

A Peroxidase Enzyme-Linked Paper Immunosorbent Technique  
(PELISA) Compared to Microimmunofluorescence (MIFA)  
for the Detection of Human Serum Antibodies to  
the Rickettsia, *Rickettsia tsutsugamushi*

Principal Investigators :                    John W. Crum, MAJ, MSC  
   Suvath Hanchalay, B.A.  
   Chirapa Eamsila, 1st LT\*

OBJECTIVES :

1. To design a technique which is technologically simple, inexpensive, demands little skill and presents potential for use in a relatively unsophisticated clinical setting.
2. To use the same antigens for the PELISA technique as for the MIFA procedure and compare the two.

BACKGROUND : Serodiagnosis of *Rickettsia tsutsugamushi* infection in clinical situations is usually accomplished by the Weil-Felix reaction detecting agglutinins to the OXK strain of *Proteus mirabilis*. The technique lacks specificity and sensitivity (1) and subsequent infections do not appear to demonstrate increased Weil-Felix titers (14). The complement fixation (CF) test does not give significant titers with acute-phase sera without endemic regional homologous strains of the rickettsia included as a part of the antigen (6, 13).

Indirect immunofluorescence (IFA) procedures have become the accepted serodiagnostic technique because of their sensitivity in detecting group antigens as well as homologous strains of the scrub typhus rickettsia (5, 7). Adaptation of the IFA test to the microimmunofluorescent (MIFA) technique placed the fluorescent microscope technique in the position of being an acceptable tool for routine serodiagnosis and studies of the disease epidemiology (3, 15, 16). The Weil-Felix reaction, complement fixation test and microimmunofluorescent procedures are all complicated by the difficulty in preparing specific antigens (4, 8, 13). A microplate enzyme-linked immunosorbent technique has been used and is reported to offer an acceptable alternative to using the MIFA procedure (5).

In most clinical situations, the only technique available for laboratory diagnosis remains the Weil-Felix reaction, this is due to the expense of equipment and lack of skilled personnel to perform the other laboratory tests. During our studies of human serum antibody to the scrub typhus rickettsia, we decided to develop an enzyme-linked immunosorbent assay (ELISA) concurrent with our MIFA studies as an alternative immunochemical laboratory procedure.

---

\* Thai Component, AFRIMS

**METHODS :** Antigen: Antigens for the Karp, Kato and Gilliam strains of *R. tsutsugamushi*, obtained from the Department of Rickettsiology, USAMRU, Institute for Medical Research, Kuala Lumpur, Malaysia, were prepared from specific pathogen free hen's eggs (SPAFAS, Inc., Norwich, Conn.) as a 20 percent yolk sac suspension in Snyder's diluent (2). Aliquots of the preparation were stored at  $-70^{\circ}\text{C}$  after determining the procedural working dilution. Working dilutions were determined, using the same diluent, which would yield approximately 1,000 organisms per 500X microscope field and titer by MIFA, to end point, with a final 1:50 dilution of high titer human sera. When testing was performed, the aliquot was thawed to  $37^{\circ}\text{C}$ , kept on wet ice, and prepared to the predetermined working dilution. The working preparations were used as equal volume pools and as single antigens for the MIFA method of Robinson (10) as well as for the ELISA technique. Negative serum controls, normal yolk sac suspensions, and serum diluent were evaluated concurrently with the human sera.

**Sera:** Human serum from Thailand and Malaysia represented indigenous persons potentially exposed to scrub typhus: positive control sera were from patients infected with *R. tsutsugamushi*. Six groups of 98, 105, 10, 50, 58, and 30 sera were designated Tablan 1, 2, 3 (TP1, TP2, TP3), Pak Chong 2, 3 (PC2, PC3) and (KLX). All were stored at  $-20^{\circ}\text{C}$  prior to testing. Serum tested by both procedures was initially diluted to 1:50 and screened against pooled antigen. Positive sera were then titered against the pool as well as against the separate antigens in 2-fold serum serial dilutions of 1:100, 1:200, 1:400, 1:800 and 1:1600. Control sera were defined as positive or negative at a 1:50 dilution. A test positive serum was read relative to the controls and positive titers as positive at 3 to 4 serial dilutions of the serum. For the MIFA procedure 28 sera and two controls were tested per microscope slide. Ten sera and two controls per slide were used for the ELISA technique.

