DEVELOPMENT AND PRELIMINARY EVALUATION OF AN IgM DOT-IMMUNOBINDING ASSAY (IgM-DIA) FOR RAPID SERODIAGNOSIS OF SCRUB TYPHUS INFECTION

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Abstract. An IgM dot-immunobinding assay (IgM-DIA) was developed for the diagnosis of scrub typhus infection. The whole cell antigens of Karp, Kato and Gilliam strains of *Rickettsia tsutsugamushi* were immobilized onto nitrocellulose paper and reacted with patients sera. The presence of IgM *R. tsutsugamushi* specific antibody in the patient sera could be detected by the observation of a visible brown dot on the nitrocellulose paper. The IgM-DIA has a sensitivity of 90.4% and specificity of 81.4% as compared to the indirect immunoperoxidase test. The IgM-DIA is rapid, simple, cost-effective, does not require microscope or incubator. It is recommended as a rapid screening test for the diagnosis of scrub typhus infection in the field or rural areas within the hyperendemic region.

INTRODUCTION

Rickettsial diseases, particularly scrub typhus, remain a major cause of febrile illness throughout the Asia Pacific region (Rapmund, 1984). In Malaysia, it has been reported that 19.3% of the febrile hospitalized patients were positive for scrub typhus infection (Brown *et al*, 1984). At present, the routine diagnosis is based on the indirect immunoperoxidase test (IIPT) (Kelly *et al*, 1988). Although the test required no special laboratory equipment except a light microscope and incubator, the teflon coated slides are expensive and the microscopic reading of slides is subjective. A periodic monitoring through proficiency survey is recommended to maintain standardization of the users. As a result, the Weil-Felix test which is of less sensitivity and specificity is still routinely used in some hospital laboratories in Malaysia and the Asia-Pacific region (Kelly *et al*, 1990).

Dot immunobinding assays (DIA) have been used for the diagnosis of various infectious diseases (Matthews and Burnie, 1991). Accordingly, we investigated the DIA as a simple serodiagnostic technique for scrub typhus infection. The detection of IgM rickettsial antibody in patients' sera was used to provide an early diagnosis for the infection.

MATERIAL AND METHODS

Antigen preparation

Prototypes of *R. tsutsugamushi* strains (Karp, Kato and Gilliam) were proliferated in SPAFAS egg yolk sac and purified as described by Eisemann and Osterman (1976). Briefly, 20% of rickettsia yolk sac suspension was subjected to centrifugation at 40,000*g* for 1 hour in a discontinuous 50% sucrose gradient using a SW 28 rotor (Beckman USA). The pellet was resuspended in Brain Heart Infusion broth (Oxoid) and recentrifuged at 12,000*g* for another hour. The pellet was then suspended in phosphate buffered saline (PBS), pH 7.3 and the protein content was determined as described by Pesce *et al* (1973). The purity of the antigen preparation was confirmed by a direct fluorescent antibody test.

Preparation of nitrocellulose paper

Nitrocellulose (NC) paper (MS1, Westboro, MA, 0.45 μm) was cut into pieces 20mm by 15mm. The paper was washed once by gentle agitation in distilled water for 5 minutes and air-dried.

Two microliters of antigen (equal proportion of Karp, Kato and Gilliam strains) of known protein content were dotted onto the NC paper and air-dried. The nonspecific sites were blocked by soaking the paper in 5% skimmed milk (Difco, US)-0.01% Tween 20 (SIGMA)-PBS for 1 hour. The paper was then air-dried prior to storage at -20°C.

Pre-selection of conditions for IgM-DIA

To determine the optimum concentration of antigen for use in the DIA, a checkerboard titration of Karp, Kato and Gilliam antigens at concentrations

ranging from 0.25 μg to 2.0 μg protein content per dot were tested with a range of dilution from known positive and control negative sera in duplicates.

Indirect immunoperoxidase test (IIPT)

The test was performed as described by Kelly *et al* (1988). The reaction was considered positive when the rickettsiae was visible as brown particles under the microscope. The reciprocal of the highest serum dilution with positive reaction is expressed as the IIPT antibody titer. An IgM titer of ≥ 1 : 50 was taken as indicative of a recent infection.

Sera

A total of 122 patient sera previously assayed by the IIPT were selected for the evaluation. Fifty-two of the sera were positive for scrub typhus at IgM titer of 1 : 50 (14), 1 : 100 (14), 1 : 200 (7) and ≥ 400 (17). Seventy sera were negative for scrub typhus by IIPT (IgM < 1 : 50) but positive for endemic or tick typhus infection (26), typhoid (24), dengue (11), legionella and leptospirosis (9).

IgM dot-immunobinding assay (IgM-DIA)

Each antigen-dotted NC paper was incubated in diluted serum in PBS for 1 hour. The antigen-dotted-NC paper was then washed 3 times with PBS and incubated with antihuman immunoglobulin M conjugated with horseradish peroxidase (Dako, Denmark) at 1 : 1,000 dilution for 1 hour. The antigen-dotted NC paper was subsequently washed three times with PBS before being submerged in a chromogenic substrate solution containing 0.05% 3, 3 diaminobenzidine tetrahydrochloride (SIGMA) and 100 μl of 3% hydrogen peroxide for 5 minutes. The reaction was stopped by washing with distilled water. All procedures were performed at room temperature.

