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Seroprevalence of Scrub Typhus Infection in Patients with Pyrexia at Some Malaria Clinics in Three Western Provinces of Thailand

Charnchudhi Chanyasanha[1], Khanittha Kaeburong[1], Mongkol Chenchittikul[2] and Dusit Sujirarat[3]

SUMMARY In Thailand, the epidemiological data on scrub typhus infection represents only "the tip of an iceberg" especially in malaria clinics where patients come to seek attention because of other febrile illnesses that may have initial clinical signs that are indistinguishable from malaria. The objectives of this study were to determine the prevalence of antibody titers to *Orientia tsutsugamushi,* and its various strains, among patients at some malaria clinics in three western provinces of Thailand. The sample was represented by 200 patients from 6 malaria clinics in Ratchaburi, Petchaburi and Kanchanaburi provinces between June and November, 1994. Blood specimens were collected with their consent. Immunofluorescent antibody assays (IFA) were used for measuring IgM and IgG antibody titers for scrub typhus infection. The results showed that the prevalence rate for scrub typhus infection (IgM and/or IgG titer ≥ 50) was 59.50% (119 cases). The immunofluorescent antibody response to various strains of *O. tsutsugamushi* showed that co-infections with the Karp, the Gilliam and the Kato strains were the most common (found in 68.10% of cases). Geometric mean antibody titers (GMT) were highest for the Karp strain, followed by the Gilliam then Kato strains. In conclusion, this study indicates that the prevalence rate of scrub typhus is not rare in these areas.

Thailand is a developing country that is still grappling with infectious diseases. Although modern medical and laboratory technology have been applied in the investigation and treatment of infectious diseases, they are still prevalent. The varied ecology and topography of Thailand are appropriate for infective organisms, but there is a lack of medical apparatus and health personnel for controlling them. Poor health behaviors and an ineffective surveillance system are also contributing factors, especially in rural areas.

Scrub typhus is caused by *Orientia tsutsugamushi* (formerly *Rickettsia tsutsugamushi*) and is endemic in rural areas of many countries in Southeast Asia, the South Pacific islands, Australia and the Asian subcontinent.[1-3] In endemic areas, scrub typhus is a major cause of febrile illness that is sometimes fatal. It is an accidental disease in humans,[4] transmitted by larval trombiculid mites (chiggers). The study sites were chosen because they have an ecology favorable for the vectors of malaria (mosquitoes) and scrub typhus (chiggers). There have been few studies of scrub typhus infection in patients in malaria clinics. For prevention and control, situational data is needed to set project priorities for this disease as well as for malaria control. The objectives of this study were to determine the prevalence rate of scrub typhus infection and to assess fluorescent antibody titers (IgM and IgG) to *O. tsutsugamushi,* and its various strains, among patients at selected malaria clinics.

From the [1]Department of Microbiology, Faculty of Public Health, Mahidol University, Bangkok, Thailand, [2]Health Sciences Research Institute, Department of Medical Sciences, Nonthaburi, Thailand and [3]Department of Epidemiology, Faculty of Public Health, Mahidol University, Bangkok, Thailand.

MATERIALS AND METHODS

Research design and sample size

This cross-sectional study was conducted to determine the prevalence rate of scrub typhus infection at malaria clinics in Kanchanaburi, Phetchaburi and Ratchaburi Provinces, Thailand, during June through November 1994. This study was conducted at 6 malaria clinics, 2 from each of 3 provinces. Sample size was calculated by using the formula ($n= Z^2 PQ/d^2$),[5] where "P" is the proportion of persons with infection attributes. In this study , the value of "P" equals 0.59,[6] Q is equal to (1-P) and "d" is the standard error and equals 0.5. When substituted into the formula, the sample size is equal to 119 cases. However, in this study 200 cases were used. The study protocol was approved by the Ethical Review Research Committee, established by the Ministry of Public Health, Thailand.

Specimen collection

All participants of the study were older than 15 years of age. Written consent was obtained from the patients or the patients' parents or guardians. A five milliliter sample of blood was collected from each case. Specimens were kept at room temperature until clotted, then the sera were separated and all single sera were kept at -20°C in a deep freeze refrigerator until laboratory analysis.

