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Original article

A comparison of two molecular methods for diagnosing leptospirosis from three different sample types in patients presenting with fever in Laos

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ABSTRACT

Objectives: To compare two molecular assays (*rrs* quantitative PCR (qPCR) versus a combined 16SrRNA and *LipL32* qPCR) on different sample types for diagnosing leptospirosis in febrile patients presenting to Mahosot Hospital, Vientiane, Laos.

Methods: Serum, buffy coat and urine samples were collected on admission, and follow-up serum ~10 days later. *Leptospira* spp. culture and microscopic agglutination tests (MAT) were performed as reference standards. Bayesian latent class modelling was performed to estimate sensitivity and specificity of each diagnostic test.

Results: In all, 787 patients were included in the analysis: 4/787 (0.5%) were *Leptospira* culture positive, 30/787 (3.8%) were MAT positive, 76/787 (9.7%) were *rrs* qPCR positive and 20/787 (2.5%) were 16SrRNA/*LipL32* qPCR positive for pathogenic *Leptospira* spp. in at least one sample. Estimated sensitivity and specificity (with 95% CI) of 16SrRNA/*LipL32* qPCR on serum (53.9% (33.3%–81.8%); 99.6% (99.2%–100%)), buffy coat (58.8% (34.4%–90.9%); 99.9% (99.6%–100%)) and urine samples (45.0% (27.0%–66.7%); 99.6% (99.3%–100%)) were comparable with those of *rrs* qPCR, except specificity of 16SrRNA/*LipL32* qPCR on urine samples was significantly higher (99.6% (99.3%–100%) vs. 92.5% (92.3%–92.8%), p <0.001). Sensitivities of MAT (16% (95% CI 6.3%–29.4%)) and culture (25% (95% CI 13.3%–44.4%)) were low. Mean positive Cq values showed that buffy coat samples were more frequently inhibitory to qPCR than either serum or urine (p <0.001).

Conclusions: Serum and urine are better samples for qPCR than buffy coat, and 16SrRNA/*LipL32* qPCR performs better than *rrs* qPCR on urine. Quantitative PCR on admission is a reliable rapid diagnostic tool, performing better than MAT or culture, with significant implications for clinical and epidemiological investigations of this global neglected disease. **K. Woods, Clin Microbiol Infect 2018;24:1017**

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Introduction

Leptospirosis is a leading cause of morbidity and mortality globally with an estimated 1 million cases and 60 000 deaths annually [1]. In South East Asia there are an estimated 55.5 cases per 100 000 annually, with an estimated mortality of 2.96/100 000 [1]. In temperate regions, leptospirosis is the third commonest infectious cause of life-threatening disease in returning travellers [2].

Leptospirosis presents as a non-specific febrile illness that can progress to serious complications [3–5] with up to 40% mortality if untreated [6]. Diagnosis is often delayed as *Leptospira* species grow slowly in culture, and the reference standard Microscopic Agglutination Test (MAT) requires acute and convalescent sera, making diagnosis retrospective by nature. Culture and MAT are therefore poor clinical diagnostic tools for leptospirosis. Furthermore, they are imperfect reference standards, necessitating the use of statistical models such as the Bayesian latent class model to determine the true accuracy of alternative *Leptospira* diagnostics [7–9].

Several molecular assays for *Leptospira* spp. have been developed, targeting housekeeping genes such as *gyrB* [10], *rrs* (16SrRNA) [11] and *secY* [12], or pathogen-specific *LipL32* [13], *ligA* and *ligB* [14], which avoid amplification of non-pathogenic *Leptospira* species. Large-scale prospective evaluations in endemic tropical settings are lacking and uncertainty remains regarding the optimum sample for molecular detection of *Leptospira* spp. with buffy coat [13,15], serum [16] and urine [13,17] all recommended.

We prospectively evaluated the *rrs* quantitative PCR (qPCR) [18] alongside an assay for 16SrRNA and *LipL32* developed by Public Health England (henceforth 16SrRNA/*LipL32* qPCR) using admission serum, buffy coat (BC) and urine samples from febrile patients presenting to Mahosot Hospital, Vientiane, Laos.

Materials and methods

Retrospective study

The 16SrRNA/*LipL32* qPCR was evaluated using stored (−80°C) admission serum and BC samples from 59 cases of leptospirosis (positive by: culture $n = 19$; MAT $n = 20$ (admission titre ≥1:400 or four-fold convalescent rise); or *rrs* qPCR on BC $n = 20$) and 83 controls (diagnoses identified in a published study [19], see Supplementary material, Table S1). Frozen DNA previously extracted from BC was used in 43/59 cases and all 83 controls, because stored samples were not available for fresh extraction.

