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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[8] and were as follows: forward 5′-GGCATACGGTATTAGCACTTA-3′, reverse 5′-GCATTAATTAGTGGCAAACG-3′, and probe ROX-5′-TAAATGTTATTCCGTACTGATGGGCAG-3′-BHQ2. The hydrolysis probe for *O. tstsugamushi* 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 $MgCl_2$, 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 typhus[8] 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 (5×10^5 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 infection 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.[9] 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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