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CHLAMYDIOLOGY AND RICKETTSIOLOGY

Optimization and Evaluation of a Multiplex Quantitative PCR Assay for Detection of Nucleic Acids in Human Blood Samples from Patients with Spotted Fever Rickettsiosis, Typhus Rickettsiosis, Scrub Typhus, Monocytic Ehrlichiosis, and Granulocytic Anaplasmosis

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ABSTRACT Spotted fever group rickettsioses (SFGR), typhus group rickettsioses (TGR), scrub typhus (caused by *Orientia tsutsugamushi*), ehrlichiosis, and anaplasmosis often present as undifferentiated fever but are not treated by agents (penicillins and cephalosporins) typically used for acute febrile illness. Inability to diagnose these infections when the patient is acutely ill leads to excess morbidity and mortality. Failure to confirm these infections retrospectively if a convalescent blood sample is not obtained also impairs epidemiologic and clinical research. We designed a multiplex real-time quantitative PCR (qPCR) assay to detect SFGR, TGR, *O. tsutsugamushi*, and infections caused by *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis* with the *ompA*, 17-kDa surface antigen gene, *tsa56*, *msp2* (*p44*), and *vlpt* gene targets, respectively. Analytical sensitivity was $\geq$2 copies/μl (linear range, 2 to 2×10^5) and specificity was 100%. Clinical sensitivities for SFGR, TGR, and *O. tsutsugamushi* were 25%, 20%, and 27%, respectively, and specificities were 98%, 99%, and 100%, respectively. Clinical sensitivities for *A. phagocytophilum* and *E. chaffeensis* were 93% and 84%, respectively, and specificities were 99% and 98%, respectively. This multiplex qPCR assay could support early clinical diagnosis and treatment, confirm acute infections in the absence of a convalescent-phase serum sample, and provide the high-throughput testing required to support large clinical and epidemiologic studies. Because replication of SFGR and TGR in endothelial cells results in very low bacteremia, optimal sensitivity of qPCR for these rickettsioses will require use of larger volumes of input DNA, which could be achieved by improved extraction of DNA from blood and/or extraction of DNA from a larger initial volume of blood.

KEYWORDS spotted fever and typhus group rickettsioses, scrub typhus, ehrlichioses, anaplasmosis, *Orientia* spp., *Rickettsia* spp., *Ehrlichia chaffeensis*, *Anaplasma phagocytophilum*, *Rickettsiales*, ticks, diagnostics, etiology of fever studies

The burden and clinical impact of rickettsioses and related infections is increasingly recognized (1–7). Spotted fever group rickettsioses (SFGR), typhus group rickettsioses (TGR), and human granulocytic anaplasmosis (HGA) are causes of acute febrile illness worldwide. Human monocytic ehrlichiosis (HME), caused by *Ehrlichia chaffeensis*, also results in acute febrile illness across the Americas, whereas scrub typhus has been found only in the Asia-Pacific triangle until recently (3, 8). Recent data suggest both increasing incidence and significant underreporting (<25%) of rickettsioses (3, 9–13). Little is known about anaplasmosis in tropical regions (14–20). The current diagnostic reference standard for agents responsible for rickettsioses (SFGR and TGR), scrub typhus (*Orientia tsutsugamushi*), and ehrlichioses (HGA and HME) is a 4-fold rise in IgG antibody

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TABLE 1 Specimens used for evaluation of multiplex PCR

