RAPID DIAGNOSIS OF SCRUB TYPHUS IN RURAL THAILAND USING POLYMERASE CHAIN REACTION

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Abstract. The aim of this study was to evaluate the use of polymerase chain reaction (PCR) amplification of the *O. tsutsugamushi* 16S rRNA gene for the diagnosis of scrub typhus in rural Thailand. A prospective study of acute febrile illness in Udon Thani, northeast Thailand, identified 183 patients as having scrub typhus on the basis of immunofluorescent antibody testing (IFA) of paired sera. A further 366 febrile patients admitted concurrently with a range of other diagnoses as negative controls. Diagnostic sensitivity and specificity of 16S rRNA PCR was 44.8% and 99.7%, respectively, compared with IFA. PCR positivity was related to duration of symptoms and presence of eschar (*P* < 0.001, both cases). PCR using primers to amplify a fragment of the 56-kd gene had a sensitivity and specificity of 29.0% and 99.2%, respectively. PCR has a high specificity but low sensitivity for the rapid diagnosis of scrub typhus in this endemic setting.

INTRODUCTION

Scrub typhus is a rickettsial infection caused by *Orientia tsutsugamushi*, a gram-negative obligate intracellular coccobacillus transmitted to humans and other vertebrates by the bite of larval stage trombiculid mites. Human infection is endemic in the Asia-Pacific region, with an estimated 1 million cases occurring annually. Scrub typhus accounts for ~20% of all febrile episodes in endemic areas and is a sporadic problem during training of military personnel. Infection commonly presents as an acute febrile illness 7–10 days after the infected chigger bite. Major presenting features are fever, severe headache, and myalgia. Other signs and symptoms include rash, lymphadenopathy, hepatosplenomegaly, cough, sore throat, abdominal pain, and central nervous system involvement. A papule may develop at the bite site that ulcerates to form a black crust or eschar associated with drainage lymphadenopathy. The presence of an eschar is highly suggestive of scrub typhus but is reported to occur in a variable proportion of patients (from 7% to 97%). In the absence of an eschar, presenting features are often indistinguishable from those of other acute febrile illnesses common in the same geographic region including leptospirosis, murine typhus, and dengue fever.

The definitive diagnosis of scrub typhus is made by the isolation of *O. tsutsugamushi* from blood. This is not practical for routine diagnostic laboratories because culture has poor sensitivity and requires growth in a cell monolayer and containment level 3 facilities, and the organism may take more than 1 month to isolate. The indirect immunofluorescent antibody (IFA) test is used widely to confirm the diagnosis using acute and convalescent sera, but by its nature provides a retrospective diagnosis. Rapid diagnosis would contribute toward acute patient management; the two diagnostic options are detection of antibodies using a bedside test and detection of organisms or their nucleic acid or antigens in blood or other clinical samples. Rapid serology tests have been described, but their use in early diagnosis is not clear. Detection of *O. tsutsugamushi* in blood during human infection using a nested polymerase chain reaction (PCR) was reported for 12 patients presenting with a febrile illness to a hospital in southern Thailand; this targeted the gene encoding the major 56-kd surface antigen. A second study reported the use of real-time PCR to detect the 47-kd gene in 10 culture-positive patients and in 7 of 17 (41%) culture-negative individuals with serologically proven scrub typhus infection. Such techniques have not been evaluated for the rapid diagnosis of a large patient population. The purpose of this study was to compare PCR amplification of a region of the 16S rRNA gene of *O. tsutsugamushi* for the early diagnosis of 183 cases of scrub typhus in an endemic area of Thailand.