Antigen

L929 cells (mouse fibroblasts) were used for antigen preparation. These cells were cultured in MEM Eagle's medium with fetal calf serum. After 3-5 days in a 37°C incubator, the cells had grown into a monolayer in the tissue culture flask. One to two days before the formation of the monolayer (approximately 80% of the cells) the medium was removed and inoculated with rickettsial seed (*O. tsutsugamushi* infected L929 cells). These seeds were removed from -80°C and thawed in a warm water bath of 37°C. The seeds were frozen and thawed another 3 times, then ground 30 times in a sterile cell grinder before use for inoculation. Each flask of L929 cells was infected with each strain of *O. tsutsugamushi* (Gilliam, Karp or Kato) and absorbed for 2 hours, with the flasks tilted every half hour. Following the incubation period, the maintenance medium was added and the cells were propagated for a few days more in a 28°C incubator. The rickettsial organisms were often released from the cells by extension and retraction of the cell cytoplasm.[7] When the cells had propagated into a monolayer, the medium was removed and the infected cells were harvested by a scraper (Greinur Labotechnik, Germany). The infected cells were suspended in a small amount of culture medium and centrifuged at 150 x *g* for 10 minutes. The sediment was resuspended in phosphate buffered saline (PBS), pH 7.3, then stored at -80°C as the antigen for the test without further purification. The antigen was frozen and thawed another 3 times to promote cells lysis in order to liberate more organisms from the cells. One microliter of pooled strain antigen was spotted individually on 21-well microscope-slides for screening. In addition, each strain of *O. tsutsugamushi* was spotted on 12-well microscope-slides for determination of antibody end-point titers in each strain. The spotted slides were fixed in chilled acetone (5 minutes), air-dried, then stored at -20°C until use for antibody detection.

Serum dilution and laboratory determination

Each serum sample was diluted with PBS to 1:25 and 1:50 solutions. The 1:50 dilution was used for screening with pooled rickettsial antigen.[8] For samples that showed positive at 1:50 dilution, a two-fold dilution of sera was subsequently done with each rickettsial antigen (Karp, Gilliam and Kato strains) to obtain an end-point titer.

Control antigen

The uninfected L929 cells were used as a control antigen. This antigen was spotted onto each slide, which was processed in the same way as the infected cells.

Control sera

Anti-rickettsial positive and negative control human sera were kindly provided by the Health Science Research Institute, Department of Medical Sciences, Ministry of Public Health. Both control sera were tested identically in the same dilutions and under the same conditions as the patients' serum specimens for every assay: control antigen, pooled strains or each strain of rickettsial antigen separately.

Antibody determination

Immunofluorescent antibody assay (IFA) was used to determine scrub typhus antibody titers.[9] Briefly, 10 µl of a patient's serum at dilution 1:50 were added to the antigen spotted slides which were then fixed for the first screening. The slides were placed in a humidified chamber to prevent evaporation and incubated at 37°C for 40 minutes. Then, the slides were removed

from the incubator, washed with PBS 3 times (5 minutes each time) and air-dried. Five microliters of diluted 1:40 Fluorescein isothiocyanate (FITC) conjugated rabbit anti-human IgG or IgM (Dako, Denmark) was dropped onto each spot and incubated at 37°C for 40 minutes (one slide for IgM FITC conjugate and the other slide for IgG FITC conjugate), washed again with PBS 3 times (5 minutes each time), then air-dried. The slides were then mounted with buffered glycerol (0.05 M carbonate buffer, pH 9.5 in fluorescence-free glycerol) and covered with a cover slip. The slides were viewed at 200x magnification, under a fluorescence microscope (Nikon: Optiphot-2) with a Philips CS 100 W-2 lamp. The positive samples were further diluted in two-fold serial dilutions to determine the end-point antibody titers with each strain of antigen. The final fluorescent antibody titer of a serum was designated as the reciprocal titer of the highest serum dilution which gave a positive fluorescent reaction.

Interpretation of the results

A positive serodiagnosis was considered as a titer with IgM and/or IgG $\geq$ 50, and seroconversion was defined as a change in antibody titer of $<$ 50 to $\geq$ 50. The mean titers in each group of classification in the results was expressed as the reciprocal geometric mean titer (GMT) and the standard deviation (SD).

RESULTS

Of the two hundred malaria clinic patients in this study, most of them were male and the average was between 25-29 years of age. The prevalence rates in males and females were not different (see Table 1). There were 119 cases (59.50%) seropositive for scrub typhus infection by IFA. The highest prevalence rate and GMT corresponded to scrub typhus infection found in the 45-49 years age group (77.77%) as well as in Petchaburi province. The infection rate for each age group are shown in Table 2. All provincial prevalence rates are summarized in Table 3. When classified by occupation, border policemen and forest officers were found to have the highest rates of infection (as shown in Table 4). The GMT were highest for Karp strain (GMT = 136). Data on cases and GMT of scrub typhus infection classified by strain, are summarized in Table 5. The results show that the co-infections with the three strains of *O.*

Table 1 Prevalence rates and geometric mean titers of scrub typhus infection among patients visiting malaria clinics, classified by sex.

