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Research Note

Genetic variants of *Orientia tsutsugamushi* identified from scrub typhus cases in Malaysia

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Abstract. We report herein the clinical presentation and diagnosis of scrub typhus in three patients attending a teaching hospital in Malaysia. Three genetic variants belonged to the Karp and Gilliam strains of *O. tsutsugamushi* were amplified from the acute blood samples of the patients by a nested polymerase chain reaction assay. The circulation of different genetic variants of *O. tsutsugamushi* strains might complicate the presentation and severity of scrub typhus. Loop-PCR is a promising diagnostic tool for rapid diagnosis of scrub typhus.

INTRODUCTION

Scrub typhus is an acute febrile illness which is prevalent in the Asia Pacific region. The causative agent, *Orientia tsutsugamushi*, is a Gram-negative obligate intracellular bacterium which is transmitted through the bites of infected *Leptotrombidium* mites. The finding of eschars has been important for making a provisional diagnosis of scrub typhus (Brown *et al.*, 1976), as this vector-borne disease can have similar clinical presentations with other tropical diseases such as dengue, malaria, murine typhus and leptospirosis. Delayed diagnosis and treatment of scrub typhus may occur resulting in life-threatening complications such as pneumonia, acute respiratory distress syndrome (ARDS), myocarditis, meningoencephalitis, acute renal failure, gastrointestinal bleeding and multi-organ failure (Wang *et al.*, 2007).

Laboratory investigations using serology and molecular approaches are important to confirm the diagnosis of scrub typhus. In a previous study, antibody prevalence of 24.9% to *O. tsutsugamushi* has been documented amongst febrile patients attending various health centers in Malaysia (Tay *et al.*, 2000). Despite the epidemiologic data, there is little documentation on the clinical aspects of scrub typhus cases in Malaysia, except for a report of two cases in children with eschars (Pau & Tan, 2008). Although Karp, Gilliam, and Kato are the major prototypes of *O. tsutsugamushi*, >20 antigenic variants have been described in previous investigations (Kelly *et al.*, 2009).

This study reports the diagnosis of scrub typhus in three adult patients using serological and molecular-based methods. The infecting strain was identified based on sequence analysis of the gene encoding the

56kDa immunodominant protein of *O. tsutsugamushi*.

CASE STUDY

Case 1. A 48-year-old lorry driver, an ex-army officer, presented with an eight-day history of fever, headache, arthralgia, myalgia and anorexia. He had been treated with amoxicillin by a general practitioner with no improvement. On admission to hospital, he was found to be alert with stable vital signs. No rash, eschar or petechiae was present on his body. His cardiovascular system and abdomen were normal. Chest X-ray (CXR) showed minimal right pleural effusion. Abdominal ultrasound revealed fatty liver changes. Except for elevated transaminases, his renal profile, white cell and platelet counts were normal (Table 1). A preliminary diagnosis of community-acquired pneumonia was made and he was treated with ceftriaxone for six days and azithromycin for five days. His fever subsided five days after commencement of azithromycin.

Case 2. A 43-year-old oil palm plantation worker was admitted to a private hospital due to fever, epigastric pain, diarrhea and vomiting for five days. He was started on ciprofloxacin. On the eighth day of his illness, he became confused and developed shortness of breath but had no cough or hemoptysis. A diagnosis of severe hospital-acquired pneumonia with possible meningitis was made and the patient was prescribed meropenem, azithromycin, metronidazole and acyclovir. On admission to our hospital, he was confused, drowsy, and restless. He was jaundiced and tachypneic (respiratory rate of 30/min) with a temperature of 37.5°C, BP 115/65, PR 96/min and an oxygen saturation of 99% on high flow mask. There was decreased air entry and crepitation was heard over the bases of the lungs. There was no evidence of meningism or focal neurological deficits. Blood investigations showed transaminitis, elevated alkaline phosphatase, bilirubin, urea and creatinine. White blood cell count was normal but the haemoglobin level and platelet count were low (Table 1). MRI of the brain

Table 1. Demographic, hematology and blood chemistry profiles of patients with scrub typhus

	Patient 1	Patient 2	Patient 3	Reference range
Demographic data				
Age (years)	48	43	64	
Ethnic group	Indian	Chinese	Malay	
Gender	Male	Male	Male	
Occupation	Lorry driver (ex-army)	Oilpalm plantation worker	Unemployed	
Blood profiles				
Hemoglobin (g/dl)	13.1	10	11.7	12-15
White blood cells (10^9/L)	9.4	6	14.7	4-10
Platelets (10^9/L)	154	81	117	150-400
Urea (mmol/L)	5.8	10.6	22.6	2.5-6.4
Creatinine (µmol/L)	114	112	517	62-115
Bilirubin (µmol/L)	19	132	14.6	3-17
Albumin level (g/L)	30	18	34	35-50
Alkaline phosphatase (U/L)	88	524	85	50-139
Alanine aminotransferase (U/L)	145	132	282	12-78
Aspartate aminotransferase (U/L)	129	168	199	15-37