MATERIALS AND METHODS

Study patients and serum samples. A prospective study of acute febrile illness was conducted between October 2000 and December 2001 in a large, 1,000-bed general hospital in Udon Thani, northeast Thailand. This facility serves a predominantly rural population of agricultural workers. Patients were enrolled into the study if they were ≥15 years of age, had fever (> 37.8°C) of unknown cause, had given written informed consent to participate, and agreed to out-patient follow-up and a further blood test at 2 weeks. Patients with a blood smear positive for malaria parasites or other definable infections such as pneumonia or urinary tract infection were excluded. Blood was drawn on admission for aerobic blood culture, serologic testing, and molecular diagnostics. A second (convalescent) serum sample was taken at ~2 weeks after presentation. Duration of symptoms before admission, antibiotic treatment at presentation, clinical features, and in-hospital outcome (survival or death) were recorded using a standardized data collection form. Blood samples were maintained at ~20°C before use. The study protocol was approved by the Ethical Review Sub-committee of the Ministry of Public Health, Thailand.
Serological testing. The IFA assay was used as the reference standard for the diagnosis of scrub typhus. This was performed on paired (acute and convalescent) sera at Siriraj Hospital using established methodology. In brief, pooled antigens of *O. tsutsugamushi* strain Kato and Gilliam were spotted on a glass slide kindly provided by The National Research Institute of Health (NIH), Ministry of Public Health, Thailand. Initial screening was performed using a dilution of 1:50, after which positives were assayed using 2-fold serial dilutions from 1:100 to 1:6,400. Antibody binding was determined using a fluorescent microscope (Olympus BX50, Olympus Corporation, Tokyo, Japan). Known positive and negative control sera were run with each experiment. A positive result for scrub typhus infection was defined as a single IFA IgM titer against *O. tsutsugamushi* of $\geq 1:400$ or a 4-fold or greater rise in IFA IgM titer, and/or a single IgG titer $\geq 1:800$ or a 4-fold or greater rise in IgG titer.

Molecular testing. Total genomic DNA was extracted from a 5-mL EDTA admission blood sample using the Nucleon BACC 3 kit (Amersham, Biosciences UK Ltd, Buckinghamshire, UK). Bacterial genomic DNA was extracted from laboratory culture using the Wizard SV Genomic DNA purification kit (Promega, Madison, WI). Primers were designed to be specific for *O. tsutsugamushi* by aligning the 16S rRNA gene sequence for *O. tsutsugamushi* strain Kato (accession no. D38624), strain Karp (accession no. D38623), strain Gilliam (accession no. L3622), *Rickettsia typhi* (accession no. L36221), *R. prowazekii* (accession no. M21789), *R. conorii* (accession no. L36104), and *R. sibirica* (accession no. D38628). Scrub typhus and leptospirosis can present with very similar clinical features and may cause a dual infection. In view of this and the fact that rapid serologic tests may show cross-reactivity between rickettsia and leptospires, the alignment further included *Leptospira interrogans* (accession no. Z12817), *L. weillii* (accession no. AY034037), and *L. borgpetersenii* (accession no. AY149231). Primers were designed using Primer 3 software and were predicted to amplify *O. tsutsugamushi* alone. The primers OT1-F (5'-CGAATTAATGCTGAGTTTGCTTAG-3') and OT1-R (5'-CCTCTCAGAATCAGCTACGATACA-3') gave a predicted amplification product of 220 bp (position 48–71 and 245–268 of sequence accession number L3622).

Verification of primer specificity was performed using genomic DNA from *O. tsutsugamushi* strain Kato, *R. typhi*, *R. prowazekii*, *R. conorii*, *R. australis*, *R. honei*, *Burkholderia pseudomallei*, *B. mallei*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus sp.*, *Aeromonas hydrophila*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Staphylococcus aureus*, and *L. interrogans* serovar *autumnalis*. The reaction mix contained 1× PCR buffer, 1.5 mmol/L MgCl$_2$, 200 μmol/L of dNTP, 1 μmol/L of each OT1-F and OT1-R primers, 1.25 U Taq Polymerase I enzyme, and 50–100 ng genomic DNA as template in a total volume of 50 μL. Amplifications were performed using a DNA engine PTC-200 (MJ Research, Watertown, MA) and the following cycling conditions: 95°C for 3 minutes (1 cycle), followed by 40 cycles of 90°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Amplification products were run on a 3% agarose gel, stained with ethidium bromide, and photographed under UV light using the Gel Doc 1000 system (Bio-Rad Laboratories, Hercules, CA).

Testing of clinical samples from patients presenting with febrile illness was performed using total DNA extracted from admission blood samples. Amplification was performed using the Rotor-Gene 3000 real time thermal cycler (Corbett Research Ltd., Sydney, Australia). The reaction mixture consisted of 1× iQ SYBR Green Supermix (Bio-Rad), 0.5 μmol/L of each OT1-F and OT1-R, 5 μL DNA sample, and sterile distilled water to a total volume of 25 μL. Cycling conditions were 95°C for 3 minutes (1 cycle), followed by 40 cycles of 90°C for 30 seconds, 60°C for 10 seconds, and 72°C for 20 seconds. After standardization, a positive result on melt curve analysis was set at 84°C. DNA extracted from *O. tsutsugamushi* strain Kato culture and *L. interrogans* serovar *Autumnalis* was used as positive and negative controls, respectively.