**MIFA procedure:** Training for the performance of the test was obtained in Kuala Lumpur using the MIFA method of Robinson (10) and was performed in Bangkok with no deviation from the essential procedural steps. The method essentially prescribes appropriately diluted antigens and controls, placed as 30 pen nib dots on a microscope slide, fixed in acetone, incubated at  $37^{\circ}\text{C}$  with an initial 1:50 (serum: phosphate buffered saline) diluted specimen per antigen dot and again incubated with a fluorescence conjugated horse anti-human immunoglobulin. Phosphate buffered saline (PBS) was utilized for all intermittent washing steps. Microscopic fluorescence examination was performed with a Leitz Orthoplan research microscope, equipped with fluorescence technique objectives, at 40X dry power. Filter and light sources were combined to produce a maximum intensity ocular emission fluorescence at 525 millimicrons.

**PELISA technique:** Antigen was applied as a single one lambda drop on a microscope slide, fixed in chloroform: methanol, dried and sandwiched with a five lambda test serum drop (diluted 1:50), incubated, washed, dried and sandwiched with an anti-human IgG peroxidase conjugate (diluted 1:400), incubated, washed and dried before direct application of a substrate saturated filter paper. The enzyme reaction was visually monitored as production of a purple-brown product and terminated by removal of the filter paper holding to remaining substrate and product produced. This paper was quickly dried and retained as a permanent record of the test. All sera, as in MIFA, were first screened against pooled

antigen. A positive serum was then titered against the pool and also titered against the individual antigens rendering separate titers for the pooled and individual antigens. Procedural details were as follows :

One lambda drops of working antigen, delivered by an Eppendorf automatic hand pipette, were spotted on slides precleaned with a mixture of chloroform N.F. (Allied Chemical, Morristown, N.J.): methanol ACS (Eastman Kodak, Rochester, N.Y.), 2:1 by volume (C:M) and air dried 1 hr. to approximately 5 mm diameter circular spots, 12 spots to a slide in 3 rows, 4 spots per row. After air drying, the spotted slides were fixed by immersion in C:M at 4°C for 10 min. and again air dried. The antigen prepared slides were immediately used or stored in a desiccation container at -20°C and used within two weeks. Slides were general laboratory use 75 mm x 25 mm glass with one frosted end (Propper Mfg Co., Inc., L.I.C., N.Y.). Serum was diluted with a 1% Tween 20: 0.5 M NaCl solution, pH adjusted to 7.4 with 0.1 M  $K_2HPO_4$  (12). Five lambda of the diluted serum was spotted at room temperature to cover the dry antigen spot on the microscope slide, maintaining an approximate 5 mm diameter circular antigen-serum surface area and incubated on a damp sponge humidified at 37°C for 30 minutes. The slide was then washed at room temperature with a pH 7.2-7.4 phosphate buffered saline (PBS) (9) three times, 5 minutes each time, by soaking in a histopathology Coplin jar which was emptied and refilled with fresh PBS between the successive soaks. Following this wash the slides were dried using an unheated hair dryer. Each slide was carefully cleaned around the antigen-serum spots with a dry cotton tipped applicator to remove any traces of residual serum which would interfere with the edge surface tension integrity of later sandwiched drop applications.