Prospective study

Study population

A total of 1471 consecutive patients presented with a febrile illness to Mahosot Hospital between 30 May and 30 November 2014, of which 811 were included. Inclusion criteria were: fever (history of fever or documented temperature ≥38°C), plus at least one of: headache, rash, myalgia, arthralgia, lymphadenopathy, meningitis, encephalitis, respiratory symptoms, jaundice, or acute renal failure. Exclusion criteria were: age <6 months; fever duration >1 month; admission diagnosis of: wound infection; diabetic foot infection; postoperative infection; abscess; parotitis; urine infection; or diarrhoea. All participants (or their parents/guardians) provided written informed consent before sample collection. Ethical approval for all investigations was granted by the Oxford Tropical Research Ethics Committee (University of Oxford, UK) and the National Ethics Committee for Health Research, Lao PDR.

Sample processing

Samples were collected at presentation from the 811 patients: serum ($n = 785$), EDTA BC ($n = 774$), blood clot ($n = 811$) and urine ($n = 644$). The BC were obtained by centrifuging EDTA blood at 3200 ***g*** for 8 min. Convalescent serum was collected 10–14 days later when possible ($n = 248$). Samples were stored at +4°C until DNA preparation.

DNA preparation. The 1.5-mL urine aliquots were centrifuged at 20 000 ***g***, retaining the pellet with 200 μL urine for DNA extraction. Manual DNA extraction was performed on BC, serum and urine using the QIAamp DNA Minikit (Qiagen, Hilden, Germany) within 7 days of sampling [19]. Ten microlitres of GFP-plasmid *Escherichia coli* control (10^8/mL) was added to each sample before extraction as a process and inhibition control.

Molecular detection. The 16SrRNA/*LipL32* qPCR includes two reaction mixes per sample: a duplex assay targeting *LipL32* and an internal control (GFP *E. coli* plasmid), and a triplex assay targeting the 16SrRNA gene. The triplex assay probes correlate with genomic variants of pathogenic, intermediate and environmental *Leptospira* strains (see Supplementary material, Fig. S1). Comparison of cycle threshold (Cq) values for these probes distinguishes pathogenic from non-pathogenic *Leptospira* spp. (Public Health England, unpublished data; see Supplementary material, Table S2). Quantitative PCRs were performed with 5 μL DNA. The *rrs* qPCR was performed as described previously [18]. Each of the 20-μL 16SrRNA and *LipL32* qPCR reaction mixes contained: 12.5 μL Fast Bluex2 Master Mix (Eurogentec, Southampton, UK), 0.5 μM of each primer and 0.125 μM of each probe. Cycling conditions were: 95°C for 5 min, then 50 cycles of: 95°C for 3 s, 60°C for 30 s, 72°C for 10 s. Each qPCR run included standard curves (~1 genome equivalent (GE)/μL – 10^3 GE/μL; Lao clinical isolate, assumed genome size ~4.7 Mb) and non-template controls (which were always negative). The qPCRs were performed in weekly batches using a Rotorgene 6000 (Qiagen) or CFX96 Touch (Bio-Rad Laboratories Ltd, Hercules, CA). Separate investigators (blinded to clinical data and other results) performed the 16SrRNA/*LipL32* qPCR (KW) and the *rrs* qPCR (WP).

Culture. Blood clots were cultured for *Leptospira* spp. (as previously described [20]). by investigators blinded to the qPCR results.

Serology. MAT was performed at the WHO/FAO/OIE Collaborating Centre for Leptospirosis Reference and Research, Queensland, Australia (see Supplementary material, Table S3). Criteria for a confirmed leptospirosis diagnosis were a single MAT titre of ≥1:400 or a four-fold convalescent rise in titre [21].

Data analysis

Result interpretation. The *rrs* qPCR was considered positive with a Cq ≤40 [22]. The 16SrRNA/*LipL32* qPCR was considered positive with a Cq ≤45 and GFP internal control Cq≤35 (see Supplementary material, Table S2). If interpretation of the 16SrRNA/*LipL32* qPCR was equivocal despite a GFP Cq within the normal range, then the 16SrRNA/*LipL32* qPCR was repeated in triplicate to obtain the final result. Only 16SrRNA/*LipL32* qPCR results indicating the detection of pathogenic *Leptospira* DNA were considered positive for the comparative analysis with the *rrs* qPCR.

Diagnostic characteristics. Sensitivity and specificity of the *rrs* and 16SrRNA/*LipL32* qPCR for diagnosing leptospirosis were calculated using MAT or culture positive as the combined reference standard. McNemar's exact test was used for statistical comparisons. Bayesian Latent Class Modelling (LCM) was performed using WinBUGS 1.4 software [23] to estimate the true accuracy of each diagnostic test as