Rapid diagnosis using conventional PCR to amplify a 483-bp region of the gene encoding the 56-kd outer membrane protein has been described previously. This methodology was applied to all clinical samples in this study. The reaction was performed using a DNA engine PTC-200 (MJ Research). Amplification products were run on a 2% agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light as before.

Testing strategy. All patients identified by IFA as having scrub typhus were selected as cases. A control group of two controls for each case was formed by selecting two patients who did not have scrub typhus who were admitted immediately after a scrub typhus case. In the event that sequential patient cases were cases, an appropriate number of consecutive controls were selected immediately after the cases. A database was created of cases and controls, after which these were randomized and blinded before testing.

Statistical analysis. Sensitivity and specificity were calculated with exact 95% confidence intervals (CIs). Proportions were compared using the Fisher exact test, and the association between PCR positivity and days of symptoms before hospital admission was analyzed by logistic regression using Stata /SE 8.0 (StataCorp., College Station, TX).

RESULTS

A total of 722 patients presented with acute febrile illness to Udon Thani hospital from October 2000 to December 2001. Of these, 183 patients (25%) had scrub typhus based on IFA testing; 156 patients were diagnosed on the basis of the IgM titer (79 with 4-fold rise in titer and 77 with single high titer), and 27 patients were diagnosed on the basis of the IgG titer (14 with 4-fold rising titer and 13 with single high titer). A further 366 patients from the same cohort were selected as controls. The diagnosis in control patients varied; the top three most common groups or diagnoses were leptospirosis (22.4%), bacterial septicemia (6.3%), and murine typhus (1.6%).

The number of days between onset of symptoms and hospital admission for patients with positive scrub typhus serology ranged from 1 to 60 days (median, 4.5 days; interquartile range [IQR], 3–7 days). An eschar was noted in 11 of 183 patients (6%). Two patients were taking antibiotics on admission that were predicted to be effective treatment of scrub typhus in this geographic area (chloramphenicol, one patient; doxycycline, one patient). Seven patients (3.8%) died as a result of scrub typhus infection during hospital admission.

Verification of 16S rRNA PCR primer specificity was performed using genomic DNA from a range of bacteria chosen
O. tsutsugamushi

Results are shown in Table 1.

- **Figure 1.** Specificity of PCR targeting 16S rRNA gene of *O. tsutsugamushi*. Lane M, 100-bp DNA ladder marker; lanes 1–14, *Aeromonas hydrophila*, *Burkholderia pseudomallei*, *Escherichia coli*, *Enterococcus sp.*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhi*, *Leptospira interrogans*, *Rickettsia australis*, *R. conorii*, *R. honei*, *R. prowazekii*, *R. typhi*; lanes 15 and 16, *O. tsutsugamushi* Udonthani isolates UT76 and UT125; lane 17, *O. tsutsugamushi* strain Kato; lane 18, no template control.

To represent those most likely to be encountered by our patient group. Amplification products were only seen for *O. tsutsugamushi* (Figure 1). PCR amplification of clinical samples was positive in 82 of 183 (44.8%) patients with positive IFA serology indicative of scrub typhus and 1 of 366 controls (0.27%) who were negative on IFA. The single patient with a negative IFA in whom *O. tsutsugamushi* DNA was detected had an eschar on presentation and is likely to represent a false-negative IFA (IgM and IgG titers were negative for paired sera in this case). None of the 27 patients who were diagnosed as having scrub typhus on the basis of IFA IgG titer alone were positive by PCR. Concordance between IFA and PCR is detailed in Table 1. The number of days of illness before admission for PCR-positive and PCR-negative cases was 7.1 ± 3.0 and 5.3 ± 4.7 (SD) days, respectively. The rate of PCR positivity was positively related to the number of symptomatic days before hospital presentation (P < 0.001) and the presence of an eschar (P < 0.001). PCR positivity was not related to death (P = 1).

The low sensitivity of our PCR assay led us to evaluate another set of previously published primers designed to amplify a fragment of the gene encoding the major outer 56-kd protein of *O. tsutsugamushi*. Results are shown in Table 1. The sensitivity of the 56-kd gene PCR was lower than that for the 16S rRNA gene PCR (P = 0.0001, McNemar test).

