survival time in swine. Bush *et al.*(1) using C^{14} labeled glycine, reported a mean survival time of 62 days in 5 growing pigs. Jensen *et al.*(2), reported an "apparent" life span of 63 days in 18 normal growing pigs using Fe^{59}. Bush *et al.*(3), using Cr^{51} determined the mean erythrocyte half-life to be 17 days in 4 normal growing pigs while Hansard and Kincaid(4) using Cr^{51} found the "average" survival time for mature swine to be 71 days.

In all previous studies the erythrocyte life span in swine has been deduced from the results of cross-transfusion experiments (homologous transfusions). However, the use of this method presupposes that the "new environment" for the injected cells will have no influence upon their survival time. Such an assumption seems not justified for various reasons. Hence, an "a priori" better method is estimation of the life span of the individual's own erythrocytes in its own circulation (autologous transfusions). This report concerns comparison of these 2 methods.

Materials and methods. Fifteen pigs varying in age from 10 to 12 weeks at the beginning of the study were used. Four of the animals were Durocs from 2 litters, 4 were Hampshires from 2 litters and the remaining 7 were Landrace-Poland crossbreeds from one litter. The pigs were fed a commercial grower ration and water *ad libitum* throughout the study.

Erythrocyte survival time was estimated by labeling the erythrocytes with Cr^{51}. Four of the animals received injections of autologous labeled cells while the other 11 received homologous labeled cells from a donor pig. Blood was withdrawn from a donor pig or from the pig to be studied and placed in special formula ACD solution.† Two microcuries of radioactive sodium chromate† were added for every 1 ml of blood obtained and the mixture incubated at 37°C with intermittent agi-

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† Abbott Laboratories, Oak Ridge, Tenn.