Organism (no.)	Laboratory (no. of samples; location)	Specimen type (no.)	Reference assay (no. positive)	Reference(s)
SFGR (20)	National Institute of Health, Ricardo Jorge (18 samples; Portugal); CDC (2 samples; USA)	Blood/buffy coat (18), DNA (2)	Conventional PCR (18), real-time PCR (2), serology (18), culture (18)	41, 42
TGR (30)	Mahidol Oxford Tropical Medicine Research Unit (20 samples; Thailand); Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (10 samples; Lao PDR)	Blood/buffy coat (10), DNA (20)	Real-time PCR (30), serology (10), culture (10)	35
O. tsutsugamushi (30)	Mahidol Oxford Tropical Medicine Research Unit (20 samples; Thailand); Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit (10 samples; Lao PDR)	Blood/buffy coat (10), DNA (20)	Real-time PCR (30), serology (10), culture (10)	35
E. chaffeensis (45)	Johns Hopkins University School of Medicine (USA); Mayo Clinic (USA); University of Texas Medical Branch (USA)	Blood (6), DNA (39)	Conventional PCR (12), real-time PCR (32), serology (10), morulae (1), culture (4)	26, 43–45
A. phagocytophilum (15)	Johns Hopkins University School of Medicine (USA)	Blood	Real-time PCR (15), serology (11), morulae (14), culture (4)	26
Bloodstream infections (17)	Johns Hopkins Hospital/University School of Medicine (USA)	Blood	Blood culture (17)	26
P. falciparum (28)	Johns Hopkins Hospital/University School of Medicine (USA)	Blood	Blood smear (28), real-time PCR (22)	36
P. vivax (5)	Johns Hopkins Hospital/University School of Medicine (USA)	Blood	Blood smear (5), real-time PCR (5)	36
E. chaffeensis (6)	USA reference laboratory (Mayo Clinic)	Blood	Real-time PCR (6)	45
Convalescent from *E. chaffeensis* or *A. phagocytophilum* (3)	Johns Hopkins University School of Medicine (USA)	Blood	Conventional PCR (3), serology (3), morulae (2), culture (3)	26

titer between paired acute- and convalescent-phase serum samples by indirect immunofluorescence (IFA), which is laborious, subjective, and intrinsically retrospective. Furthermore, IFA cannot absolutely distinguish between SFGR and TGR or between *Anaplasma phagocytophilum* and *E. chaffeensis* without testing for both agents of rickettsioses and ehrlichioses because of serologic cross-reactions. Rapid sensitive and specific detection of rickettsial agents could avert needless morbidity and mortality (21, 22) related to failure to prescribe needed doxycycline (5). High-throughput assays are needed to support large epidemiologic and clinical studies to define the global burden, distribution, and clinical features of rickettsioses and related infections, including the pathogenicity of different species of SFGR (23, 24). To allow high-throughput simultaneous assessment of multiple potential pathogens, including coinfections, we developed a quantitative, multiplex 5′-nuclease quantitative real-time PCR (qPCR) assay to rapidly detect and distinguish SFGR, TGR, *O. tsutsugamushi*, *A. phagocytophilum*, and *E. chaffeensis*.

MATERIALS AND METHODS

Samples. We used specimens from patients with acute febrile illness in whom rickettsioses were independently confirmed by ≥1 reference method as follows: 4-fold IgG increase in antibody titer, PCR using different primers, culture, or observation of morulae in blood leukocytes (for *A. phagocytophilum* and *E. chaffeensis*). These patients included 20 with SFGR, 30 with TGR, 30 with *O. tsutsugamushi* infection, 15 with *A. phagocytophilum* infection, and 45 with *E. chaffeensis* infection. Control samples were from patients with other etiologies of acute febrile illness (including 17 with bloodstream infections, 33 with blood smear and PCR-confirmed malaria [28 caused by *Plasmodium falciparum* and 5 caused by *Plasmodium vivax*], and 6 with PCR-confirmed *Ehrlichia ewingii* infection) or those from patients convalescent from *A. phagocytophilum* (1) or *E. chaffeensis* infections (2) (Table 1). Depending upon origin, sample types included EDTA-anticoagulated blood, buffy coat, mononuclear cells, and DNA prepared from whole blood or buffy coat. For blood and buffy coat samples, DNA was prepared using the QIAamp DNA blood minikit (Qiagen), starting with 200 μl and resuspended into 200 μl buffer AE or water as recommended. For samples received as DNA, methods included the QIAamp blood minikit, QIAsym-

