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The increasing prevalence of Endemic Typhus in Kuala Lumpur and an evaluation of a diagnostic ELISA dot test for the detection of antibodies to Rickettsia typhi

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ABSTRACT

A seroepidemiology study was done in response to the recent increase of Endemic Typhus cases diagnosed at University Hospital. The serosurvey was based on doctors' request for the Weil Felix (WF) or the Indirect Immunoperoxidase (IIP) test in Pyrexia of Unknown Origin (PUO) patients for the years 1991 to 1997. Over the 7 years, we found that the incidence of Endemic typhus is increasing with gender (male: female = 2:1), age (20-40 years) and race distribution (Indians > Malay > Chinese) that reflects socioeconomic circumstances. A commercially available ELISA dot assay [INDX (E2R3) Dip-S-Ticks], for the detection of antibodies against *R. typhi* was compared with the indirect immunoperoxidase test (IIP). The ELISA assay was done against 219 IIP tested sera. The Dip-S-Ticks was found to be comparable to the IIP with a sensitivity of 91.7% and specificity of 92.8% at cut-off titres of >1:80 IIP.

Keywords: Endemic typhus, epidemiology, ELISA, IIP

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INTRODUCTION

Endemic (Murine) Typhus Fever is an "urban", flea born typhus infection that has over the past few years become a growing public health concern. *Rickettsia typhi* the microorganism responsible is an intracellular parasite that requires an arthropod vector/reservoir and one or several warm blooded animal hosts, classically rodents. It is transmitted to man by blood sucking arthropods, either directly of through infected faeces/urine which is deposited onto the human host during the feeding process[1]. This can then be inoculated through broken skin (bite site or abrasions post scratching) or through aerosols of infected urine/faeces. Once infected, it multiplies itself in the endothelial cells of small blood vessels and produces systemic vasculities. Hence, clinically it typically presents with fever, vasculitic rash, varying degree of multiorgan vasculities, and in certain cases vascular occlusion and Disseminated Intravascular Coagulation.

Whilst interest in the developed nations on Rickettsiology are waning with better public health measures and disease control, it could become one of growing importance in developing/industrialising nations. It is known, for obvious reasons, that Endemic Typhus thrives with rapid growth and development. Overcrowding, poor sanitation and public health measures, and clearing of urban/suburban land (lalang) for construction allow for both, rodent population to increase as well as exposes man into the zoonotic cycle within our encroachment. The influx of migrant workers and their unhygienic living condition is another factor to consider. Despite waning interest in endemic typhus, the disease continues to have a significant impact accounting for up to 8.5% of admission due to acute infective febrile illness[2].

Clinically, Endemic Typhus is usually a benign condition which typically presents itself as fever (pyrexia of unknown origin), headache and rash with practically negligible mortality. However, this triad presentation was only seen in 12.5% of Endemic Typhus patients in a study done in America in 1991[3]. Sporadic cases with significant morbidity have also been reported, notably with complications of endocarditis, splenic rupture, meningitis and renal impairment[4]. In practice Endemic typhus usually presents itself as non specific pyrexia of unknown origin in an outpatient setting. A cocktail of test for PUO is then performed and the patient treated empirically pending results. Although some patients resolve spontaneously, due to the non specific nature of ET it is important to have a rapid, effective and accessible diagnostic test so that appropriate treatment can be initiated. Tetracycline and chloramphenicol are effective Rickettsiostatic antibiotics. Fluroquinolones, macrolides and rafimpin have also been reported to be effective[5] but the optimal duration of treatment has not been established and antibiotic resistance may emerge with unrestricted or improper use of antibiotics. Sulfonamides also enhances rickettsial growth and should be avoided in this case[6].

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Literature search has also shown that the ecology of Endemic Typhus is evolving. In USA/Los Angeles, seroepidemiological studies have shown that squirrels, but particularly cats and opossums have a high seropositivity and a low seropositivity was found in commensal rodents[7]. These animals have also been shown to be heavily infested with cat flea (ctenocephalides felis). This is contrary to the classical rodent-flea-rodent cycle and is of clinical importance in our setting where small domestic animals, stray cats and dogs are prevalent.