Anti-human IgG (heavy chain) conjugate was obtained from Microbiological Associates, Walkersville, MD. as a conjugated horseradish peroxidase IgG Fraction prepared in rabbits. The working peroxidase conjugate was prepared as a 1:400 dilution in the Tween NaCl diluent. Aliquots of the diluted working conjugate were stored at -20°C until use. Working conjugate, at room temperature, was delivered as a 5 lambda drop onto the slide antigen-serum spot, maintaining a 5 mm diameter spot-surface contact, kept humidified at 37°C for 30 minutes, and followed by washing and drying as performed in the application of serum samples to the antigen spot. The conjugate dilution of 1:400 was determined by titration to be optimal. Substrate was a freshly prepared mixture of 9 parts, 80 mg% aq., of 5-Amino Salicylic acid (ICN Pharmaceutical Inc., Plainview, N.Y.) pH, adjusted to 6 with 1N NaOH and 1 part 0.05%  $H_2O_2$  aq. (Mallinckrodt, Inc., St. Louis, MO.) added as an oxidizer (11). Filter paper (Whatman ML ashless #41) was cut to the same dimensions as the microscope slide, saturated with 500 lambda of the prepared substrate, and carefully overlaid on the slide. Production of the purple-brown substrate product is visually monitored at the control spots site for approximately 4 to 6 minutes, then the filter paper is removed and air dried. A test positive or negative serum is visually determined relative to the controls. A positive titer was defined to be a 3 to 4 fold dilution of serum. Positive and negative sera controls were tested on each slide.

**RESULTS :** The MIFA procedure and our peroxidase paper enzyme-linked immunosorbent assay (PELISA) using an enzyme conjugate were studied in ability to detect antibody in 351 human sera. An agreement of 96.3% with the MIFA procedure in the screening of antibody at a 1:50 serum dilution against a heterologous

antigen pool of Karp, Kato and Gilliam strains of the scrub typhus rickettsia was obtained (Table I). Of the sera screened positive for antibody, 2% more were detected by PELISA. Of negative sera, 2% more were detected by the MIFA than by PELISA.

In performing titers, by 2-fold serial dilutions of the serum, comparison was made as agreement or disagreement between the 2 methods as being within one or two dilutions (Table II). Titer agreement within 2 dilutions was 94.6% for the pool, 93.7% for the Karp strain, 93.7% for Kato and 92.0% for Gilliam. These agreement percentages represent serum with detectable antibody as well as those without detectable antibody levels. Positive antibody detection ranged from the initial 1:50 dilution to 1:1600, all in 2-fold serial dilutions.

Figure 1 is an enlarged photograph and code description (Table III) of the dry PELISA 75 x 22 mm microscope slide size filter paper strips. Strip I represents only positive and negative control sera with all except I/1/C, I/1/D, I/2/A, I/3/A and I/3/B being positive. Strip II represents actual test sera and four controls, II/2/C and II/2/D being a positive and negative control sera and II/3/C and II/3/D being uninfected no-antigen normal yolk sac reagent controls tested against a positive and negative control serum. Strip III represents tested sera and a positive and negative control sera at III/3/C and III/3/D.

#### REFERENCES :

1. Berman, S.J., and W.D. Kundin. 1973. Scrub typhus in South Vietnam: a study of 87 cases. *Ann. Intern. Med.* 79: 26-30.
2. Bovarnick, M.R., J.C. Miller, and J.C. Snyder. 1950. The influence of certain salts, amino acids, sugars and proteins on the stability of rickettsia. *J. Bacteriol.* 59: 509-522.
3. Brown, G.W., D.M. Robinson, D.L. Huxsoll, T.S. Ng, K.J. Lim, and G. Sannasey. 1976. Scrub typhus: a common cause of illness in indigenous populations. *Trans. R. Soc. Trop. Med. Hyg.* 70: 444-448.
4. Dasch, G.A., and E. Weiss. 1978. Factors affecting the viability of *Rickettsia tsutsugamushi* purified from yolk sacs and L cells. In J. Kazar, R.A. Ormsbee, and I.N. Tarasevich (ed.), *Rickettsiae and rickettsial diseases*. Veda Publishing House of the Slovak Academy of Sciences, Bratislava. 115-127.
5. Elisberg, B.L., and F.M. Bozeman. 1966. Serological diagnosis of rickettsial diseases by indirect immunofluorescence. *Arch. Inst. Pasteur Tunis.* 43: 193-204.
6. Elisberg, B.L., J.M. Campbell, and F.M. Bozeman. 1968. Antigenic diversity of *Rickettsia tsutsugamushi*: epidemiologic and etiologic significance. *J. Hyg. Epidemiol. Microbiol. Immunol.* 12: 18-25.

