

## DETECTION OF ORIENTIA TSUTSUGAMUSHI IN CHIGGERS AND TISSUES OF SMALL MAMMALS USING POLYMERASE CHAIN REACTIONS

### ABSTRACT

This study is to detect the presence of *Orientia tsutsugamushi* in chiggers and host target organs of scrub typhus such as liver, spleen as well as blood. Nine locations of Peninsular Malaysia were chosen; Slim River, Perak; Janda Baik, Pahang; Seremban, Negeri Sembilan; Hulu Langat, Selangor; Raub, Pahang; Bukit Panchor, Pulau Pinang; Gunung Inas, and Ulu Sedim, Kedah and Setiu, Terengganu. Collection sites included oil palm plantations, rubber estates, secondary forests, housing estates and coastal areas. All samples were processed by a standard method for extraction of DNA, followed by amplification of DNA using nested polymerase chain reactions (PCR) and then visualized by gel electrophoresis. A total of 465 chiggers, *Leptotrombidium deliense* extracted from 11 species of hosts *Callosciurus* spp, *Leopoldamys sabanus*, *Maxomys rajah*, *Maxomys whiteheadi*, *Niviventer cremoriventori*, *Rattus rattus*, *Rattus tanezumi*, *Rattus tiomanicus*, *Rhinosciurus laticaudatus*, *Sundamys muelleri*, *Tupaia glis* and *Tupaia minor*. All chiggers and animal tissues were found negative with scrub typhus except for a blood sample from a rodent, *L. sabanus* from Sungai Sedim, Kedah. The finding showed that the prevalence of scrub typhus in Peninsular Malaysia was very low. More study and survey need to be done.

**KEYWORDS:** *Orientia tsutsugamushi*, chiggers, tissues, small mammals, PCR.

### 1. INTRODUCTION

Scrub typhus, caused by antigenically disparate isolates of *Orientia tsutsugamushi*, is a widely distributed mite-borne human disease in the Asia Pacific region<sup>1</sup>. It is distributed throughout the Asia Pacific rim, being endemic in Korea, China, Taiwan, Japan, Pakistan, India, Thailand, Malaysia, and northern portions of Australia<sup>2</sup>. It is a mild to fatal disease depending on both the *O. tsutsugamushi* strain encountered and the genetic background and physical condition of the patient. The disease typically present with fever, headache, maculopapular rash, eschar (pathogenic lesion), lymphadenopathy and central nervous system involvement. This disease is caused by infection with *O. tsutsugamushi* following the bite of an infected trombiculid mite. The usual hosts for chiggers are species of field mice, rats, tree shrew and other small mammals that are peculiar to the zoogeographic regions and specific habitat. Majority of the vector borne disease survived in nature by utilizing animals as their vertebrate hosts<sup>3</sup>. Scrub typhus encountered in grassy fields along the banks of rivers in neglected or abandoned rice fields, gardens and plantation in over grown man-made clearings, in forests or jungles and in the junctional shrubby fringe between field and forest.

Study by Tay et al,<sup>4</sup> found that majority of the patients who lived and worked mainly in rural areas in Malaysia had antibody against rickettsiae. That included people in the agricultural sector such as rubber trappers, farmers or oil palm plantation workers. The study also reported the detection of *O. tsutsugamushi* from those patients' blood samples using polymerase chain approach<sup>[4]</sup>. Another study reported that 66% of rubber estate workers in Slim River, Malaysia were seropositives for at least one rickettsia and had antibody to more than one rickettsiae<sup>5</sup>. The findings of these studies suggested that rickettsial infections are endemic in certain parts of Malaysia.