Sex	No. of tests (cases)	No. of positive (cases)	Prevalence rate	GMT± SD
Male	137	83	60.58	91 ± 3.02
Female	63	36	57.14	100 ± 4.00
Total	200	119	59.50	95 ± 3.35

GMT = Geometric mean titers, SD = Standard deviation

Table 2 Prevalence rates and geometric mean titers of scrub typhus infection among patients visiting malaria clinics, classified by age group

Age group (year)	No. of tests (cases)	No. of positive (cases)	Prevalence rate	GMT± SD
15 - 19	28	15	53.57	87 ± 2.61
20 - 24	28	14	50.00	77 ± 2.66
25 - 29	47	27	57.44	97 ± 3.87
30 - 34	31	19	61.29	75 ± 2.71
35 - 39	25	17	68.00	105 ± 4.01
40 - 44	21	14	66.66	166 ± 3.88
45 - 49	9	7	77.77	149 ± 6.62
50 - 54	1	0	0	-
55 - 59	9	5	55.55	66 ± 2.32
≥ 60	1	1	100.00	50 ± 0.00
Total	200	119	100.00	95 ± 3.35

GMT = Geometric mean titers, SD = Standard deviation

Table 3 Prevalence rates and geometric mean titers of scrub typhus infection among patients visiting malaria clinics, classified by province.

Provinces	No. of tests (cases)	No. of positive (cases)	Prevalence rate	GMT ± SD
Ratchaburi	80	50	62.50	83 ± 3.20
Petchaburi	65	42	64.61	131 ± 4.00
Kanchanaburi	55	27	49.09	71 ± 2.50
Total	200	119	59.50	95 ± 3.35

GMT = Geometric mean titers, SD = Standard deviation

Table 4 Prevalence rates and geometric mean titers of scrub typhus infection among patients visiting malaria clinics, classified by occupation.

Occupation	No. of tests (cases)	No. of positive (cases)	Prevalence rate	GMT ± SD
-Border policeman and forest officer	20	13	65.00	88 ± 3.30
-Agriculture	113	71	62.83	84 ± 3.10
-Search for food orgoods from the forest	54	31	57.40	112 ± 3.80
-Others	13	4	30.76	119 ± 3.60
Total	200	119	59.50	95 ± 3.35

GMT = Geometric mean titers, SD = Standard deviation

Table 5 Number of cases and geometric mean titers of scrub typhus infection classified by strains of *O. tsutsugamushi.*

Strains	No. of positive cases	GMT ± SD
Gilliam	99	126 ± 3.01
Karp	99	136 ± 3.09
Kato	94	108 ± 3.15

GMT = Geometric mean titers, SD = Standard deviation

tsutsugamushi (Gilliam, Karp and Kato) were antigenically dominant at 68.10% as shown in Fig. 1.

DISCUSSION

The prevalence rate was calculated from positive serum samples of titer ≥ 50. A titer less than 50 was observed in our samples for those who probably had no exposure to scrub typhus infection. This titer has been used in a number of studies as a cut off for positive samples.[10-14] Hence, we used a titer of ≥ 50 as a criterion for positive serum samples indicative of scrub typhus infection, either previous or recent. In a study on the longevity of antibodies in patients with scrub typhus in Peninsular Malaysia, it was found that the antibodies to *O. tsutsugamushi* can persist for at least 12 months.[15] Our cut off value is specific for the group of individuals studied in the period examined. Previous reports from areas not endemic for scrub typhus have found in people IFA titers of less than 25.[16] Therefore, our cut off value is applicable in similar groups, especially in endemic areas where exposure to scrub typhus exists.

The results of this study show that the prevalence rate of scrub typhus infection in patients visiting malaria clinics in three western provinces of Thailand is 59.50%, as demonstrated by positive antibody titers to *O. tsutsugamushi*. Previous studies on its prevalence in Thailand were reported for central Thailand in 1982 and found to be 69%,[17] and in 1983 for northern Thailand it was found to be 59.50%.[6] Therefore, the prevalence rate found in this study is in accordance with previously reported results. Furthermore, this study fo-