Negative serum (IgM titer < 1 : 50), low positive serum (IgM titer 1 : 50) and high positive serum (IgM titer 1 : 400) were included as control sera in each test. The intensity of the brown dot developed from the test sera were compared with the control sera.

Test sera were graded as positive (+) if the intensity of the brown dot was similar or stronger than the dots developed for the low positive control serum. The sera were classified as indeterminate (±) if visibly distinguished brown dots were seen but with lesser intensity as compared to the low positive control serum. The negative sera demonstrated fairly visible or no dots on NC paper.

RESULTS

Preselection of antigen concentration and sera dilution

The optimum antigen concentration per dot and the appropriate serum dilution for the IgM-DIA was determined by a checkerboard titration. The color intensity of the antigen-antibody complex increased with the concentration of antigen dotted on the NC paper and decreased with serum dilution.

It was found that 0.5 μg antigen per dot and sera dilution at 1 : 400 were optimal for the IgM-DIA (Fig 1) and these conditions were subsequently used.

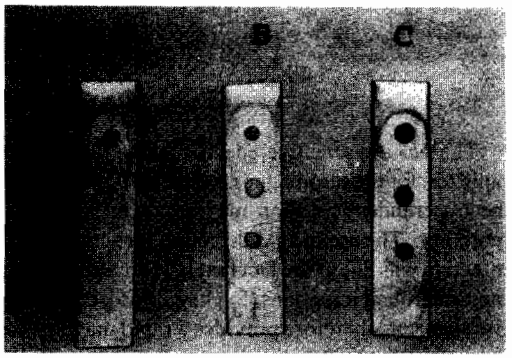

Fig 1–Color deveopment of dots on which equal proportion of Karp, Kato and Gilliam strains of known protein content were dotted. The concentration of rickettsial protein per dot were 0.25, 0.5, 1 and 2 μg from the bottom, reacted with control sera at 1 : 400 dilution. (A = negative control; B = low positive; C = high positive).

Evaluation of the IgM-DIA

The result of the DIA tested against 122 patient sera were shown in Table 1. Of the 52 positive sera, all (90.4%) were positive except 2 (both IgM titer were at 1 : 50) and 3 were indeterminate (at IgM titer of 1 : 100). All sera of IgM titer ≥ 1 : 200 were also positive by the IgM-DIA. Of the negative sera, five were positive (a typhoid case and 4 non-scrub typhus

Table 1

IgM dot-immunobinding assay (IgM-DIA) on 122 patient sera.

Cases	No. tested	IgM dot-immunobinding assay		
		+	±	-
Scrub typhus	52	47	3	2
Other rickettsial infections	26	4	2	20
Typhoid	24	1	2	21
Dengue	11	-	1	10
*Other infections	9	-	3	6

+ = positive; ± = indeterminate; - = negative.
* Includes legionella and leptospira infections (9).

rickettsial infections) and 8 were indeterminate. The sensitivity and specificity of the test was thus calculated to be 90.4 and 81.4% respectively.

DISCUSSION

The lack of a sensitive, specific, cost-effective and rapid test for the serodiagnosis of scrub typhus within the hyperendemic region may result in significant mortality. The use of IgM detection for scrub typhus infection was due to the fact that IgM appears early in most microbial infections (Svehag, 1965), has a short life span, and therefore provides a rapid diagnosis of recent or current infection.

The IgM-DIA employed in this study was based on the detection of antigen-antibody complex on nitrocellulose membrane. A mixture of Karp, Kato and Gilliam strains of *R. tsutsugamushi* was used as the immobilized antigen as cross-reactive antigenicity among *R. tsutsugamushi* strains were observed in this country (Shirai *et al*, 1979). Purified antigen was used to eliminate nonspecific serologic reaction. The color intensity of the dot depended on the antigen concentration and the sera dilution. The optimal conditions determined for the IgM-DIA were 0.5 μg antigen protein concentration per dot and a 400 fold serum dilution.

Of the 52 IIPT positive sera, 2 were negative and 3 were indeterminate (all at IgM titer of ≤ 1 : 100 by the IgM-DIA. This discrepancy may be due to the presence of antibody in the sample reacting with any one of the Karp, Kato and Gilliam antigen but not with all three, hence resulting in a very faint dot. A second serum obtained after a few days of infection showing stronger color intensity on the NC paper may confirm the diagnosis of the infection.

The IgM-DIA has a sensitivity of 90.4 and specificity of 81.4% as compared to the Indirect Immunoperoxidase test. This IgM-DIA was more reliable as a screening test as compared to the Weil-Felix test which has a sensitivity and specificity of approximately 50% (Kelly *et al*, 1990). It was rapid as the test took about 5 hours and was particularly valuable for screening large numbers of sera. The test was simple and could be performed at room temperature without the use of laboratory equipment, such as microscope and incubator. Reading of the test results could easily be distinguished by the naked eye.

The test is therefore recommended for field studies and diagnosis of scrub typhus in rural areas. For further confirmation a quantitative test such as the indirect immunoperoxidase test should be performed.

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