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Comparison of Indirect Immunofluorescence Assays for Diagnosis of Scrub Typhus and Murine Typhus Using Venous Blood and Finger Prick Filter Paper Blood Spots

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Abstract

We performed indirect immunofluorescence assays (IFAs) to compare levels of IgM and IgG antibodies to *Orientia tsutsugamushi* and *Rickettsia typhi* in admission-phase serum samples and filter paper blood spots (assayed immediately and stored at 5.4°C and 29°C for 30 days) collected on the same day from 53 adults with suspected scrub typhus and murine typhus admitted to Mahosot Hospital Vientiane, Lao People's Democratic Republic. The sensitivities and specificities of admission-phase filter paper blood spots in comparison to paired sera were between 91% and 95% and 87% and 100%, respectively, for the diagnosis of scrub typhus and murine typhus. The classification of patients as having or not having typhus did not significantly differ after storage of

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the blood spots for 30 days ($P > 0.4$) at 5.4°C and 29°C. Because filter paper blood samples do not require sophisticated and expensive storage and transport, they may be an appropriate specimen collection technique for the diagnosis of rickettsial disease in the rural tropics.

Introduction

Rickettsioses are increasingly recognized as common causes of undifferentiated fever in the Asiatic tropics, with recent studies from Nepal, Lao People's Democratic Republic (Laos), and Thailand highlighting their clinical importance.¹⁻⁴ At Mahosot Hospital in Vientiane, Laos, 115 (27%) of 427 adults with negative blood cultures had serologic evidence of acute rickettsial disease: *Orientia tsutsugamushi* (14.8%), *Rickettsia typhi* (9.6%), and spotted fever group (2.6%).¹ However, clinical discrimination of scrub typhus and murine typhus from other undifferentiated fevers, such as dengue, malaria, and leptospirosis, is often difficult.^{1,5,6} Laboratory diagnosis is crucial but is conventionally based on serologic analysis with indirect immunofluorescence assay (IFA) detection of specific IgM and IgG antibodies.^{4,5} However, in comparison with the large burden of disease, few laboratories are able to perform these assays in disease-endemic countries, and slides, microscopes, and training required are expensive.

Filter paper blood spots have been used in the diagnosis of infectious diseases since 1961 when Sadun and Anderson described their use in the fluorescent antibody diagnosis of schistosomiasis.^{7,8} In 1963, Guthrie and Susie developed the now widespread use of whole blood obtained by heel pricks blotted on to filter paper for neonatal metabolic disease screening, such as for congenital hypothyroidism.⁹ Filter paper blood spot-based assays have since been developed for diagnosis of a wide range of infectious diseases by detection of antibodies and organism-specific genes by the polymerase chain reaction.⁹⁻¹¹

Rural tropical areas often have insufficient access to diagnostic laboratory facilities and difficulties in transporting frozen specimens. Therefore, filter paper blood spots may be an economic and simple alternative to sending frozen diagnostic specimens to a centralized laboratory.

Three studies have examined the use of filter paper blood spots in serologic diagnosis of rickettsial disease.¹²⁻¹⁴ In a comparison of IFA titers between serum samples obtained by venipuncture and blood from nine patients with antibodies to *R. conorii* that was anticoagulated with EDTA and placed on filter papers stored at room temperature and 4°C, all had either the same or one dilution lower titers, except one sample for which the IFA titer was two dilutions lower when the sample was stored at room temperature.¹⁴ In a comparison of IFA titers from serum samples and whole blood on filter paper in the diagnosis of *O. tsutsugamushi* infection, only 4 (2%) of 173 samples were incorrectly classified as being above or below the screening titer.¹³ However, these studies variably had small sample sizes, anti-coagulated blood was used, blood was applied with a pipette (which is not practical in the field), murine typhus was not included, and statistical tests were not used to assess accuracy.

We conducted a pilot study among adults with suspected murine typhus and scrub typhus admitted to Mahosot Hospital to answer the following questions. First, do IgM and IgG murine and scrub typhus antibody titers, as determined by IFA, significantly differ between serum samples and finger-pick filter paper blood spots? Second, do IgM and IgG murine and scrub typhus antibody titers significantly differ between fingerpick filter paper blood spots stored for one month at 4°C and at 30°C?

