

Molecular Detection of Pathogens in Negative Blood Cultures in the Lao People's Democratic Republic

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Abstract. Bloodstream infections cause substantial morbidity and mortality. However, despite clinical suspicion of such infections, blood cultures are often negative. We investigated blood cultures that were negative after 5 days of incubation for the presence of bacterial pathogens using specific (*Rickettsia* spp. and *Leptospira* spp.) and a broad-range 16S rRNA PCR. From 190 samples, 53 (27.9%) were positive for bacterial DNA. There was also a high background incidence of dengue (90/112 patient serum positive, 80.4%). Twelve samples (6.3%) were positive for *Rickettsia* spp., including two *Rickettsia typhi*. The 16S rRNA PCR gave 41 positives; *Escherichia coli* and *Klebsiella pneumoniae* were identified in 11 and eight samples, respectively, and one *Leptospira* species was detected. Molecular investigation of negative blood cultures can identify potential pathogens that will otherwise be missed by routine culture. Patient management would have been influenced in all 53 patients for whom a bacterial organism was identified, and 2.3–6.1% of patients would likely have had an altered final outcome. These findings warrant further study, particularly to determine the cost–benefit for routine use, ways of implementation, and timing of PCR for organisms such as *Rickettsia* and *Leptospira*, which are important pathogens in rural Asia.

INTRODUCTION

Bloodstream infections (BSIs) cause significant global morbidity and mortality.¹ Bloodstream infections are confirmed through performing broth culture of a patient's blood for 5–7 days to detect bacteremia. This is regarded as the gold standard,^{2,3} and allows pathogen identification and susceptibility testing to guide treatment. Nonetheless, blood cultures are often negative. In the Lao Peoples' Democratic Republic (Lao PDR), one prospective series found 16.7% of blood cultures from inpatients were positive, yet only 10.8% were clinically relevant (the remainder is likely positive because of contamination).⁴ The main reasons for negativity may be that the patients had non-bacteremic illness or bacteria were present intermittently or at very low blood density. However, an additional explanation is that the causative microorganisms are delicate, fastidious, non-viable, slow growing at low densities, or uncultivable in blood culture medium. This is often seen with bacteria such as *Streptococcus pneumoniae*, *Leptospira* spp., *Rickettsia* spp., and *Orientia* spp.,^{5,6} or when antimicrobial treatment has been started before blood collection, which may kill or inhibit pathogen growth.³ These diagnoses may therefore be missed and may be referred to as “false negatives.” Timely, appropriate, and targeted antimicrobial treatment is imperative to ensure patient survival in suspected BSI,⁷ so there is a need to minimize and identify false negatives early. Previous studies have looked at specifically detecting *Orientia tsutsugamushi*, *Rickettsia* spp., and *Leptospira* spp. in hemoculture fluid to evaluate alternative methods of diagnosis, proving somewhat successful for *O. tsutsugamushi* and *Leptospira* spp. but not for *Rickettsia* spp.,^{8,9} but no

general evaluation of a broader range of bacteria was conducted. We investigated 5-day negative blood cultures for the presence of bacterial pathogens using polymerase chain reaction (PCR).

MATERIALS AND METHODS

Ethical approval for use of blood samples was granted by the Oxford Tropical Research Ethics Committee, the University of Oxford (Oxford, United Kingdom), and the National Ethics Committee for Health Research, Lao PDR. Study patients provided written informed consent. The study was conducted between June and July 2019. Blood cultures from patients suspected of having a systemic infection were processed routinely at Mahosot Hospital Microbiology Laboratory, Vientiane, Lao PDR. Samples collected in BD BACTEC Plus Aerobic/F culture vials (Becton Dickinson, Franklin Lakes, NJ) were incubated using the BACTEC (Becton Dickinson, New Jersey) system and reported as negative after 5 days. Negative blood cultures were randomly selected from discarded culture bottles for further testing, with the only selection criterion being samples from adults (≥ 15 years old). DNA extraction was performed on 0.5 mL of blood culture fluid using QIAamp DNA Mini Kit (Qiagen, Manchester, United Kingdom) according to Method 5 by Villumsen et al.¹⁰ Both broad and genus- or species-specific PCRs were performed (Table 1). PCR selections were based on common endemic organisms in the region. Quantitative PCRs (qPCRs) targeting *Rickettsia* spp. 17-kDa,¹¹ *Rickettsia typhi ompB*,¹² and *Leptospira* spp. *rs* gene¹³ were performed on CFX96 Real-Time System (Bio-Rad, Bangkok, Thailand). Conventional PCRs (cPCRs) targeting *O. tsutsugamushi* 56-kDa gene¹⁴ and pan-bacterial 16S rRNA¹⁵ were performed on a C1000 Touch Thermal Cycler (Bio-Rad, Thailand); products were visualized on a 1% agarose gel. PCR products from 16S rRNA-positive samples were sent for sequencing (Macrogen, Seoul, South Korea), and resulting sequences were compared against GenBank via BLASTn submission (<https://blast.ncbi.nlm.nih.gov>).

