PATIENT AND SAMPLE-RELATED FACTORS THAT EFFECT THE SUCCESS OF *IN VITRO* ISOLATION OF *ORIENTIA TSUTSUGAMUSHI*

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Abstract. *Orientia tsutsugamushi* is the causative agent of scrub typhus infection, a major cause of human disease in rural areas of Southeast Asia. Twenty-six blood samples collected from patients with serologically proven scrub typhus during a six month period were sent to Bangkok (535 km from the clinical site) by road at ambient temperature (average daily temperature range: 27.1-29.1ºC) for attempted *in vitro* isolation in Vero cells. *O. tsutsugamushi* was isolated from 12 samples (sensitivity 46.7%) with the time to isolation ranging from 16 to 37 days [median 27 days, inter-quartile range (IQR) 22.5-33.5 days]. Patient factors such as days of fever and *O. tsutsugamushi* IgM antibody titer, transport factors such as transit time, and isolate genotype (Karp and Gilliam/Kawasaki) were assessed to determine their influence on the outcome of *in vitro* isolation. None of the factors significantly influenced the isolation outcome. This study demonstrates that *O. tsutsugamushi* can often be isolated *in vitro* from the blood of scrub typhus patients when transported at ambient tropical temperatures for many days.

INTRODUCTION

Orientia tsutsugamushi is the causative agent of scrub typhus infection, a major cause of human disease in rural areas of Southeast Asia. Scrub typhus infections are largely confined to the Asia-Pacific region, where the pathogen is maintained and transmitted to humans by the bite of the larval stage of the *Leptotrombidium* spp mite (Watt and Parola, 2003). The mainstay of therapy is treatment with doxycycline although treatment failure attributed to antibiotic resistance has been reported in northern Thailand (Watt *et al,* 1996).

Isolation of the infecting *O. tsutsugamushi* organism has been performed in mice and mammalian cell cultures and allows for genetic and antigenic characterization and the determination of antibiotic susceptibility. Furthermore, isolation of *O. tsutsugamushi* provides the raw materials for diagnostic assays. *In vitro* isolation of *O. tsutsugamushi* has been predominantly performed in embryonated chicken eggs, Vero (African green monkey kidney) or L (mouse fibroblast) cells, though isolation is complicated by biocontainment issues and contamination with adventitious microorganisms.

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For reasons of low-sensitivity and expediency, *in vitro* isolation is rarely used in the clinical setting for the diagnosis of scrub typhus infection, but the ability to culture is imperative for drug susceptibility testing and studies of molecular epidemiology, as well as providing a source of reference cultures for diagnostic tests in an endemic area.

This study describes the *in vitro* isolation of *O. tsutsugamushi* from scrub typhus patients in Thailand and the patient and sample transport related factors that influence a successful outcome.

MATERIALS AND METHODS

Patient specimens

Patients were recruited from a clinical investigation site located in Udon Thani in northeast Thailand (located 565 km from Bangkok). Samples were collected between June to November 2004 (the rainy season) from patients with suspected scrub typhus infection following a positive IgM antibody result by immunochromatographic rapid assay (Panbio, Australia) (Wilkinson *et al,* 2003) or on strong clinical suspicion (*eg*, presence of eschar). Details of samples are presented in Table 1. Following informed consent, approximately 10 ml of whole blood was collected from patients into blood tubes containing K_3-EDTA (Vaccutainer, Becton-Dickinson, USA).

Transport of specimens

Samples were transported by road from Udon Thani to Bangkok for attempted *in vitro* isolation. A sample collected in the morning in Udon Thani would arrive in Bangkok the morning of the following day, but samples collected in the afternoon normally did not arrive in Bangkok until two days later. The blood tube was placed in a well-padded microbiologically secure container conforming to IATA packing instruction 650 and transported at ambient temperature without refrigeration. On some occasions, transport of the specimens was delayed because of weekends (*ie*, collected on a Friday and arriving on a Monday) or delays by the transport company. Transit times for the specimens from Udon Thani to Bangkok are presented in Table 1.

Isolation of *O. tsutsugamushi*

In vitro isolation of *O. tsutsugamushi* was performed at a biocontainment level 3 laboratory in Bangkok. To ensure the integrity of samples and isolates, all manipulations were performed separately in a Class II biological safety cabinet to prevent cross-contamination and all culture flasks were assigned a unique code with full descriptors to prevent mis-identification. *In vitro* isolation was performed as follows:Blood samples were centrifuged at 3,000*g* for 10 minutes and 1ml of buffy coat collected by aspiration and mixed with 1ml of growth medium (RPMI 1640 medium containing 10mM HEPES (PAA, Austria) supplemented with 10% (v/v) fetal calf serum (PAA, Austria). The sample was then divided and inoculated into two 25 cm^2 tissue culture flasks containing 90% confluent Vero cell monolayers (ATCC CCL81). The flasks were centrifuged for 30 minutes at 500*g* at ambient temperature (approximately 25ºC) to facilitate infection of the monolayer. Following centrifugation the flasks were incubated at 37ºC for 60 minutes, the inoculum removed, the monolayer washed twice and 10 ml of fresh growth medium added and incubated in a 5% CO_2 environment at 37ºC for 30 days. To replenish the spent growth medium, approximately 60% of the growth media was removed and replaced every 4 days. The presence or absence of *O. tsutsugamushi* was checked by IFA every 7 days. IFA-positive cultures were re-passaged onto new Vero cell monolayers. At 30 days post-inoculation, IFA-negative cultures were re-passaged onto new Vero cell cultures and monitored using the same procedure. Cultures negative at the completion of the second passage were considered negative for the presence of *O. tsutsugamushi* and discarded.