Patients And Methods

Study site, patients, and clinical procedures

A prospective evaluation of serologic diagnosis of scrub typhus and murine typhus was undertaken in patients admitted to the Adult Infectious Disease Ward at Mahosot Hospital from December 2005 through May 2006. Consecutive patients > 15 years of age with suspected typhus (fever, headache, and/or myalgia) were recruited if they gave informed written consent, were smear negative for malaria if from a malaria-endemic area, and had a fever $\geq 37.5^{\circ}\text{C}$. The study was reviewed and approved by the National Ethics Committee for Health Research of the Lao People's Democratic Republic and the Oxford Tropical Research Ethics Committee, United Kingdom.

Five milliliters of whole blood for serum was collected from patients on admission and after 14 days and stored at -80°C . Three dried blood spots were collected onto Proteinsaver™ (Whatman, Maidstone, United Kingdom) filter paper strips. Experiments with aspirated whole blood demonstrated that 75 μL of whole blood dropped onto this paper covered a circle with a diameter of approximately 13 mm. Therefore, after finger prick with a lancet, a drop of blood was expressed onto a filter paper strip to fill a 13-mm diameter circle stenciled by pressure onto the paper. The strips were left to air dry at room temperature for four hours. One blood spot was then processed for IFA (below) and two blood spots were stored in sealed plastic bags containing silica gel crystals in either a refrigerator (4°C) or incubator (30°C) for 30 days. The actual temperature in the refrigerator and incubator was recorded every morning.

Laboratory procedures

In processing serum samples, 4 μL of serum was diluted to 1:25 in a microtitration plate with autoclaved phosphate-buffered saline (PBS) plus 3% skimmed milk powder. These sera were serially diluted two-fold from 1:25 to 1:12,800. A 2- μL aliquot of each serum dilution was aspirated from the wells, being careful to prevent crosscontamination, added to IFA slides coated with antigen from *O. tsutsugamushi* strains Karp, Kato, and Gilliam serotypes or *R. typhi* strain Wilmington (Australian Rickettsial Reference Laboratory, Geelong, Victoria, Australia), and incubated in a moist chamber at 37°C for 1 hour. Slides were then washed three times (5 minutes/wash) with autoclaved PBS. After washing and drying, the slides were treated with specific fluorescein isothiocyanate-conjugated goat anti-human gamma chain immunoglobulin (Sigma Aldrich, Germany), incubated for 30 minutes at 37°C, washed three times (5 minutes/ wash) with autoclaved PBS, and mounted in buffered glycerol (90% [v/v] glycerol and 10% PBS).

A cardpunch was used to cut 6-mm diameter discs from the blood-impregnated filter paper blood spots, halfway between the center and the edge of the blood spot. These spots were eluted overnight in 250 μ L of autoclaved PBS at 37°C. Saturated discs were equivalent to a 1:25 dilution of serum (calculated using the method of Fenollar and Raoult¹⁴). Eluted samples were serially diluted in sterile PBS to 1:12,800. Eluted samples were stored at -80°C. The same procedures were used with serum and filter paper samples stored in the refrigerator and the incubator for 30 days. The 14-day convalescent phase serum sample and the 30-day storage blood spots were tested on the same day. The IFA slides were read with an ECLIPSE E600 microscope (Nikon Co., Tokyo, Japan) by two observers (RP and SDB) who were blinded to the other's results.

There is considerable cross-reactivity between *R. typhi* and *R. prowazekii*,⁵ but *R. prowazekii* has not been reported in mainland Southeast Asia, and cross-absorption and Western blot studies confirmed that the serologic responses in patients in Laos were to *R. typhi*.¹ The end point of each IFA titer was defined as the lowest serum concentration demonstrating definite fluorescence. A positive result was defined as an IgM or IgG titer 1:400 or a four-fold increase in titer.¹⁵

Statistical analysis

Analysis was performed using STATA version 8 (STATA Corp., College Station, TX). Categorical variables were compared using McNemar's test. The Kappa test was performed and interpreted according to the procedure of Landis and Koch.¹⁶ Sensitivity, specificity, negative predictive value, and positive predictive value were calculated.