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TABLE 1
Details of PCRs performed

Pathogen	Name	Sequence 5'-3'	Primer/Probe	Target region	Ref
<i>Orientia tsutsugamushi</i>	56 kDa-RTS-8	AGGATTAGAGTGTGGTCCTT	Forward	56-kDa outer membrane protein (omp)	(14)
	56 kDa-RTS-9	ACAGATGCACTATTAGGCAA	Reverse		
<i>Rickettsia</i> spp.	R17K128F2	GGGCGGTATGAAYAAACAAG	Forward	17-kDa surface antigen	(11)
	R17K238R	CCTACACCTACTCCVACAAG	Reverse		
	R17K202TAQP	FAM-CCGAATTGAGAACCAA	Probe		
		GTAATGC-TAMRA			
<i>Rickettsia typhi</i>	Rt557F	TGGTATTACTGCTCAACAAGCT	Forward	ompB	(12)
	Rt678R	CAGTAAAGTCTATTGATCCTACACC	Reverse		
	Rt640BP	FAM-CGCGAT	Probe		
		CGTTAATAGCAGCACCAGCATT TCGCG-BHQ1			
<i>Leptospira</i> spp.	Lepto-F	CCC CGTCCGATTAG	Forward	rrs gene	(13)
	Lepto-R	TCCATTGTGGCCGRACAC	Reverse		
	Lepto-probe	6-FAM-CTCACCAAGGCG ACGATCGGTAGC-BHQ1	Probe		
Pan-bacterial	27F/V1-F	AGAGTTTGATCMTGGCTCAG	Forward	16S rRNA	(15)
	518R/V3-R	GTATTACCGCGGCTGCTGGCA	Reverse		

Patient records were checked, and along with other laboratory results (including PCR results for rickettsial organisms from ethylenediaminetetraacetic (EDTA) buffy coat, and *Leptospira* spp., dengue virus and Zika virus from serum^{11,13,16,17}), discharge diagnosis and antimicrobial usage were linked to PCR results.

RESULTS

One-hundred ninety, day-5 negative blood culture samples were studied from 189 patients. Of these, 53 (27.9%) were positive by PCR for bacterial DNA. Twelve (6.3%) were positive for *Rickettsia* spp. (qPCR quantification cycle [Cq] = 34.5–38.5), of which two samples were further identified as *R. typhi* (Cq = 30.8–36.8). One sample (0.5%) was positive for *Leptospira* sp. (Cq = 34.1). Pan-bacterial 16S rRNA cPCR gave 41 (21.6%) positives; of these, 30 PCR products were suitable for sequencing. *Escherichia coli* was identified in 11 samples (identity 100%), *Klebsiella pneumoniae* in eight (99–100%), *Rheinheimera* spp. in five (99–100%), *Pantoea vagans* in two (99–100%), one sample was identified as *Erwinia gerundensis* (100%), one as *Plesiomonas shigelloides* (100%), and one as a *Yersinia* sp. (99%) (Figure 1). One sample was suspected

to be a polymicrobial infection from mixed sequencing results. One sample was positive for both *Rickettsia* spp. and *K. pneumoniae*. *Orientia tsutsugamushi* was not detected. Where a genus- or species-specific identification was made, 34 were classed as “potentially clinically significant,” five as “not significant,” and two of “uncertain clinical significance” (see Figure 1). PCR of buffy coat samples for rickettsial pathogens and *Leptospira* PCR (on serum) was only requested in one patient, who was negative (the blood culture fluid was also negative for any bacteria for this patient). Dengue investigations were requested for 112 of the patients in this series; 90 were positive for dengue virus RNA in serum (90/112, 80.4%), of whom 23 (25.6%) also had a positive bacterial PCR from the negative blood culture fluid. Bacteria identified in dengue-positive patients include *Rickettsia* spp. (21.7%), *Leptospira* spp. (4.3%), *E. gerundensis* (4.3%), *K. pneumoniae* (21.7%), *E. coli* (26.1%), and *P. shigelloides* (4.3%). Of the five *Rickettsia* spp. positives who were also positive for dengue, one patient had an admission diagnosis of “Rickettsial disease/dengue without warning signs.” However, the odds ratio (OR) of the likelihood of association between PCR-positive blood