Several methods have been developed for detection of rickettsiae in arthropod samples. The direct immunofluorescent assay (DFAT) has been widely reported for detection of rickettsiae from arthropods samples<sup>6</sup>. However, the method requires specific antibody against rickettsiae, which is usually not available except in reference laboratory. Polymerase chain reaction (PCR) approach is a highly sensitive technique for detection of rickettsiae. Kelly<sup>6</sup> develop a method for detecting and characterizing the DNA of *O. tsutsugamushi* in chiggers (larval trombiculid mite) by PCR. Nested PCR is described as a very sensitive technique to detect *O. tsutsugamushi* DNA, making it a useful tool for early diagnosis of scrub typhus<sup>7</sup>. Three procedures for extracting DNA from frozen

chiggers were compared by evaluating the yield of PCR applicant obtained with nine oligonucleotide primers pairs derived from the rickettsial 22kD, 47kD, groESL. 56kD and 110kD antigen genes. Although extracts and primer pairs differed in amplification efficiency, *O. tsutsugamushi* DNA was successfully detected in extracts seeded with known amount of Karp-strain rickettsiae. PCR was also employed for detection of *O. tsutsugamushi* DNA from chiggers for the first time. Both adults and larval stages of chiggers application of the polymerase chain reaction which uses specific oligonucleotide primers and Taq polymerase to synthesize copies quantities of DNA from single template, proved to be valuable new approach to the detection and identification of pathogenic rickettsial within infected vectors. The purpose of this study is to detect the presence of *O. tsutsugamushi* in chiggers and host target organs of scrub typhus such as liver, spleen as well as blood using nested PCR.

## 2. MATERIALS AND METHODS

### 3. Study areas

Selection of study areas was based on the presence of scrub typhus reported in Peninsular Malaysia<sup>6</sup>. Nine locations were chosen: there are Slim River in Perak, Janda Baik in Pahang, Seremban in Negeri Sembilan, Hulu Langat in Selangor, Raub in Pahang, Bukit Panchor in Pulau Pinang, Gunung Inas and Ulu Sedim in Kedah and Setiu in Terengganu. Collection sites included oil palm plantations, rubber estates, secondary forests, housing estates and coastal areas.

### 4. Host

### 5. Trapping of small mammals

Trapping of small mammals with wire traps was conducted simultaneously over five days using 100 traps (each measuring 20x20x30 cm) per site. Traps were laid in undergrowth along random transect lines at approximately five meters intervals. Wire traps were baited with bananas and checked every morning. Baits were replenished every day.

### 6. Processing animals

Traps with animals inside were each placed in cloth bags and brought back to field laboratory (complete with microscopes, dissecting sets, chemicals etc) for further processing. Animals were anesthetized with Zoletil 50 (Virbac Laboratories, France) prior to identification of the species. Cardiac puncture was then done to collect 3 ml blood using appropriate humane procedure in EDTA tubes and stored at -20°C until further use. Animals were then dissected to collect livers and spleens.

### 7. Blood samples

Freeze-thawed 2.5 µL small mammals whole blood were used as a template in PCR.

### 8. DNA extraction

### 9. DNA extraction from chigger

Chiggers preserved in alcohol with known identification and locations of collection were rinsed with distilled water for five times. A group of 15 chiggers were used for extracting DNAs. DNAs were extracted by using QIAmp DNA Mini Kit (Qiagen) according to manufacturer's instruction. The DNAs were then stored at 4°C and used as templates for PCR or -20°C for longer periods.

## 10. DNA extraction from animal tissues

Ten milligrams of each tissue of small mammals were used for DNA extraction. The DNAs were extracted using QIAmp DNA Mini Kit (QiAgen) according to manufacturer's instruction and were stored at 4°C and used as templates for PCR or -20°C for longer periods.

## 11. Primers

Nested PCR were performed as described by Furuya et al<sup>8</sup>. PCR amplification of the Sta56 gene was performed by using species specific primers, p34 (5'- TCA AGC TTA TTG CTA GTG CAA TGT CTG C-3'), p55 (5'- AGG GAT CCC TGC TGC TGT GCT TGC TGG G-3'), p10 (5'- GAT CAA GCT TCC TCA GCC TAC TAT AAT TGC C-3'), p11 (5'- CTA GGG ATC CCG ACA VGAT GCA CTA TTA GGC-3').