7. Elisberg, B.L., C.F. Needy, and F.M. Bozeman. 1978. Antigenic inter-relationships among strains of *Rickettsia tsutsugamushi*. In J. Kazar, R.A. Ormsbee, and I.N. Tarasevich (ed.), *Rickettsiae and Rickettsial diseases*. Veda Publishing House of the Slovak Academy of Sciences, Bratislava. 253-262.
8. Kobayashi, Y., K. Nagai, and N. Tachibana. 1969. Purification of complement-fixing antigens of *Rickettsia orientalis* by ether extraction. *Am. J. Trop. Med. Hyg.* 18: 942-952.
9. Lewis, V.J., W.L. Thacker, and S.H. Mitchell. 1977. Enzyme-linked immunosorbent assay for Chlamydial antibodies. *J. Clin. Micro.* 6: 507-510.
10. Robinson, D.M., G. Brown, E. Gan, and D.L. Huxsoll. 1976. Adaptation of a microimmunofluorescence test to the study of human *Rickettsia tsutsugamushi* antibody. *Am. J. Trop. Med. Hyg.* 25: 900-905.
11. Ruitenbergh, E.J., P.A. Steerenberg, B.J.M. Brosi, and J. Buys. 1974. Serodiagnosis of *Trichinella spiralis* infections in pigs by enzyme-linked immunosorbent assays. *Bull. W.H.O.* 51: 108-109.
12. Saunders, G.C., and E.H. Clinard. 1976. Rapid micromethod of screening for antibodies to disease agents using the indirect enzyme-labeled antibody test. *J. Clin. Micro.* 3: 604-609.
13. Shishido, A. 1964. Strain variation of *Rickettsia orientalis* in the complement fixation test. *Jpn. J. Med. Sci.* 17: 59-72.
14. Smadel, J.E., H.L. Ley, Jr., F.H. Diercks, and J.A.P. Cameron. 1952. Persistence of *Rickettsia tsutsugamushi* in tissues of patients recovered from scrub typhus. *Am. J. Trop. Hyg.* 56: 294-302.
15. Van Peenen, P.F.D., C.M. Ho, and A.L. Bourgeois. 1977. Indirect immunofluorescence antibodies in natural and acquired *Rickettsia tsutsugamushi* infections of Philippine rodents. *Infect. Immun.* 15: 813-816.
16. Walker, J.S., E. Gan, C.T. Chye, and I. Muul. 1973. Involvement of small mammals in the transmission of scrub typhus in Malaysia: isolation and serological evidence. *Trans. R. Soc. Trop. Med.* 67: 838-845.

Table I. Comparative Screen (1:50 serum dilution) Agreement and Disagreement Between the MIFA and ELISA Methods for Detection of Serum Antibody to Scrub Typhus Antigens (n = 351)

Study groups	Total sera	MIFA		ELISA	
		Positive	Negative	Positive	Negative
TP1	98	66	32	69	29
TP2	105	78	27	82	23
TP3	10	3	7	5	5
PC2	50	0	50	0	50
PC3	58	3	55	2	56
KLX	30	21	9	20	10
<u>Disagreements</u>					
TP1	98		3	3	
TP2	105		4	4	
TP3	10		2	2	
PC2	50				
PC3	55	2	1	1	2
KLX	30	1			1
<u>Totals</u>					
Disagreement		3	10	10	3
Agreement		168	170	168	170
Sera	351	171	180	178	173

Agreement : 96.3%

Disagreement : 3.7%

Antibody positive : ELISA greater than MIFA : 2%

Antibody negative : MIFA greater than ELISA : 2%

Table II. Comparative Agreement and Disagreement Between the MIFA and ELISA methods for the Detection of Serum Antibody to Scrub Typhus Antigens