Clinical significance		Frequency						% identity ^c
		0	2	4	6	8	10	
Significant	<i>Escherichia coli</i>	[Bar chart showing frequency of 11]						100%
	<i>Klebsiella pneumoniae</i>	[Bar chart showing frequency of 8]						99.3-100%
	<i>Leptospira</i> sp. ^a	[Bar chart showing frequency of 1]						n/a
	<i>Pantoea vagans</i>	[Bar chart showing frequency of 2]						99.7-100%
	<i>Plesiomonas shigelloides</i>	[Bar chart showing frequency of 1]						100%
	<i>Rickettsia</i> sp. ^a	[Bar chart showing frequency of 10]						n/a
	<i>Rickettsia typhi</i> ^a	[Bar chart showing frequency of 2]						n/a
Uncertain clinical significance	<i>Erwinia gerundensis</i>	[Bar chart showing frequency of 1]						100%
	<i>Yersinia</i> sp.	[Bar chart showing frequency of 1]						99.7%
Not significant	<i>Rheinheimera</i> sp.	[Bar chart showing frequency of 5]						99.3-100%
Unknown	No identification ^b	[Bar chart showing frequency of 10]						

FIGURE 1. Numbers of organisms identified by genus- and species-specific PCRs, 16S rRNA PCR, and sequencing (including percent identity match), *n* = 53.

^a Identification by genus- and species-specific PCRs.

^b 16S rRNA PCR-positive, but no genus or species identification could be made.

^c Percent identity match by sequencing of 16S rRNA PCR product. n/a = not applicable, samples were identified by genus- or species-specific PCRs, not 16S rRNA and were not subjected to BLAST analysis.

culture and the presence of dengue infection was not statistically significant (OR: 1.46; 95% CI: 0.41–6.55; $P = 0.778$). Of 111 patients also tested for Zika virus, all were negative, and patients were not screened for Chikungunya virus. Vientiane is not a malaria-endemic region. According to patient records, four (7.5%) PCR-positive patients had taken ceftriaxone before sampling (organisms identified included *Rickettsia* sp., *E. coli*, and *P. vagans*) and 15 (28.3%) had not taken antimicrobials, and for 34 (64.2%) patients, this was unknown. Only two patients (one *Leptospira* sp.-positive and one *Rickettsia* sp.-positive) received doxycycline. No other *Rickettsia* spp.-positive patients received a tetracycline.

Where available, patient records were reviewed to evaluate if the patient management, diagnosis, or outcome would have been influenced or changed by the PCR result (presence/absence of bacteria) or species identification. The positive 16S PCR result would probably have influenced patient management considerations (i.e., the attending clinician would have checked current specific management or considered possible changes to management) in all 53 patients (100%), whether or not a specific species identification had been made. Where patient management information was available, the majority of patients (55.8%, 24/43) would likely have had a change in management (i.e., given antibiotics when they originally did not receive any, or had a change in antibiotic class given). In particular, three patients identified as *Rickettsia* sp. positive had not been given a tetracycline; if these data had been available during patient admission, they would have had a change in management. Where a genus- or species-specific identification was made, management would likely have been influenced in 90.2% (37/41) of patients, and management likely have been changed in 69.7% (23/33) of patients. The identification of any bacteria present would have likely changed the final diagnosis in 55.8% (24/43) of patients: 90.9% (30/33) when a genus- or species-specific identification was made. Overall, a review of patient outcomes suggested that a positive 16S rRNA cPCR result would have changed the outcome in only one patient (2.3%, $n = 43$), and a genus- or species-specific identification in two patients (6.1%, $n = 33$).