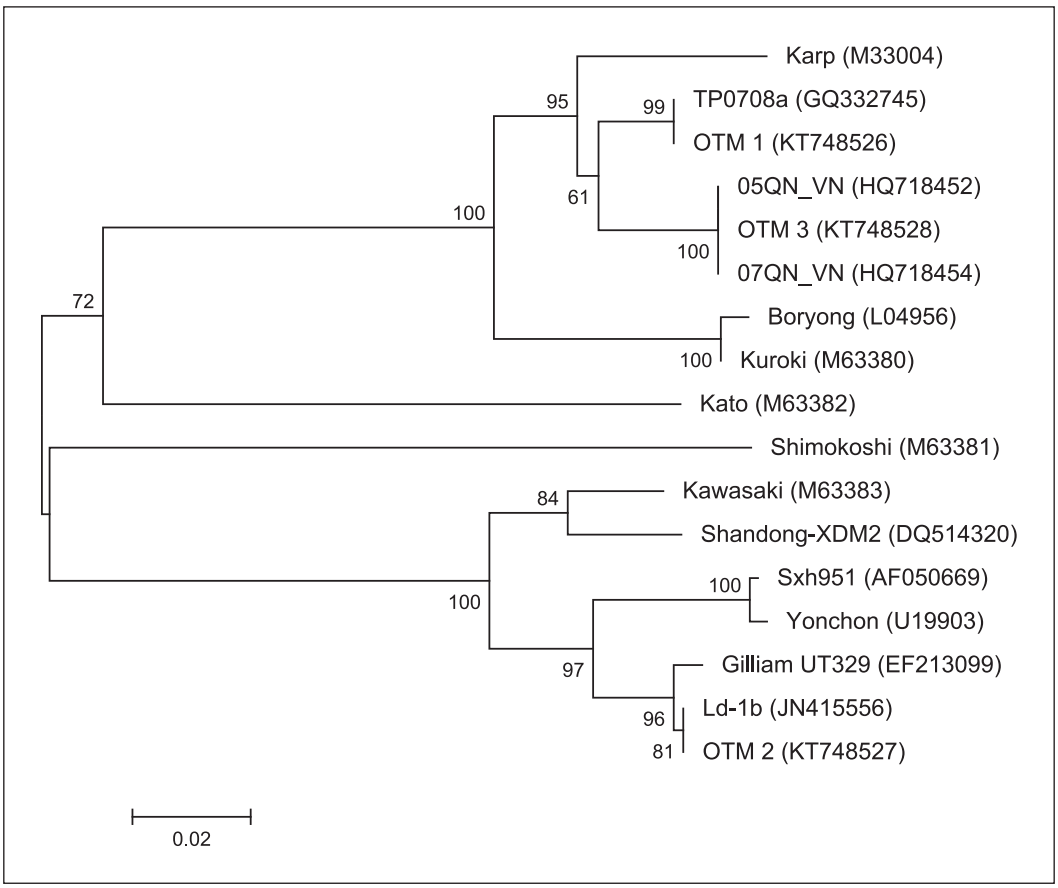

Figure 1. Dendrogram was constructed based on the sequence homologies of the amplified fragments (372-404 nucleotides) derived from the 56 kDa gene of *O. tsutsugamushi* strains. The numbers at nodes indicate bootstrap values. Bar shows genetic distance of 0.02.

was normal. Abdominal ultrasound revealed hepatosplenomegaly. CXR showed bilateral diffuse alveolar shadowing suggestive of acute respiratory distress syndrome (ARDS) or pulmonary hemorrhage. Lumbar puncture was carried out and the opening pressure was 32 cm H2O. CSF showed presence of pleocytosis, lymphocytes of 55%, high protein and low glucose levels. A provisional diagnosis of severe leptospirosis with ARDS and meningoencephalitis was made. He was given meropenam for eight days, azithromycin for seven days and acyclovir for five days. His fever subsided nine days after commencement of antibiotics.

Case 3. A 64-year-old unemployed man presented with fever of two-week duration with chills, rigors, headache, body ache, vomiting and cough with whitish sputum and was admitted to our hospital. He gave a history of hypertension, dyslipidemia and recent travel to a village within Malaysia two weeks prior to the onset of fever. On examination he was alert, vital signs were stable and his temperature was 38.3°C. There was no jaundice, rash or eschar. He had evidence of left parapneumonic effusion of his lungs. Blood investigations revealed elevated levels of transaminases, urea and creatinine. He had high white blood cell and low platelet counts (Table 1). A diagnosis of severe sepsis secondary to community-acquired pneumonia was made and he was treated with ceftriaxone and azithromycin for eight and four days, respectively. He improved clinically, biochemically and radiologically second day after the treatment. His peripheral blood counts, renal and liver

function tests reverted to normal by the fifth day of treatment.