Table 1
Details of patient sample and *in vitro* isolation characteristics.

Patient ID	Isolation	Fever (days)	Reciprocal IgM titer[a]	*O. tsutsugamushi* strain	Time to Isolate (Days)	Transit time (Days)
UT144	+	10	51,200	Gilliam/Kawasaki	36	2
UT150	+	7	1,600	Karp	31	4
UT177	+	5	3,200	Karp	28	1
UT203	-	7	3,200	Neg	NI[b]	2
UT206	-	3	400	Neg	NI	3
UT219	+	7	3,200	Karp	37	2
UT278	-	11	12,800	Neg	NI	1
UT295	-	6	12,800	Neg	NI	1
UT316	+	7	25,600	Karp	25	2
UT167	+	4	51,200	Karp	26	1
UT169	+	4	3,200	Karp	23	2
UT176	+	5	1,600	Karp	36	2
UT187	-	7	51,200	Neg	NI	2
UT196	+	5	51,200	Gilliam/Kawasaki	29	1
UT210	-	18	51,200	Neg	NI	1
UT213	+	5	12,800	Karp	16	9
UT221	+	14	800	Karp	22	2
UT224	-	7	51,200	Neg	NI	3
UT258	-	2	51,200	Neg	NI	2
UT263	-	14	12,800	Neg	NI	2
UT267	-	7	3,200	Neg	NI	1
UT269	-	7	51,200	Neg	NI	2
UT272	-	4	6,400	Neg	NI	1
UT302	+	4	12,800	TA763	21	1
UT-A	-	5	51,200	Neg	NI	1
UT-B	-	5	6,400	Neg	NI	1

[a]Endpoint determined using IFA; [b]Not isolated

Indirect immunofluorescence assay

Whole cell lysates of *O. tsutsugamushi* prototype strains (Karp, Kato and Gilliam), from sonicated tissue culture cells, were spotted onto 40-well Teflon-coated microscope slides, air-dried and fixed in cold acetone for 10 minutes. The slides were used immediately or stored at -20ºC until required. Patient sera were serially 2-fold diluted from 1:50 to 1:51,200 in PBS containing 2% (w/v) skim milk powder (SMP) and incubated in a humidified atmosphere for 30 minutes at 37ºC followed by 3 washing cycles in PBS. Anti-human IgM FITC conjugate (Jackson, USA) diluted in PBS-SMP diluent containing 0.00125% (w/v) Evans Blue counterstain was applied to all wells and incubated in a humidified atmosphere for 30 minutes at 37ºC. The cells were examined by fluorescence microscopy at a magnification of x 400 and the binding endpoint-titer was determined as the highest reciprocal dilution displaying fluorescence.

Data analysis

Multiple linear regression was performed using Stata™ Version 8.0 (StataCorp, College Station, Tx, USA) to determine factors influencing the success of *in vitro* isolation of *O. tsutsugamushi.* The factors investigated were

external factors (sample transit times), patient factors (days of fever, antibody titer at admission) and isolation factors (days to isolation, strain). Student's *t*-test was used to compare groups (transit time, antibody titers, days of fever, strain, time to isolation) for significant difference. Pearson correlation (r) was used to determine the degree to which two factors were related. The strain of *O. tsutsugamushi* isolates was determined by phylogenetic analysis of the entire 56kDa protein gene open reading frame (approximately 1.6 Kb) compared with Karp, Kato, Gilliam and Kawasaki reference sequences stored on GenBank (S.D. Blacksell, unpublished data).

RESULTS

O. tsutsugamushi in vitro isolation

Twenty-six patient samples were sent to Bangkok, and *O. tsutsugamushi* was successfully isolated from 12 (46.2%) of these (Table 1). The time to *in vitro* isolation ranged from 16 to 37 days [median 27 days, inter-quartile range (IQR) 22.5-33.5 days].

Climatic information

Average daily and minimum morning and maximum afternoon temperatures for Udon Thani Province are presented in Fig 1. Average daily temperatures ranged from 27.1 (November) to 29.1ºC (June).