Currently, Endemic Typhus can be serologically diagnosed at the University Hospital using three methods: (l) The Weil Felix method, which has poor sensitivity and specificity (this test has been terminated since 1996). (2) Indirect fluorescent antibody test (IFA), the Gold Standard which is highly sensitive and specific but requires a fluorescent microscope and has to be read immediately before the fluorescein fades and (3) Indirect Immunoperoxidase (IIP) test which has been shown to be comparable to IFA in serodiagnosis of typhus. Isolation of rickettsiae is possible up to the 12th day of infection, but is technically difficult, time consuming and hazardous. This is not a viable diagnostic alternative.

In this study we investigated the epidemiology of Endemic Typhus at University Hospital and then proceeded to evaluate the INDX Dip-S-Ticks as an alternative diagnostic method to the IIP. The Dip-S-Tick utilises an enzyme-linked immunassay (ELISA) dot technique to detect serum antibodies. It is commercially produced by Integrated Diagnostics, Inc. Baltimore, MD, (USA) and has the advantage of being fast (can be completed in 1 hr.) and simple, requiring only a heated water bath (50°C), clarifier vessels (holding 50 ml of water), distilled water, pipettes and a timer beyond the provided kit.

MATERIALS AND METHODS

I. Hospital based screening

Patients attending the University Hospital from 1991-1997 who presented with pyrexia of unknown origin with a query of possible typhus infection were selected. This made up 17,428 samples for which either the WF or the IIP or both were carried out. All seropositive cases were recorded and patient demographics including age, sex and race were noted. A seropositive sample is defined as: 1. For paired sample: a four fold rise in serum antibody titre. 2. Single serum sample: An IgM titre of 1:160, and 1gG titre of 1:640 by IIP.

II. Patient sera

214 samples were selected to evaluate the INDX Dip-S-Ticks. Out of these samples, 145 sera were from patients diagnosed with Acute Endemic Typhus using IIP test. The remaining 69 sera made up the negative control panel and represents the following diseases, Dengue (10), Typhoid (10), Leptospira (10), Hepatitis B (10), Hepatitis A (9), CMV (6), Rubella (2), Herpes (1), Mumps (1), and Healthy/normal sera (10).

Out of the 145 Acute Endemic Typhus sera, there were 62 paired samples and 26 single samples. All sera were provided by the Virology and Serology Unit of the University Hospital and numbered according to the original lab number. It was tested using the INDX Dip-S-Ticks and read independently.

III. Indirect Immunoperoxidase Test

This uses the Yamamoto *et al.* (1982) method previously described[8,9]. The antigen slides are provided on a monthly basis from Institute of Medical Research (IMR), Kuala Lumpur and stored at -20 °C. Each teflon coated slide has two rows of 6 wells. Each well has 4 dots consisting of *O. tsutsugamushi, R. typhi, R. siberica* and normal yolk sac as a negative control. All antigens are propagated in normal yolk sac. The serum is serially diluted (six times) from 1:80 to 1:2560. 10µl of each dilution is placed on each well on both rows and allowed to incubate in a moist chamber at 37°C for 30 min. After which it is washed with PBS 3 times for 5 minutes each time and air dried. This is followed by the conjugation step where 10µl of HRP conjugated to anti-Human IgG is added to the first row and anti IgM to the second row. The slide is then incubated as above and washed 3 times, first 2 times with PBS and the 3rd wash with water, air dried, immersed in DAB substrate at 37°C for 10 min. in the dark, followed by a quick wash. Finally, the slide is counter-stained with methylene-blue solution, washed, dried, cover glassed and examined under light microscope at 40X objective.

