Short Report: Molecular Confirmation of Co-Infection by Pathogenic *Leptospira* spp. and *Orientia tsutsugamushi* in Patients with Acute Febrile Illness in Thailand

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Abstract. Leptospirosis and scrub typhus are major causes of acute febrile illness in rural Asia, where co-infection is reported to occur based on serologic evidence. We re-examined whether co-infection occurs by using a molecular approach. A duplex real-time polymerase chain reaction was developed that targeted a specific 16S ribosomal RNA gene of pathogenic *Leptospira* spp. and *Orientia tsutsugamushi*. Of 82 patients with an acute febrile illness who had dual infection on the basis of serologic tests, 5 (6%) had polymerase chain reaction results positive for both pathogens. We conclude that dual infection occurs, but that serologic tests may overestimate the frequency of co-infections.

Leptospirosis and scrub typhus are major causes of acute febrile illness in the Asia-Pacific region.1,2 Leptospirosis is caused by pathogenic *Leptospira* spp., and scrub typhus is caused by the gram-negative obligate intracellular bacterium *Orientia tsutsugamushi*. Because both infections affect agricultural workers and have similar clinical features, including fever, myalgia, headache, and lymphadenopathy, they are difficult to distinguish on clinical grounds alone. Co-infection with leptospirosis and scrub typhus was first reported in rice farmers who were hospitalized with leptospirosis in northeastern Thailand; with 9 (40%) of 22 patients were also seropositive for scrub typhus.3 Dual infection has also been reported in Taiwan and India.4–6 A study from Thailand reported that 103 (12.2%) of 845 patients with an acute febrile illness had dual infection, of which 33 were attributed to leptospirosis and scrub typhus.2 All previous studies have relied on serologic tests, and the possibility remains that co-infection represents cross-reactivity between serologic assays, or an acute infection by one pathogen after a recent infection by another pathogen. The aim of this study was to determine if dual infection in Thai patients on the basis of serologic testing could be confirmed by a molecular method.

The study protocol was approved by the Ethics Committee of the Faculty of Tropical Medicine, Mahidol University, Thailand (MUTM 2010-033-01). A duplex real-time polymerase chain reaction (PCR) was developed for the 16S ribosomal RNA (rRNA) gene. Primers and hydrolysis probe targeting the 16S rRNA gene of pathogenic *Leptospira* spp. were based on a reported TaqMan assay.7 These primers generated an 88-basepair product (positions 205–220 and 240–263 of *L. interrogans* 16S rRNA gene sequence; GenBank accession no. AY631894). Primers and hydrolysis probe targeting the 16S rRNA gene of *O. tsutsugamushi* were modified from those of a previous study and were as follows: forward 5'-GGCATAACGCTTACTGCCTA-3', reverse 5'-GATTGTGCAAACG-3', and probe ROX-5'-TAAA TGTTATTCCGTACTGATGGGCAG-3'-BHQ2. The hydrolysis probe for *O. tsutsugamushi* was labeled with ROX so that this probe could be used in a single reaction with the hydrolysis probe for *Leptospira* spp. (6-FAM). The modified primers amplified a 92-basepair product (positions 53–72 and 125–145 of the 16S rRNA gene of *O. tsutsugamushi* strain Boryong; GenBank sequence accession no. NC_009488). The assay was optimized and performed in a 20-μL single reaction containing 5 μL DNA, 1× QUANTIPROBES (Quantimix Easy Probes Kit; Biotools, Madrid, Spain), 8 mM MgCl2, 0.15 μM of each primer, and 0.1 μM of each probe. Cycling conditions were at 95°C for 8 minutes (1 cycle), followed by 50 cycles at 95°C for 10 sec and 60°C for 1 minute.

The PCR amplification efficiencies and detection limits of the assay were determined by using a linearized plasmid pG16S described for scrub typhus8 and genomic DNA of *L. interrogans* serovar Lai for leptospirosis. DNA concentration was determined by using the Quanti-IT High-Sensitivity DNA Assay Kit (Invitrogen, Carlsbad, CA) and the Rotor-Gene 3000 by using the DNA concentration measurement mode. Serially diluted DNA for each pathogen was used as a template in four triplicate calibration curves.

The mean PCR efficiency was 0.88 (95% confidence interval [CI] = 0.81–0.93) for *L. interrogans* and 0.97 (95% CI = 0.96–0.99) for *O. tsutsugamushi*. The calibration curve for *Leptospira* spp. had a mean slope of −3.7 (95% CI = −3.8 to −3.48) and a y intercept of 38.2 (95% CI = 36.1–40.2), and that for *O. tsutsugamushi* had a mean slope of −3.3 (95% CI = −3.5 to −3.2) and a y intercept of 37.5 (95% CI = 36.4–38.8). Cycle quantification ranged from 19.9 to 36 (interquartile range = 22.7–34.5) for *O. tsutsugamushi*, and from 14.5 to 32.2 (interquartile range = 18.7–35.9) for *Leptospira* spp. The calibration curve showed a linear dynamic range over five orders of magnitude (5 × 10² to 5 copies/μL) for both pathogens. The limit of detection of a duplex quantitative PCR was five genome equivalents for *Leptospira* genomic DNA and five copies for the *O. tsutsugamushi* plasmid. The mean coefficient of variation for the quantification calibrator for leptospirosis and scrub typhus was 0.1%.