Influence of transit time on *O. tsutsugamushi* isolation

The transit time for all samples ranged from 1 to 9 days (median 2 days, IQR 1-2 days) (Table 1). For samples where isolation was successful (n=12), transit times ranged from 1 to 9 days (median 2 days, IQR 1-2 days), compared with samples that did not yield *O. tsutsugamushi* (n = 14) which ranged from 1 to 3 days (median 1.5 days, IQR 1-2 days) (p=0.23). There was no significant correlation between time to isolation and sample transit time when all observations were assessed (r = -0.415) as well as when the outlying 9-day transit (UT213) observation was removed (r = 0.297).

Influence of admission antibody titer on *O. tsutsugamushi* isolation

The IgM reciprocal antibody titer on admission for all samples ranged from 400 to 51,200 (median 12,800, IQR 3,200-51,200) (Table 1). For isolate-positive samples the reciprocal antibody titer ranged from 800 to 51,200 (median 8,000, IQR 2,400-38,400) and for isolate-negative samples ranged from 400 to 51,200 (median 12,800, IQR 6,400-51,200). There was no significant difference in IgM reciprocal antibody titer between the two groups (p=0.37). Examining isolate-positive samples only, there was no significant correlation between time to isolation and IgM antibody titer (r = 0.11).

Influence of days of fever on *O. tsutsugamushi* isolation

Duration of fever for all patients ranged from 1 to 18 days (median 6.5 days, IQR 5-7 days) (Table 1). For isolate-positive samples

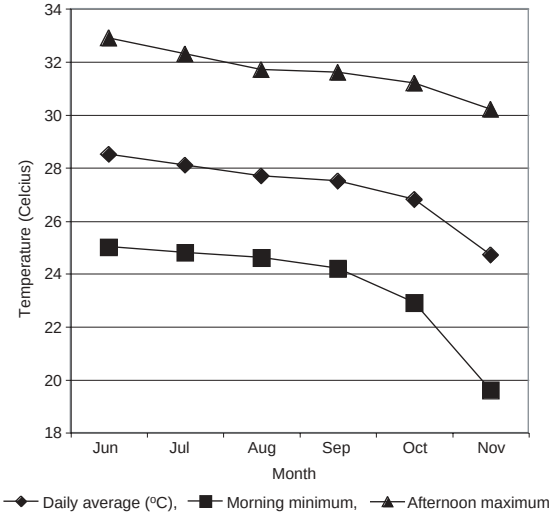

Fig 1–Average monthly temperatures for Udon Thani Province. Source: Thai Meteorological Department.

the days of fever ranged from 4 to 14 days (median 7 days, IQR 5-7 days) and isolate-negative samples ranged from 2 to 18 days (median 5 days, IQR 4.5-7 days) with no significant difference between the two groups (p=0.53). Examining isolate positive cases only, there was no significant correlation between time to isolation and days of fever (r = 0.12).

Influence of *O. tsutsugamushi* genotype on isolation

Nine of the *O. tsutsugamushi* isolates belonged to the Karp group and the remaining isolates belonged to the Gilliam/Kawasaki (n=2) and TA763 (n=1) groups. There was no significant difference in the time to isolation between the groups (p=0.24).

DISCUSSION

This study has examined patient and transport-related factors that influence the successful *in vitro* isolation of *O. tsutsugamushi* from scrub typhus patients. This is the first description of the successful *in vitro* isolation of *O. tsutsugamushi* from patients following extended storage and transport of samples at ambient tropical temperature.

The sensitivity of *in vitro* isolation of *O. tsutsugamushi* in this study was 46.2% using the local positivity cut-off for IFA IgM titer of ≥1:400 IgM titer IFA (Coleman *et al,* 2002). Patient-related factors including IgM antibody titer and days of fever had no significant influence on the success of isolation or on the time to positivity in culture. There was no statistically significant difference in *O. tsutsugamushi* isolation outcome with regard to days of sample transit, although a previous study has demonstrated that delays have a detrimental affect on *Rickettsia conorii* isolation using the shell vial method (La Scola and Raoult, 1996).

Sample transport temperature may influence isolation outcome. Samples in this study were transported at ambient temperature rather than frozen. Average daily temperatures ranged from 27.1 to 29.1ºC during the period of the study. High ambient temperatures, typical in tropical areas where scrub typhus is endemic, may have aided isolation because *O. tsutsugamushi* continues to replicates at 28ºC (Hanson, 1987) and may explain the positive isolation following a sample transit of 9 days (isolate UT213). Other studies (La Scola and Raoult, 1996; Vestris *et al,* 2003; Gouriet *et al,* 2005) have described much lower *in vitro* isolation sensitivity rates (<10%) for various intracellular pathogens than that reported here when patient samples were transported in a frozen state.

The methodology presented here demonstrates that blood samples from scrub typhus patients can be left for several days at ambient tropical temperature without significantly affecting the *in vitro* culture outcome of *O. tsutsugamushi* in mammalian cells. Further studies are required to improve the success of *in vitro* culture under tropical conditions in endemic areas, such as the use of β-lactam group antibiotics to reduce bacterial contamination.

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