DISCUSSION

Potential bacterial pathogens were successfully identified by PCR in over 25% of blood cultures that had been reported as negative after 5 days; over 5% contained rickettsial DNA. However, this must be interpreted with caution, given the high incidence of acute dengue in this series. Any bacterial organism cultured from a sterile site (such as blood) is a potential pathogen, although in the Lao PDR, contaminants such as coagulase-negative staphylococci are frequently isolated from positive blood cultures.⁴ For samples containing *E. coli*, *K. pneumoniae*, *R. typhi*, or *Leptospira* spp. DNA, the findings were considered potentially clinically significant. These bacteria are unlikely contaminants, and most patients will develop febrile illnesses within days of infection.^{18,19} *Escherichia coli* and *K. pneumoniae* grow readily in blood culture media, are common isolates from blood cultures,²⁰ and have the potential to cause serious illness.²¹ That these pathogens did not grow in these bottles, leading to a false negative, could be due to several reasons, such as organism death or growth inhibition from incorrect sample handling or prior antimicrobial treatment, very low circulating bacterial loads, or transient DNAemia (e.g., secondary to dengue infection). Although plausible, inappropriate handling of samples, such as storage conditions of inoculated bottles before

blood culture,²² is an unlikely reason for a negative blood culture in this study as the time between sample collection and inoculating blood culture bottles is generally short in Mahosot Hospital (bottles are inoculated at the bed-side and transferred to the laboratory within a few hours). The use of incorrect blood volumes for inoculating blood culture bottles may also be discounted as the mean blood volume used was not statistically significant between PCR-positive (mean: 10.15 mL; IQR: 9.68–10.62 mL) and PCR-negative (mean: 9.84 mL; IQR: 9.39–10.29 mL) blood cultures ($P = 0.434$). A more likely reason is a low bacterial inoculum (assuming the initial presence of viable organisms) due to antimicrobial treatment before blood culture, with the selective pressure of antimicrobials suppressing bacterial growth.²³ Only 7.5% of the PCR-positive patients reported taking antimicrobials before sampling. However, given the widespread practice of self-medication with antimicrobials in the Lao PDR and ready availability of over-the-counter antimicrobials, many patients are likely to have self-treated.^{24,25} Patient records indicated that 25 of the PCR-positive patients had other samples taken for culture (other blood cultures, pus, urine, stool, and cerebrospinal fluid). Of these, seven patients (28%) had positive cultures. One patient whose blood culture was PCR positive for *Klebsiella pneumoniae* also had *K. pneumoniae* identified from two blood cultures taken within the previous 6 months. There were no other bacterial DNA PCR-positive samples which grew the same bacteria from a previous blood culture.

Although it has been shown that leptospires and rickettsial organisms such as *O. tsutsugamushi* remain viable in blood culture^{8,9,26} and may even benefit diagnosis through enhancement of organism numbers, standard blood culture detection systems are unable to reveal their presence. This study has confirmed those findings, with the detection of both *Leptospira* spp. and *Rickettsia* spp., including *R. typhi* by PCR. Our results suggest that routine PCR of negative blood cultures for these organisms might be valuable in some settings.

Patients in this study had blood cultures collected because they displayed symptoms/signs of BSI. However, the relationship between the finding of bacterial DNA and the clinical manifestations cannot be proven—it could be casual and not causative. Not all patients in the study may have been genuinely bacteremic, and some of the bacterial species identified are of uncertain or doubtful clinical significance, such as *Rheinheimera* spp. The clinical manifestations observed in patients could be due to other non-BSI etiologies (in many cases, this may have been due to dengue) because BSI symptoms are mostly nonspecific. For patients with a PCR-positive but blood culture-negative result, interpretation has to be carried out together with the analysis of patient's clinical records to determine its clinical significance. In addition, these so-called false negatives may not be clinically significant if the bacteria detected are too low in concentration to cause disease, such as a self-limiting transient infection.³ That said, clinical review suggests that a positive PCR from negative hemoculture fluids would have influenced patient management in all 53 patients, and in 90.2% of cases when a genus or species identification was made. In addition, between 55.8% and 69.7% of patients would probably have had a change in clinical management, although only 2.3–6.1% of patients would likely have had an altered final outcome.

The finding of *Rickettsia* spp. and *Leptospira* spp. DNA in negative blood culture affirms the potential of this technique to detect such fastidious bacteria.^{8,9,25} The detection of Enterobacteriaceae, such as *E. coli* and *K. pneumoniae*, in negative blood cultures

shows that a multipronged approach may improve diagnosis, even for bacteria normally considered fast growing in broth media.

These findings warrant further study (with comparison of patients who are dengue PCR positive and negative) and discussion, particularly over routine use and timing of PCR for organisms such as *Rickettsia* and *Leptospira*, which are important pathogens in Southeast Asia, and the role of dengue in bacterial infections. This will inevitably involve balancing clinical, financial, and logistical considerations, especially in low-resource settings such as the Lao PDR.

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