IV. The INDX Dip-S-Ticks

The INDX Dip-S-Ticks uses a dot blot immunoassay as described by Weddle *et al.* (l995)[10]. The Dip-S-Ticks contains 6 windows with antigens bounded to the windows by a nitrocellulose membrane. The first window is the IgG/IgM positive and negative control. The remaining 5 windows contains the following antigens *Ehrlichia sennetsu* (ES), *Human granulocytic ehrlichia* (HGE), *R. rickettsii* (tick typhus), *R.typhi* (Endemic Typhus), and *Orientia Tsutsugamushi* (Scrub typhus) at a concentration which will provide a positive test at the specified titres (Fig. 1.) Four reagents (the sample diluent, enhancer, conjugate G+M and the developer) are provided with reaction cuvettes. An optional reagent proverb G is also provided for an additional

Fig. 1 The INDX E2R3 Dip-S-Tick.

IgG/IgM positive	
Enrlichia sennetsu	1:64 IFA
Human Granulocytic Ehrlichia	1:80 IFA
R. rickettsii	1:16 - 1:32 IFA
R. typhi	1:32 - 1:64 IFA
O. tsutsugamushi	1:400 Karp/Kato/Gilliam IIP

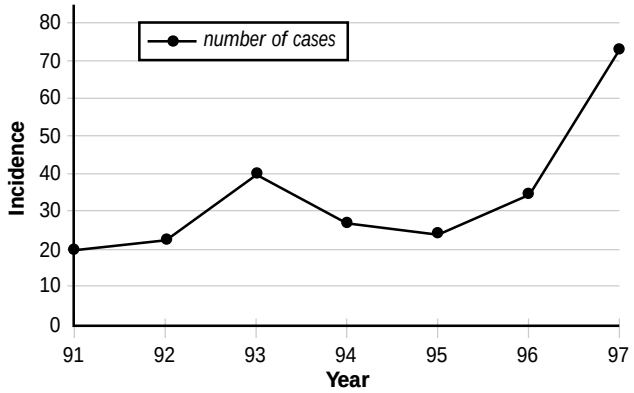

Fig. 2 Annual incidence of Endemic Typhus for Years 1991-1997.

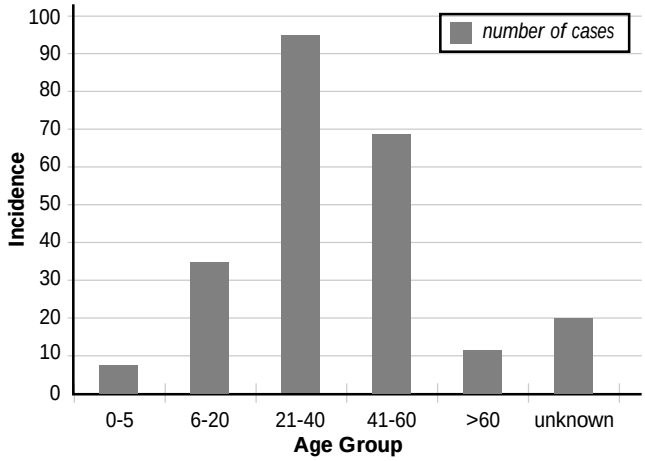

Fig. 3 Age Group incidence of Endemic Typhus for Years 1991-1997.

Table I. Race and Gender incidence of Endemic Typhus (1991-1997)

Race	Male No. (%)	Female No. (%)	Total No. (%)
Malay	43	21	64 (27)
Indian	55	40	95 (39)
Chinese	34	22	56 (24)
Others	19	4	23 (10)
Total	151 (63)	87 (37)	238

step when assaying for only IgM. In this study, we had screened for both IgG and IgM. This assay is performed in a 50°C waterbath. Two ml of each reagent is dispensed into 4 separate reaction cavettes. In the first cuvette containing the sample diluent, 10µl serum is added to make a serum dilution of 1:200. The assay strip is pre-wetted in the clarifier vessel with distilled water for 4 min., then immersed in turn into the 4 reagent for the specified amounts of time i.e. the diluted serum (5 min.), the enhancer (5 min.), the conjugate G+M (12 min.) and finally the developer (5 min.). Between each reagent the strip is washed by dipping motion in a clarifier vessel with clean distilled water. Finally the assay strip is blotted with a paper towel and allowed to air dry. The positive control must be positive before interpretation. A dot with an easily seen, distinct border and a white or pale gray colour perimeter indicates a positive test. The results were read according to the intensity of staining from a scale of 0 (non reactive) to 4. A borderline reaction was recorded as ±.