Results

Patients

Fifty-three adults with clinically suspected typhus were recruited. Analysis of admission-phase and convalescent-phase serum samples by IFA indicated that 23 (43%) patients had murine typhus and 15 patients (28%) had scrub typhus. Seven patients (13%) without serologic evidence of typhus had blood cultures positive for *Salmonella enterica* serovar Typhi. No etiologic diagnosis was made in 8 (15%) patients. Of 23 patients with murine typhus, the median (range) age was 24 (15–46) years, duration of illness was 13.5 (7–21) days, and admission tympanic temperature was 38.5°C (36.0–40.0°C). The equivalent results for those with scrub typhus were 26.5 (17–54) years, 14.5 (9–21) days, and 38.3°C (36.0–40.0°C). A history of fever, headache, myalgia, and rash were present in 100%, 100%, 61%, and 13% of those with murine typhus, respectively, and 100%, 100%, 60%, and 6% of those with scrub typhus, respectively.

Inter-observer agreement

Kappa statistics for IFA detection of IgM and IgG antibodies against *R. typhi* and *O. tsutsugamushi* between the two IFA readers for admission-phase serum samples and filter paper samples (Table 1) were classified as fair or moderate agreement.¹⁶ Because one of the IFA readers (SDB) had more experience in reading IFA results, his results were used in the subsequent analysis.

IgM and IgG antibody titers for murine and scrub typhus determined by IFA in serum samples and fingerpick filter paper blood spots

Murine typhus IFA titers on admission-phase serum samples and filter paper samples were the same or showed a ± 2 -fold difference in 50 (94%) of 53 patients for IgM antibodies and in 48 (91%) of 53 patients for IgG antibodies. Scrub typhus group IFA titers on admission-phase serum samples and filter paper samples had the same titer or a ± 2 -fold difference in 47 (89%) of 53 patients for IgM antibodies and in 50 (94%) of 53 patients for IgG antibodies. The sensitivity and specificity of the classification of patients as having murine typhus by IgM IFA using admission-phase filter paper blood spots in comparison to paired serum samples were 91% and 100%, respectively, with a positive predictive value of 100% and a negative predictive value of 80% (Table 1). The equivalent results for scrub typhus were a sensitivity of 95%, a specificity of 88%, a positive predictive value of 95%, and a negative predictive value of 87%. There were no statistically significant differences between the classifications of patients as having murine or scrub typhus by the two techniques ($P > 0.5$, by McNemar's test).

IgM and IgG antibody titers for murine and scrub typhus in fingerpick filter paper blood spots stored at 4°C and 30°C for one month

Recorded median (range) daily temperatures were 5.4°C (3.2–8.9°C) in the refrigerator and 29.0°C (22.1–36.1°C) in the incubator. Samples from three patients could not be analyzed at 30 days. Therefore, the sample size for the storage comparison is 50.

In comparison to IFA assays performed immediately, admission murine typhus IFA titers for blood spots kept for 30 days at 5.4°C were the same or showed a ± 2 -fold difference in 44 (88%) of 50 patients for IgM antibodies and in 38 (76%) of 50 patients for IgG antibodies. Samples kept at 29.0°C had the same titers or a ± 2 -fold difference in 48 (96%) of 50 patients for IgM antibodies and in 44 (88%) of 50 patients for IgG antibodies. When compared with IFA assays performed immediately, the admission scrub typhus group IFA titers for blood spots kept for 30 days at 5.4°C were the same or showed a ± 2 -fold difference in 42 (84%) of 50 patients for IgM antibodies and in 46 (92%) of 50 patients for IgG antibodies. Samples kept at 29°C had the same titers or a ± 2 -fold difference in 43 (86%) of 50 patients for IgM antibodies and in 47 (94%) of 50 patients for IgG antibodies. There were no statistically significant differences between classification of patients as having murine typhus and scrub typhus between filter papers tested on admission and after storage at the two temperatures ($P > 0.6$ and $P > 0.4$, respectively, by McNemar's test).