## 12. PCR

### 13. Amplification of pathogen in chiggers

The amplification was performed in a volume of 50uL. A mixture of 50uL solution containing 5 uL 10X PCR Buffer, 3 uL MgCl<sub>2</sub>, 1 uL of each dNTP and primers (p34 and p55 for first PCR/ p10 and p11 for second PCR), 0.25 uL taq and 2 uL chigger's DNA as template. The amplification program for initial denaturation consisted at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 2 min and extension at 70°C for 2 min. This process was followed by final extension at 70°C for 5 min and holding temperature at 12°C using an Eppendorf Master Cycler Personal machine.

### 14. PCR Amplification of pathogen in blood samples

The PCR was performed using KAPA Blood PCR Kit (Kapa Biosystems Inc. USA) according to the manufacturer's manual. A mixture of 25 µL solution containing 2X Kappa Blood PCR mix, 0.5 µM of each primer and 2.5 µL blood DNA template was prepared. The amplification program for initial denaturation consisted at 94°C for 2 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 2 min and extension at 70°C for 2 min. This process was followed by final extension at 70°C for 5 min and holding temperature at 12°C using an Eppendorf Master Cycler Personal machine.

## 15. Electrophoresis

The amplification products underwent electrophoresis in 1.5% agarose gel containing ethidium bromide at 90V for 1 hour and visualized under ultraviolet (UV) light. DNA from reference strains Karp and Gilliam were used as positive control (supplied by Bacteriology Unit, IMR) and distilled water was used as a negative control in each amplification. A 100 bp DNA ladder (Bioron, Germany) was used as the standard marker for comparison.

## 16. Gene sequencing

PCR products from gel were excised with a sterile gel cutter and purified using 5 Prime PCR Agarose Gel Extract Mini Kit (Hamburg, Germany) according to the manufacturer's procedure. DNA sequencing in both directions was done in the presence of the ABI PRISM ready reaction big dye terminator cycle sequencing kit (Applied Biosystems, Foster City, California, USA), following the manufacturer's manual.

## 17. BLAST search

To identify the pathogen, the obtained unknown sequences were compared with those already deposited in the GenBank database using the BLAST program in Basic Local Alignment Search Tool searches (<http://www.ncbi.nlm.nih.gov/BLAST/>)<sup>9</sup> to identify sequences with high similarity.

## 18. RESULTS AND DISCUSSION

Eighty-eight (88) small mammals comprising of 11 species were trapped from nine locations in Peninsular Malaysia (Table 1). A total of 465 chiggers identified as *Leptotrombidium deliense* were screened and all were found negative with scrub typhus. Eighty-eight samples for each blood, liver and spleen samples were also screened and 1.14% of blood sample was detected positive with *O. tsutsugamushi* (strain Gilliam) with 481bp (Figure 1). The blood was from *L. sabanus* caught in Sungai Sedim, Kedah. The similarity of gene sequence of the positive sample in this study with corresponding sequence in GenBank was 98% *O. tsutsugamushi*

This study was undertaken with aim of detecting the *O. tsutsugamushi* infection from tissues of small mammals and chiggers; *L. deliense*. The selected areas were reported positive with scrub typhus. Malaysia Vector Borne Disease Sector (No. 1/2011)<sup>10</sup> reported that, total cases of scrub typhus in Malaysia since 2006-2010 were 55 cases; it's showed that scrub typhus still present in Malaysia. A study by Blacksell et al<sup>11</sup> have been conducted and determined that the ***O. tsutsugamushi*** Karp strain is the dominant strain causing human infections in Thailand with lower proportions of Gilliam. In this study, over the 264 tissues samples, only one blood sample was positive with *O. tsutsugamushi* with Gilliam strain. By molecular methods, strains phylogenetically similar to Karp make up ~40% of all genotyped isolates, followed by Gilliam strain<sup>12</sup>. This study showed very low prevalence and may be due to many factors. No scrub typhus detected from the *L. deliense* as well as liver and spleen. The negative detection of scrub typhus from tissues and chiggers could be due to the absence or low numbers of scrub typhus in these samples. The sensitivity and specificity of PCR system used in this study confirmed the negative detection of *O. tsutsugamushi* from the samples.