V. Data Analysis

All 214 sera were previously determined by the respective diagnostic laboratories at University Hospital. The Dip-S-Ticks test on these sera were performed and separately read, in a double blind manner, independent of their identity.

The Dip-S-Tick and IIP test were compared using the Spearman rank correlation coefficient to see if there is an association between IIP titres versus Dip-S-tick staining intensity. Sensitivity and specificity for the Dip-S-Tick test were calculated based on IIP cutoff titre values >80 dilution, using standard equations: % Sensitivity = true positives/(true positives + false negative)X100 and % Specificity = true negatives/(false positive + true negative)X100. The positive predictive value (PPV) is calculated as the number of true positives/all positive tests and the efficiency of test (accuracy) as (true positive + true negative)/number of total samples.

RESULTS

I. Epidemiology

Between the years 1991-1997 a total of 17,428 WF ± IIP tests were performed with an average of 2,895 test per year. Out of these requests, there were 238 seropositive cases. Although the annual request for IIP/WF tests have been constant, the incidence of Endemic Typhus have shown a two to three fold increase in the past 2 years (Fig. 2).

These 238 seropositive cases were grouped according to age, race and gender. The resulting data shows that Endemic Typhus predominantly affects age group

20-40 and 40-60 year old age groups (Fig. 3) and has a male to female ratio of 2:1 (Table I). Race distribution indicates that it affects Indians more than the other races (Table I). Monthly incidence over the 7 years showed no evidence of a seasonal and cultural/festival variation (Fig. 4).

II. Evaluation of the INDX Dip-S-Ticks

The positive control in all 214 sera was positive indicating that all sera were tested correctly. Table II shows the result of the Dip-S-Ticks test and staining intensity on the 145 Active Endemic Typhus sera and the 69 negative control panel.

Active Endemic Typhus Sera (145):

Out of these 145 sera, 12 samples had titres of <160 by the IIP. These samples were taken during the acute phase of the illness and subsequently seroconverted i.e. showed a greater than 4 fold rise in titre levels. Using the Dip-S-Ticks test, two of these samples stained 1+, the remainder 10 being equivocal (+) or negative (Table II). Of the remaining 133 sera samples with IIP titre of >160, the Dip-S-Tick stained positive except for two sera. One sample which stained negative had an IIP IgG titre of >1600 and IIP IgM of 1:800 the other sample stained equivocal (±) and had IgG and IgM titre of 1:400 and 1:800 respectively.

These samples also showed a positive correlation between titre levels and staining intensity with equivocal stains for borderline IIP titres and 4+ stains for high titres (Fig. 5). The correlation is stronger when IIP IgG titres were used ($r_s = 0.652$, $P<0.0005$) compared to combined IgG+IgM ($r_s = 0.632$, $P < 0.0005$) and IgM ($r_s = 0.504$ $P < 0.0005$). It should be noted that the staining intensity is a subjective appraisal. For the higher titres cross reactivity was noted with *R. rickettsii* (64 out of 131 INDX positive samples, i.e. 49%) and *O.tsutsugamushi* (3 out of 131 INDX positive samples, i.e. 2.3%) but at a lower intensity than with the *R.typhi* stains. Cross-reactivity was also noted in the IIP test, mainly to *R. siberica*, a different strain of Rickettsia that is presumed to be the causative pathogen for Tick Typhus in Malaysia.

Negative Control Sera Panel:

Out of the 69 control sera tested, there are 5 cross reactive samples that stained with an intensity of 1+ to *R. typhi* or *R.Rickettsii* i.e. Typhoid (2), Hepatitis A (1) and Hepatitis B (1), and intensity of 4+ to Ehrlichia i.e. CMV/SLE(1).

From these results, the sensitivity and specificity of the INDX Dip-S-Ticks can be calculated to be 91.7% and 92.8% respectively, and a positive predictive value (PPV) of 96.4% and efficiency of test of 92.1%.