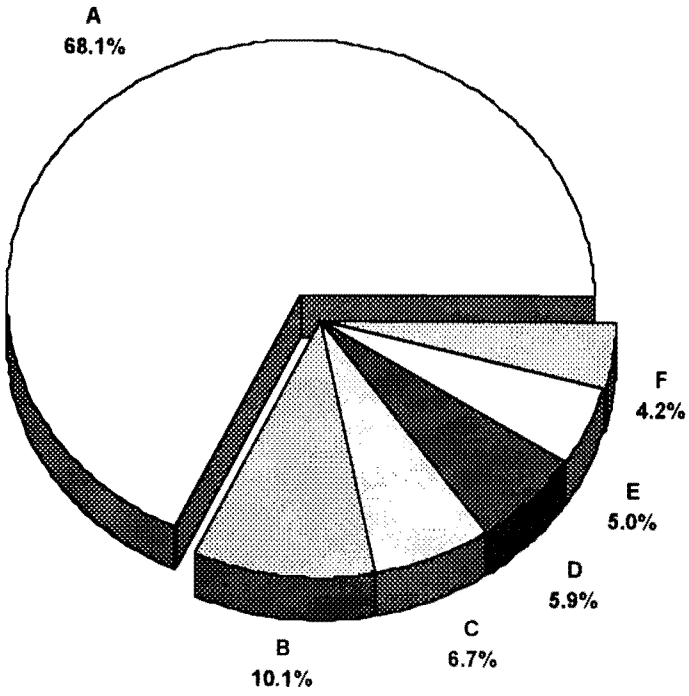

Fig. 1 Percent IFA antibody responses to the various strains of *O. tsutsugamushi.*

cused on patients who resided in rural areas where there is an increased chance of contact with chiggers. The rate found in this study confirms that scrub typhus is essentially a rural disease, which is sometimes called "rural typhus" due to its etiological and ecological nature. However, urban populations can also be infected as a result of extensive travelling.[18-20] A previous study in 1991 from blood donors in Bangkok found the prevalence rate to be 13.39%,[21] while in 1994 in suburban Bangkok the prevalence was 21%.[22] Elsewhere in Asia, seroprevalence varies between countries. For example, in Sabah, in the eastern part of Malaysia, the prevalence rate was found to be 0.8%,[23] while in Peninsular Malaysia it was reported to be 35%,[24] which was approximately the same as that found on Pescadores Island, Republic of China.[10]

With respect to individual strains of *O. tsutsugamushi*, the monovalent strain response to the strains Karp, Kato or Gilliam, were rather low. It is possible that these cases represented primary infection. Meanwhile, most of the positive assays were represented by trivalent (Karp-Gilliam-Kato 68.1%) and bivalent (Karp-Gilliam 5.0%, and Karp-Kato 4.2%) responses, which may be indicative of secondary scrub typhus infection. Interestingly, no cases were found bivalently positive for Kato-Gilliam strains. We can conclude that the Karp, Gilliam and Kato strains are still predominantly found in all three provinces. The GMT for the Karp strain were the highest among the three *O. tsutsugamushi* strains. In previous reports in Thailand, it was stated that the Karp and the Karp-Gilliam strains were dominant.[25] Previous studies on positive rickettsial isolations from rats, chiggers and humans reported that cases should be found throughout Thailand, including some northern provinces as well as in Pakistan[26] and Malaysia,[27-28] and that the Karp strain is still antigenically dominant in all of these areas. Our data presents the GMT and SD values in the tables. The SD values indicate the dispersion or variation of the data. Thus, for a GMT value of the estimated 95% confidence interval (CI) is easily derived by substituting the SD in the formula.[29] The formula is: 95% CI of GMT = antilog [log GMT $\pm$ 1.96 (SD/$\sqrt{n}$)].

The IFA test for serodiagnosis and serological surveys of scrub typhus have been shown to be both highly sensitive and specific.[16] These tests found no cross reactions with other organisms, such as the etiological agents of typhoid, paratyphoid, malaria, leptospirosis and flavivirus infections.[30] The IFA test is generally accepted as one of the standard methods and has been

recommended by the World Health Organization (WHO) as the routine serological diagnostic method and serological survey tool for scrub typhus antibody detection. [WHO Meeting of The Task Force on the Serological Diagnosis of Tsutsugamushi Disease (Scrub typhus), Nishiharacho, Okinawa, Japan, 24 November 1986]. The IFA standard for serodiagnosis of scrub typhus infection has not been widely used within the endemic regions due to a general lack of fluorescent microscopes. In the past, most febrile patients were admitted to hospitals which lacked sophisticated laboratory facilities and were subsequently treated without the benefit of technologically advanced analytical procedures. More recently, many regional hospitals have acquired fluorescent microscopes. Scrub typhus antigens should be rapidly distributed to these centers for diagnostic measures to proceed. If the public service system network were fully operational, diagnosis of scrub typhus would be more common due to the transfer of specimens and laboratory reports.

After assessing the data, we concluded that scrub typhus is not rare in Thailand, and it is probably grossly under-reported. Our specimens, which were collected during June to November, half of the year, provide results that indicate many people who live in these three western provinces of Thailand have been infected with scrub typhus infection.

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