Less numbers of small mammals caught over nine localities with different ecologies also contribute to low number of scrub typhus detected. There are many possibilities that can cause this to occur. Kwa<sup>13</sup> reported that, when the plantations mature, the ensuing ecological succession causes a decline in vector populations and disease rates. As the trees grow into maturity, the palm canopy closes and grass stops growing on the ground beneath, depriving the Trombiculid chiggers of their natural habitat<sup>13</sup>. Without the grass breeding sites, *L. deliense* declines to less than 1% of the total chigger population, ultimately having been replaced by *Ascoschoengastia indica*, a non-vector nest-dwelling mite. The versatile rodent *R. tiomanicus* adapts to the absence of grass by building nests among the litter and fallen palm fronds and continues to breed but, because of the decline in vector mites, harbors less rickettsial pathogens. In the other way round, decreasing of scrub typhus may be because of decreasing of small mammals population due to the use of pesticide in oil palm and rubber plantation and also housing estate. Lack of small mammals population would lead to lack of vector. Further research need to be done by survey more trapping areas.

## 19. CONCLUSION

From the study we can conclude that the prevalence of scrub typhus in selected places in Peninsular Malaysia is low and under control.

## 20. ACKNOWLEDGEMENTS

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## 21. REFERENCES

1. Ruang-areerate T, Jeamwattanalert P, Rodkvamtook W, Richards AL, Sunyakumthorn P, Gaywee J. Genotype diversity and distribution of *Orientia tsutsugamushi* causing scrub typhus in Thailand. *J. Clin. Microbiol* 2011; 49(7): 2584-2589.
2. Sexton DJ. 2011. Scrub typhus: Treatment and Prevention. [Online] Available from: [www.uptodate.com](http://www.uptodate.com). (Cited on 2011, October 10)
3. Artsob H. Vector-borne diseases. *Encycl Public Health* 2011; 5:1-3.
4. Tay ST, Kamalanathan M and Rohani MY. *Borrelia burgdorferi* (Strain B. Afzelii) antibodies among Malaysian Blood Donors and patients. *Southeast Asian J Trop Med Pub Health* 2002; 33(4): 787-793.
5. Tay ST, Kamalanathan M, Koay AS. Seroepidemiologic survey of *Orientia tsutsugamushi*, *Rickettsia typhi* and TT118 spotted fever group rickettsiae in rubber estate workers in Malaysia. *Am J Trop Med Hyg.* 1999; 61:73-77.
6. Kelly DJ. Serologic diagnosis of rickettsial diseases. *Clin. Immunol. Newsl* 1994; 14:1-5.
7. Fournier PE, Siritantikorn S, Rolain JM, Suputtamongkol Y, Hoontrakul S, Charoenwat S, et al. Detection of new genotypes of *Orientia tsutsugamushi* infecting humans in Thailand. *Clin Microbiol Infect* 2008;14:168-73.
8. Furuya Y, Yamamoto S, Katayama T, Kawamura A Jr. Serotype-specific amplification of *Rickettsia tsutsugamushi* DNA by nest-PCR. *Journal of Clinical Microbiology* 1993; 31(6): 1637-1640.
9. NCBI. National center for biotechnology information. 2010. BLAST, a basic local alignment search tool. [Online] Available from: <http://blast.ncbi.nlm.nih.gov>
10. Technical Meeting Malaysia Vector Borne Disease Sector Report. 2011
11. Blacksell SD, Luksameetanasan R, Kalambaheti T, Aukkanit N, et al. Genetic typing of the 56-kDa type-specific antigen gene of contemporary *Orientia tsutsugamushi* isolates causing human scrub typhus at two sites in north-eastern and western Thailand. *FEMS Immunol Med Microbiol.* 2008;52:335-342.
12. Kelly DJ, Fuerst PA, Ching WM, Richards AL. Scrub typhus: The geographic distribution of phenotypic and genotypic variants of *Orientia tsutsugamushi*. *Clin. Infect. Dis.* 2009; Suppl 3: S203-30
13. Kwa BH. Environmental change, development and vectorborne disease: Malaysia's experience with filariasis, scrub typhus and dengue. *Environ dev Sustain* 2008; 10:209-217.