To further explore the basis for the low sensitivity of PCR amplification of the 16S rRNA gene, we tested 16 blood samples taken on the day of admission from Thai patients presenting with unexplained fever during 2003–2004 who were proven by us to be culture positive for *O. tsutsugamushi*. The 16S rRNA PCR was positive for 13 of 16 (81.3%) samples (data not shown). To detect the presence of PCR inhibitors in the DNA sample, the three samples that were PCR negative were spiked with known concentrations of *O. tsutsugamushi* genomic DNA. The 16S rRNA PCR detected spiked DNA samples at the same sensitivity level as genomic *O. tsutsugamushi* DNA, suggesting that PCR inhibitors in the extracted sample DNA did not contribute to reduce sensitivity.

### DISCUSSION

The results of this large study indicate that PCR lacks sensitivity for the rapid diagnosis of scrub typhus in an endemic region. Our results are much poorer than anticipated based on previous smaller scale studies, but are consistent with a recent study that reported a sensitivity of 41% for culture-negative individuals with serologically proven scrub typhus infection. An important possible explanation for our low sensitivity is the presence of PCR inhibitors, although these were not detected in this study. There are several alternative explanations. *O. tsutsugamushi* is an obligate intracellular bacterium, and PCR may be detecting cell-associated organisms, the shedding of which may be stochastic. The use of EDTA blood may be sub-optimal for a variety of reasons. Bacterial DNA will be effectively “diluted” by the presence of host DNA in the total genomic DNA preparation, which is likely to be far in excess of bacterial DNA. Furthermore, if *O. tsutsugamushi* circulate in detached endothelium or monocytes, they would be greatly diluted compared with testing Buffy coat. Extraction of bacterial DNA may also be inefficient if saturation or clogging of the DNA extraction kit occurs. An additional factor is that the bacterial copy number may be low. One study of seven samples reported that copy number varied from ~1,000 to nearly 29,000/μL of blood, but this has not been examined in a large, unselected patient population.

A further explanation for low PCR sensitivity is that the IFA titers used to diagnose scrub typhus in this study may lack specificity in this highly endemic setting. None of the patients diagnosed as having scrub typhus on the basis of IgG alone were positive by PCR, suggesting that this assay may be a poor predictor of disease or that reinfections are associated with more rapid clearance of the bacteria. IgM seems to be more specific, in that 30/79 patients (38%) with a fold rising titer used to diagnose scrub typhus in this study may lack specificity in this highly endemic setting. None of the patients diagnosed as having scrub typhus on the basis of IgG alone were positive by PCR, suggesting that this assay may be a poor predictor of disease or that reinfections are associated with more rapid clearance of the bacteria. IgM seems to be more specific, in that 30/79 patients (38%) with a fold rising IgM titer and 52/77 patients (68%) with a single high IgM titer were PCR positive.

The lower sensitivity of PCR amplification of a fragment of the 56-kd gene compared with PCR targeting the 16S rRNA gene may relate to the technology used (conventional PCR versus real-time PCR, respectively). The 56-kd gene also contains regions of genetic polymorphism, and it is possible that failure of amplification is related to variability in sequence in the region of primer annealing.

The results of this study are unlikely to alter routine clinical practice, because the PCR described here does not offer sufficient accuracy on which to base a definitive diagnosis. The high specificity of the 16S rRNA PCR could be used by research studies in which entry criteria depended on a definitive diagnosis. The single case with positive PCR but negative IFA serology indicative of scrub typhus and 1 of 366 samples was positive in 82 of 183 (44.8%) patients with positive IFA titers used to diagnose scrub typhus in this study may lack specificity in this highly endemic setting. None of the patients diagnosed as having scrub typhus on the basis of IgG alone were positive by PCR, suggesting that this assay may be a poor predictor of disease or that reinfections are associated with more rapid clearance of the bacteria. IgM seems to be more specific, in that 30/79 patients (38%) with a fold rising IgM titer and 52/77 patients (68%) with a single high IgM titer were PCR positive.

Table 1: Sensitivity and specificity of two PCR assays applied to admission sera for the diagnosis of scrub typhus in comparison with IFA of paired sera

<table>
<thead>
<tr>
<th>Assay</th>
<th>No. positive (%)</th>
<th>Percent sensitivity (95% CI)</th>
<th>Percent specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA PCR</td>
<td>82 (44.8)</td>
<td>44.8 (37.5–52.3)</td>
<td>99.7 (98.5–100)</td>
</tr>
<tr>
<td>56-kd gene PCR</td>
<td>53 (29.0)</td>
<td>29.0 (22.5–36.1)</td>
<td>99.2 (97.6–99.8)</td>
</tr>
</tbody>
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The extent and patterns of shedding of rickettsia in the blood-
stream and optimal timing for culture, together with techniques that increase the sensitivity of molecular techniques for the detection of bacterial DNA.

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