Discussion

This study suggests that the IFA detection of rickettsial antibodies in blood samples on filter paper at a positive cutoff value of 1:400 in Laos is a potential alternative method to an IFA with serum samples for classifying patients as having scrub or murine typhus. The sensitivity and specificity of admission-phase filter paper blood spots in comparison with paired serum samples for patient diagnosis were between 95% and 100% and 86% and 87%, respectively. Although storage for 30 days led to a decrease in accuracy, storage at 29°C did not lead to a significantly greater reduction in accuracy than storage at 5.4°C. As noted by

Gan and others, the duration of storage, rather than the temperature of storage, is likely to be an important factor.¹³ A temperature of 29°C is a common ambient temperature in Laos, which suggests that filter papers can be stored for a month at ambient indoor temperatures in this country before rickettsial IFAs are conducted. This finding is in contrast to work on the use of filter paper blood spots in serologic diagnosis of dengue, in which storage in a refrigerator was recommended.¹⁷ However, refrigerated storage will encourage condensation of water on the paper, which can potentially change the results.¹⁸ Limitations of the current study include the relatively small sample size and no assessment of the practical aspects of paper specimen transport.

To our knowledge, this is the first study to report kappa statistics for inter-observer agreement for a rickettsial IFA, and the results confirm that the results of this test are inherently subjective, as reflected in categories of fair and moderate agreement.⁴ We are not aware of any attempt to standardize techniques and slides between laboratories or to determine locally appropriate cutoff titers. These procedures are urgently required.

Serologic diagnosis of rickettsial disease using blood spots on filter paper may be a useful technique in countries lacking infrastructure for diagnosis of fever in rural areas or transport of frozen specimens. Placing finger prick blood samples onto filter paper obviates the need for needles, syringes, and bloodcollecting tubes and reduces transport costs enormously. With the increased cost and administration of transporting specimens nationally and internationally, use of filter paper may also facilitate transport of specimens between laboratories and in quality assurance programs. Although there have been many reports of diagnosis of infectious diseases using filter paper blood spots, we are not aware of any large-scale public health programs that use them; their potential has not been fulfilled. Further research is required on optimization of the filter paper type, practical implementation of filter paper diagnostic services, and accuracy of rickettsial gene and antigen detection using filter papers.

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Table 1
Sensitivity, specificity, and positive and negative predictive values of indirect immunofluorescent assays for the diagnosis of scrub typhus and murine typhus by using admission filter paper blood spots*

Disease	Antibody type	Sensitivity, % (95% CI)	Specificity, % (95% CI)	PPV, % (95% CI)	NPV, % (95% CI)	Kappa statistic (95% CI)	
						FP	Serum
Scrub typhus	IgM	95(76-100)	88 (48-100)	95 (76-100)	87 (48-100)	0.55 (0.32-0.78)	0.67 (0.40-0.94)
	IgG	90(70-99)	100(59-100)	100 (83-100)	77 (40-97)	0.66 (0.43-0.89)	0.61 (0.36-0.86)
Murine typhus	IgM	91 (72-99)	100(63-100)	100(84-100)	80 (45-98)	0.56 (0.33-0.79)	0.59 (0.36-0.82)
	IgG	92 (73-99)	100(54-100)	100(85-100)	75 (35-97)	0.69 (0.44-0.94)	0.65 (0.42-0.88)

* CI = confidence interval; PPV = positive predictive value; NPV = negative predictive value; FP = filter paper blood spots. The comparator gold standard was diagnosis based on indirect immunofluorescent assays (IFAs) using paired admission-phase and convalescent-phase serum samples. Positive results were defined as IgM or IgG titers $\geq 1:400$ or a four-fold increase in titer.¹⁵ Kappa statistics (95% CI) for inter-observer agreement between the two readers for murine typhus and scrub typhus IFA on admission-phase serum samples and FPs are also given.