Location	Species of small mammals caught	No of small mammals caught
Slim River, Perak	<i>Rattus tiomanicus</i>	10
	<i>Rattus tanezumi</i>	1
Janda Baik, Pahang	<i>Tupaia glis</i>	2
	<i>Leopoldamys sabanus</i>	2
Seremban, Negeri Sembilan	<i>Rattus tanezumi</i>	15
	<i>Callosciurus spp</i>	1
Hulu Langat, Selangor	<i>Sundamys muelleri</i>	1
	<i>Rhinosciurus laticaudatus</i>	2
	<i>Maxomys rajah</i>	1
Raub, Pahang	<i>Leopoldamys sabanus</i>	1
	<i>Rattus tanezumi</i>	5
Bukit Panchor, Pulau Pinang	<i>Maxomys rajah</i>	1
	<i>Rattus tanezumi</i>	2
Gunung Inas, Kedah	<i>Leopoldamys sabanus</i>	4
	<i>Maxomys whiteheadi</i>	2
	<i>Sundamys muelleri</i>	3
	<i>Niviventer cremoriventer</i>	2
	<i>Maxomys rajah</i>	2
Ulu Sedim, Kedah	<i>Sundamys muelleri</i>	4
	<i>Tupaia glis</i>	3
	<i>Rattus tiomanicus</i>	1
	<i>Rattus rattus</i>	1
	<i>Maxomys whiteheadi</i>	1
Setiu, Terengganu	<i>Tupaia minor</i>	1
	<i>Rattus tiomanicus</i>	8
	<i>Tupaia glis</i>	11

Table1: Trapping locations and the species of small mammals caught in Peninsular Malaysia

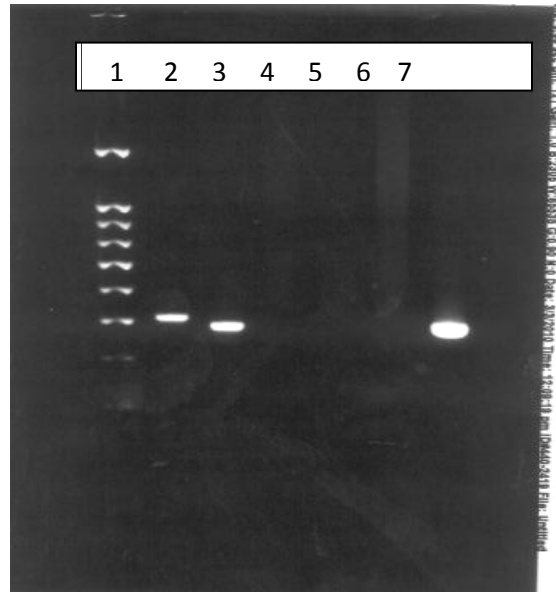


Figure 1. Agarose gel electrophoresis of amplified DNA by Nested PCR with serotype specific primers for strains Karp [lane 2 (507bp)] and Gilliam [lane 3 (481bp)]. Lane 4-6 contained negative blood samples and lane 7 contained positive blood sample with scrub typhus. Lane 1 contained 100bp ladder marker.

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