Blood samples were collected for laboratory investigations for all patients during admission. The sera were analysed for IgG and IgM antibodies against *O. tsutsugamushi*, using a commercial serological assay (Scrub Typhus Detect IgG and IgM ELISA System, INBIOS International, Inc. USA) in accordance with the manufacturer's instructions. For molecular investigation, DNA was extracted from patients' clotted blood using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany). A nested PCR assay was performed targeting the 56 kDa gene of *O. tsutsugamushi*, as described previously (Furuya *et al.*, 1993). All PCR products were sequenced and compared for similarity with other sequences in the GeneBank database. Loop-isothermal DNA amplification method was performed on the positive samples as described by Paris *et al.* (2011), using a Loopamp DNA amplification kit (Eiken Chemical Co., Ltd., Tokyo, Japan). Briefly, two microliters of DNA template were added with 40 pmol each of FIP and BIP primers, 5 pmol each of F3 and B3 primers, 20 pmol each of Loop-F and Loop-B primers, and 1 µl of fluorescent detection reagent (Eiken Chemical Co., Ltd., Tokyo, Japan). The reaction mixture was first incubated at 63°C for 60 min, and then at 80°C for 5 min. The detection of fluorescence signal was confirmed by electrophoresis of the amplified products on a 2% agarose gel.

IgG and IgM antibodies against *O. tsutsugamushi* were detected above the ELISA cut-off values for all three patients. *O. tsutsugamushi* DNA was amplified from acute blood samples of the patients. Sequence analysis of the amplified DNA from patient 1 (OTM1, Genbank accession no. KT748526) demonstrated 100% matching with that of TP0708a strain isolated from the blood of a patient from Taiwan (Genbank accession no. GQ332745, Lu *et al.*, 2010). The sequence obtained from patient 2 (OTM2, Genbank accession no. KT748527) was identical to a chigger strain (Ld-1b) from Thailand (Genbank accession no. JN415556, Takhampunya *et al.*, unpublished). The amplified product obtained from patient 3 (OTM3, Genbank accession no. KT748528) was identical to those reported from two human samples (07QN_VN and 05QN_VN) from Vietnam (Genbank accession no. HQ718454 and HQ718452, Duong *et al.*, 2013). Dendrogram constructed based on the sequences of the amplified fragments (372-404 nucleotides) from the bacterial 56 kDa gene revealed that the infecting *O. tsutsugamushi* strains were closely related to those of Karp (OTM 1 and 3, 92.3 and 88.3 % identities, respectively) and Gilliam (OTM 2, 99.1% identities) prototypes, two of the most common *O. tsutsugamushi* strains reported so far. The presence of different genetic variants of *O. tsutsugamushi* might complicate the presentation and severity of scrub typhus in our patients. Isolation of the organism is required to determine the virulence of the infecting strains. Although mixed infection caused by different genetic variants of *O. tsutsugamushi* may occur in scrub typhus patients (Zhang *et al.*, 2014), the PCR technique used in this study only allowed amplification of the predominant genotype.

Diagnosis and management of scrub typhus cases may be delayed especially in non-tertiary care setting, as serological and molecular diagnostic approaches such as PCR and sequencing are usually not available. In this study, positive signal for loop-PCR was detected from the acute blood samples and confirmed by the detection of amplified products using agarose gel electrophoresis. As expensive equipment such as a thermal cycler is not required, this study shows the possible application of loop-PCR technique in clinical settings, as the method is rapid, accurate and suitable for point-of-care testing (Paris *et al.*, 2011).

All the patients in this study had a history of recent or previous exposure to bushes and scrubland areas. The absence of classical eschars may be due to the difficulty in differentiating eschars from lesions/ulcers due to other causes, particularly in patients with dark complexion (Brown *et al.*, 1976). The observation of high levels of alkaline phosphatase, alanine aminotransferase and aspartate aminotransferase in all our patients is in agreement with previous findings

whereby transaminase elevation (more than twice normal) has been reported in 90% of patients with scrub typhus (Varghese *et al.*, 2006). Elevated levels of bilirubin and urea suggestive of renal and hepatic dysfunction were noted in two patients (patient 2 and 3) with low haemoglobin level and platelet counts (<140,000/mm3). It has been reported that almost two third (62.5%) of patients with elevated IgM antibodies to *O. tsutsugamushi* in India had low platelet counts (Varghese *et al.*, 2006). Hypoalbuminemia in scrub typhus as noted in our patients has been associated with high complication rates, prolonged hospitalization, and necessity for more aggressive patient management (Lee *et al.*, 2010). Besides febrile illnesses, all our patients had respiratory infections; with patient 2 presented withARDS, a severe complication of scrub typhus (Chen *et al.*, 2012).

In this study, our patients responded well to azithromycin which is an alternative therapy for scrub typhus particularly when infected by doxycycline-resistant *O. tsutsugamushi* strains (Strickman *et al.*, 1995). Loop-PCR is a promising method for rapid diagnosis of scrub typhus. This study also suggests that as patients with scrub typhus may present with variable clinical manifestations, high index of suspicion and prompt empirical treatment is essential for patients from endemic areas with acute febrile illness even in the absence of the classical findings of eschar.

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