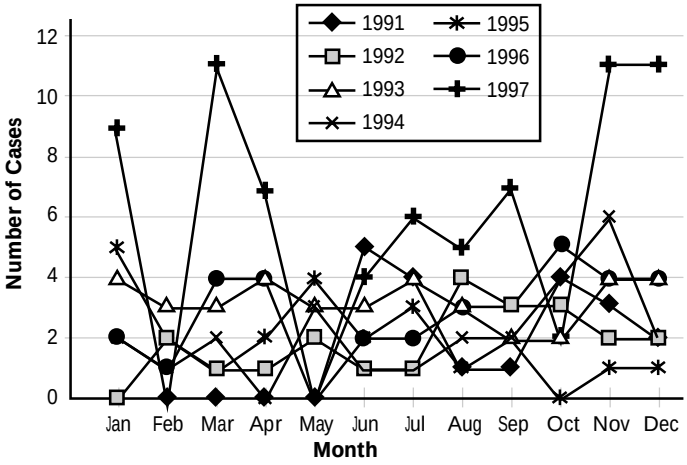

Fig. 4 Incidence of Endemic Typhus on a monthly basis for Years 1991-1997.

Table II. INDX Dip-S-Tick result on 214 sera samples

Total no. tested (n=214)		E2R3 INDX Dip-S-Ticks results					
			Positive results				
Endemic typhus sera (n=145)		negative results	RT	RR	OT	HGE	ES
IIP positive*	133	2	131	64(✚)	3(✚)	0	0
IIP negative	12	10	2	0	0	0	0
Others (n=69) Negative Control							
Dengue	10	10	0	0	0	0	0
Typhoid	10	8	0	0	2	0	0
Leptospira	10	10	0	0	0	0	0
Hep B	10	9	1	0	0	0	0
Hep A	9	8	1	0	0	0	0
Other viralº	10	9	0	0	0	0	1
Normal/Healthy	10	10	0	0	0	0	0

RT – *R. typhi;* **RR –** *R. rickettsii;* **OT –** *Orientia Tsutsugamushi;*
ES – *Ehrlichia sennets;* **HGE –** *Human granulocytic ehrlichia;*
Hep B – *Hepatitis B;* **Hep A –** *Hepetitis A.*

* *IIP positive is defined as IgG>640 and IgM>160.*

º *This is a combination of Herpes, Rubella, Cytomegalovirus/Systemic Lupus Erythematosus and Mumps. One sample (CMV/SLE) stained strongly positive for ES but cross reacted with all other rickettsial species.*

(✚) *Of the 131 RT positives by the INDX Dip-S-Ticks 64 cross-reacted with RR and with OT.*

However the staining intensity was much lower (1+) compared with (4+).

DISCUSSION

University Hospital is a tertiary hospital located in a suburban region close to Kuala Lumpur. The location is partly residential and partly industrial and there are nearby satellite regions of new development – both residential and commercial involving massive clearing up of land. The population in K.L. and surrounding suburban region is approximately 3 million. On average, UH admits 35000 - 40000 patients annually. It also has a busy outpatient department. The epidemiological study was done in this setting, based on requests for the IIP test at the University Hospital from 1991-1997.

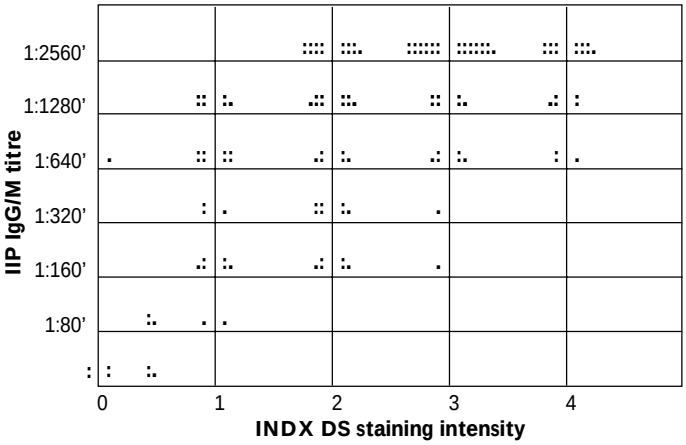

Fig. 5 Association between minimum endpoints of IIP IgG and IgM titres and Dip-S-Ticks intensity staining for each serum.