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The analytical specificity of the duplex PCR was evaluated by using genomic DNA isolated from one clinical isolate of each of the following species: *Rickettsia typhi*, *Staphylococcus aureus*, *Enterococcus sp.*, *Escherichia coli*, *Salmonella enterica serovar Typhi*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Burkholderia pseudomallei*. These species were selected because they represent common causes of serious infections in Southeast Asia. Genomic DNA of *O. tsutsugamushi* and *R. typhi* was extracted from infected laboratory tissue cultures by using the Wizard® SV Genomic DNA Purification Kit (Promega, Madison, WI). Genomic DNA was extracted from the remaining species from laboratory cultures by using the Wizard® Genomic DNA Extraction Kit (Promega) with the addition of 5 μL (10 mg/mL) of lysostaphin during the extraction of *S. aureus* DNA. None of the isolate tests showed a false-positive result.

Diagnostic sensitivities and specificities of the assay were determined by using patients selected from a cohort study of acute febrile illness conducted at a hospital in northeastern Thailand during October 2000–December 2001, which has been described.9 Blood samples were obtained at admission for *Leptospira* spp. culture, serologic testing, and molecular diagnostic tests, and a second (convalescent) sample was obtained for serologic testing approximately two weeks later.

Diagnosis of leptospirosis was based on a positive *Leptospira* culture and/or positive microscopic agglutination test (MAT) result (defined as a four-fold increase in MAT titer between acute-phase and convalescence-phase samples or a single titer ≥1:400). Diagnosis of scrub typhus was based on a positive fluorescent antibody assay (IFA) result (defined as a four-fold increase in IgM and IgG titer in a scrub typhus IFA between acute-phase and convalescence-phase samples or an IgM titer ≥1:400 and an IgG titer ≥1:800).

A case–control study was conducted from the original cohort and consisted of 100 patients with laboratory confirmed leptospirosis alone (24 of whom were culture positive for *Leptospira* spp.), 100 patients with scrub typhus alone, and 150 controls. The controls were randomly selected from patients with negative laboratory test results for both infections, and had the following diagnoses: dengue fever (n = 16); murine typhus (n = 7); bacterial septicemia caused by *Escherichia coli* (n = 5), *Klebsiella pneumoniae* (n = 2), *Klebsiella oxytoca* (n = 1), *Corynebacterium jeikeium* (n = 1), *Enterococcus* sp. (n = 1), or *Pseudomonas aeruginosa* (n = 1); melioidosis (n = 1); human immunodeficiency virus–related infection (n = 1); Japanese encephalitis (n = 1); Q fever (n = 1); other diagnoses (n = 19); or an unknown diagnosis (n = 93).

DNA was extracted from 5 mL of admission blood samples (containing EDTA) obtained during the clinical fever study as described.8 Each sample was assayed in duplicate in the duplex PCR. A positive result for one or both duplicate samples for a given species was interpreted as positive. The PCR result was positive for 59 of 100 leptospirosis monoinfection cases (diagnostic sensitivity = 59.0, 95% CI = 48.7–68.7) and for 62 of 100 scrub typhus monoinfection cases (diagnostic sensitivity = 62.0, 95% CI = 51.7–71.5). The PCR result was negative for leptospirosis for 138 of 150 controls (diagnostic specificity = 92.0, 95% CI = 86.4–95.8) and negative for scrub typhus for 139 of 150 controls (diagnostic specificity = 92.7, 95% CI = 87.3–96.3).

The assay was then applied to all patients in the acute febrile illness cohort study who had been defined as having dual infections and had samples available for testing (n = 82). A four-fold increase in scrub typhus IFA titer was observed for 64 patients (78%), and a high single titer was observed for 18 patients (22%). Leptospirosis was diagnosed on the basis of positive results for culture and MAT for five patients (6%), positive results for culture and negative results for MAT for three patients (4%), and negative culture results and positive results for MAT for 74 patients (90%). The duplex PCR results for these 82 patients were as follows: 43 (52%) were positive for leptospirosis, 9 (11%) were positive for scrub typhus, 5 (6%) were positive for leptospirosis and scrub typhus, and 25 (30%) were negative for leptospirosis and scrub typhus.

Our findings confirm that co-infection occurs, albeit at a low frequency (6%). Possible explanations for the difference observed between serologic and molecular results include low sensitivity of the molecular assay, failure to test a sample obtained during the window of bacteremia in leptospirosis, serologic cross-reactivity, and acute infection caused by one pathogen in the background of a recent but not active infection caused by the second pathogen. The assay described could represent a useful diagnostic assay to detect both pathogens in a single test.

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