Study groups	Pool 1:50 screen	Pool titer 1dil/2dil	Karp titer 1dil/2dil	Kato titer 1dil/2dil	Gilliam titer 1dil/2dil
TP1 agree	95	71/93	77/91	80/87	75/84
disagree	3	27/5	21/7	18/11	23/14
TP2 agree	101	80/97	84/97	86/98	81/98
disagree	4	25/8	21/8	19/7	24/7
TP3 agree	8	7/8	8/8	7/7	6/8
disagree	2	3/2	2/2	3/3	4/2
PC2 agree	50	50/50	50/50	50/50	50/50
disagree	-	-/-	-/-	-/-	-/-
PC3 agree	55	55/55	55/55	57/57	55/55
disagree	3	3/3	3/3	1/1	3/3
KLX agree	29	20/29	24/28	24/29	26/28
disagree	1	10/1	6/2	6/1	4/2
TOTAL agree	383	283/332	298/329	304/328	298/323
disagree	13	68/19	53/22	47/23	58/28
% agree	96.3	80.6/94.6	84.9/93.7	86.6/93.4	83.5/92.0
% disagree	3.7	19.4/5.4	15.1/6.3	13.4/6.6	16.5/8.0

The screening is performed against the three antigen pool at a 1:50 dilution. Results are expressed as in agreement or disagreement within one or two serum dilutions between the methods.

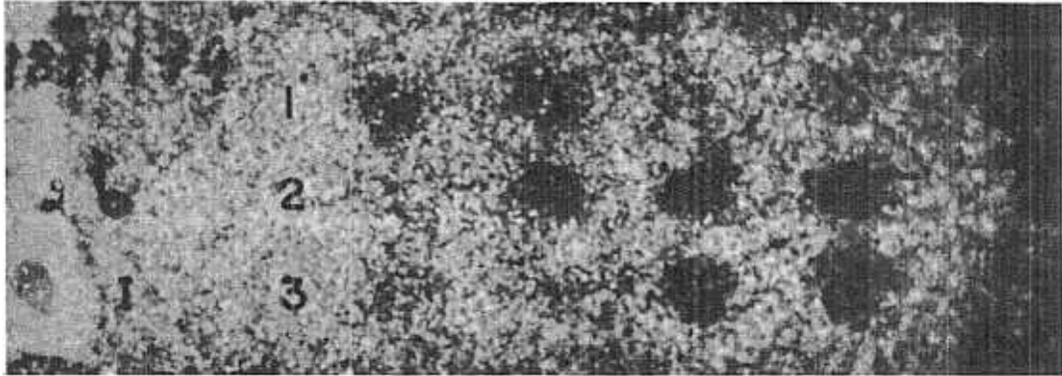
Table III. Enlarged photographs of 3 PELISA results

positive: +, negative: -, serum control: C

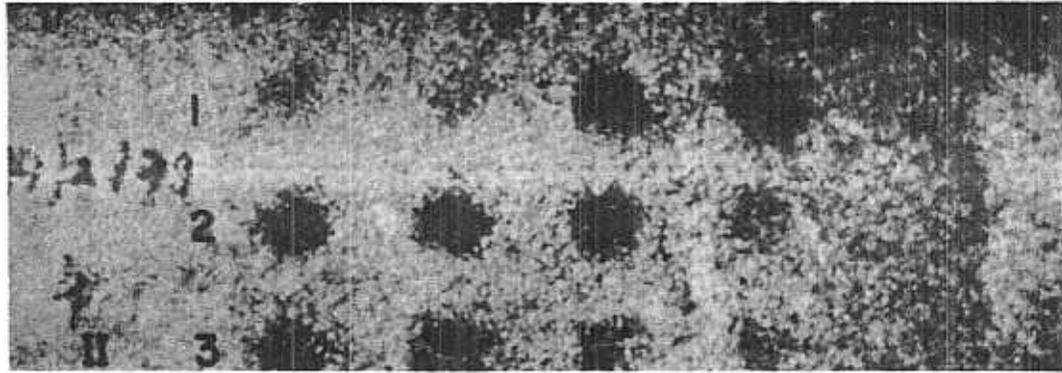
		A	B	C	D
I.	1.	+C	+C	-C	-C
	2.	-C	+C	+C	+C
	3.	-C	-C	+C	+C
II.	1	-	-	+	+
	2.	+	+	+C	-C
	3.	+	+	-C	-C
III.	1.	-	-	-	-
	2.	+	+	+	+
	3.	-	-	+C	-C

II/3/C and D are reagent, no antigen, normal yolk sac controls tested against a positive and negative control sera.

A B C D



A B C D



A B C D