Each dot (.) represent a Dip-S-Tick against a known IIP IgG/IgM value.

Our serosurvey over 7 years recorded 238 seropositive cases, but over the past two years there have been a two to three fold increase in the seroincidence of acute Endemic Typhus in UH. When Endemic Typhus was first recognised in Malaysia in 1926, it was sporadic and uncommon with a reported annual country incidence of 36155 (mean of 100) from the years 1968-1974 (monthly Statistical Bulletin of West Malaysia, 1976)[11]. Today, 73 cases were serologically diagnosed at UH for the year 1997. Although this does not suggest national prevalence, it may perhaps indicate a growing trend. A multicentric survey should therefore be carried out to ascertain the current status in Malaysia.

Monthly trends show that Endemic Typhus occurs throughout the year without a seasonal or cultural distribution (Fig. 4). This is possibly more an indication of urban rodent population. Patient demographics indicates that Endemic Typhus is a reflection of socioeconomic circumstances and as previously described by Audy[12,13], is an "occupational disease and that differences in age, gender and race can be explained by behaviour or occupation."

As mentioned earlier, Endemic Typhus is usually a non specific and non life threatening illness but it may be associated with significant morbidity and with possible fatality. It's disease course may be prolonged without effective antibiotic use. Hence a reliable laboratory diagnostic assay that is accessible, easily stored and, simple to perform and interpreted would be an important ancillary arm in any clinical center.

The IIP although sensitive and specific, is not commercially available and requires a trained technician and various laboratory equipment to run the test. It involves the use of 3,3 Diaminobenzidine (DAB) substrate for the peroxidase reaction. This is a carcinogen and has to be handled with caution. Although the steps to the IIP test are simple, it is more labour intensive and takes longer to perform. The quality of the slides may vary significantly resulting in difficulty in slide interpretation and eye strain. In contrast the INDX Dip-S-Tick is a faster test that can be easily performed in almost any setting (The provided kit contains all reagents to run the sandwich ELISA dot test). The test can also be done using finger prick blood, citrated blood or serum. On the down side, it does not give an end point titre and an additional step have to be done for IgM determination.

Previous evaluation of *R.typhi* Dip-S-Ticks on 340 serum samples "at cutoff titres of $\geq$1:64 and $\geq$1:128 gave a sensitivity of 88.2% and 91.4% and specifities of 91.8% and 87.7% respectively[14]." Our study on 214 samples at cutoff titres of 1:80 IIP for *R. typhi*, have a sensitivity and specificity of 91.7% and 92.8% respectively.

Although cross reactivity occurred in approximately half of the test, the intensity of the reaction is in every case lighter. This percentage is higher when compared with previous serosurvey study using IIP which showed that the "presence of antibody to more than one species were detected in 31.3% of febrile patients indicating the possibility of coinfection or cross seroreactivities[12]". Clinically however, *Rickettsial* species differentiation is not an important issue in the treatment. This is because the same anti-Rickettsial antibiotics are used, at this point for all Rickettsial infections.

The dipstick evaluated is the E_2R_3 Multi-Test dipstick that tests for 5 antigens (Fig. 1). The possibility of designing a Multi-Test dipstick with an appropriate combination of pathogens for this region can narrow down the number of laboratory tests requested by a physician. This is significant today with limited financial resources and expanding battery of available diagnostic tests.

In conclusion, our epidemiology study have shown that the incidence of Endemic Typhus is increasing in line with our socioeconomic development and, the evaluated INDX Dip-S-Tick is a viable and practical alternative for the diagnosis of Endemic Typhus. However, an appropriate or broader combination of diagnostic tests in a single dipstick for PUO in this region, may be a pragmatic and perhaps economical alternative especially in a GP or rural setting. Physicians and scientists in turn have to be aware which test would give them the greatest yield. Hence, the need to re-educate ourselves regarding the evolving nature and prevalence of diseases.

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