TABLE 2 Primers and probes used for multiplex PCR

Target pathogen	Accession no.	Target gene	Forward primer	Reverse primer	Probe
SFGR	NC_009882.1	SFGR *ompA* consensus	TTGTCAGGCTCTGAAGCTAAAC	AGCACCTGCCGTTGTGATATC	FAM-TAGCCGCAGTCCCTACAACACCGC-BHQ1
TGR	M28481	*R. typhi* 17-kDa antigen gene TGR optimized	ACTTGGTTCTCAATTCGGTCAC	CAGACTTGCACCGATTTGTCC	TXR-TGCCCCAAGTAATGCGCCTACACC-BHQ1
O. tsutsugamushi	AY836148.1	*Orientia tsutsugamushi* strain Kato 56-kDa antigen gene	GGTGGTAATGCTTTCGCTAATCAG	TGCTGCTTCTTGCGCCTGTAG	CY3/HEX-TGCTGCTGTTGCTGCCCTTGCC-BHQ1
E. chaffeensis	AF181986	*vlpt*	CTAATTCTGATTTACACGAGTCTTC	GCATCATCTTCGAATTGAACTTC	TET-TTGAGTTACCTGGTCC-BHQ1
A. phagocytophilum	Many	*msp2* (5′ conserved domain)	GAAGATGAWGCTGATACAGTA	CAACHGCCTTAGCAAACT	Cy5-TTATCAGTCTGTCCAGTAACA-BHQ1

phony DNA midikit, Qiagen DNA extraction kit (Qiagen), the Wizard SV genomic DNA purification system (Promega, Madison, WI, USA), and IsoQuick extraction kit (ORCA Research, Bothell, WA).

Multiplex PCR assay development. As previously reported (25, 26), we used AlleleID 6 (Premier Biosoft, Palo Alto, CA) software to design primers and probes. Since the conditions for amplification are standardized and the specific design of primers and probes identical, the assays were run similarly. However, there were a number of smaller changes, including the quantity of DNA input used, the range of standards and controls tested, and the total number of highly pedigreed samples tested. Our gene targets included a SFGR consensus (23 members of SFGR) *ompA* sequence, the genus-wide 17-kDa lipoprotein gene optimized for *Rickettsia typhi* (TGR), a consensus conserved region of the *O. tsutsugamushi* 56-kDa major outer membrane protein gene, the *A. phagocytophilum msp2* (*p44*) gene, and the *E. chaffeensis vlpt* gene (sequences in Table 2).

The assay was run as either a pentaplex (Bio-Rad CFX384 PCR instrument) or separate triplex and duplex assays (Bio-Rad IQ5 PCR instrument). DNA from 200 μl of blood was reconstituted in buffer to 200 μl, and 1 to 3 μl of blood/buffy coat DNA was used for all PCR assays. Quantitative results were adjusted for input volume. Controls and standards included DNA from microscopically quantified bacterial cells or infected mammalian cells (positive controls), DNA obtained from the blood of healthy human study participants (negative controls), and no template controls. Plasmid-cloned amplicons were used to generate a standard curve for quantification (10^0 to 10^5 copies per reaction). Standards were accepted only when the curve's *R* value was $>$0.90, the PCR efficiency was 85 to 115%, and the limit of detection was $\leq$10 copies per reaction. Samples run in duplicate or triplicate were accepted as positive only if $\geq$2 were positive. After initial experiences showed that triplicate was rarely contributory, we reduced the assay to duplicates.

Each measurement was adjusted for input volume or dilution/concentration effects of DNA elution to obtain a final measurement in bacteria per milliliter blood. We used the endpoint analysis program in the CFX Manager wherein the relative fluorescent units (RFUs) for each sample or control are calculated over the final 5 of 40 cycles to establish cutoffs. The positive cutoff value was calculated by identifying the average RFUs for the negative controls for each analyte/fluor and by adding a percentage of the range of RFUs on each plate for each analyte/fluor (highest to lowest RFU = range). We used data generated by testing 101 samples (14 *A. phagocytophilum* samples, 5 *E. chaffeensis* samples, 20 *O. tsutsugamushi* samples, 6 SFGR samples, 20 TGR samples, 3 convalescent samples of *A. phagocytophilum* and *E. chaffeensis*, 23 *P. falciparum* samples, and 20 negative controls) to compare the receiver operator characteristic (ROC) curves (*x* axis, 1 specificity; *y* axis, sensitivity at each cutoff) (27) for cutoffs set at 2.5, 3, 4, 5, 7.5, 10, 12.5, and 15% of the plate RFU range above the average negative sample RFUs for the analyte and plate. All final results were calculated using the cutoff selected for each pathogen analyte/fluor combination from the ROC curves. Multiple small informal comparisons suggested no differences in detection sensitivity between singleplex versus multiplex testing for each of the analytes (25). To formally determine this, DNA samples from blood obtained from 14 patients with *A. phagocytophilum* and 66 non-*A. phagocytophilum* "negative" controls (8 with SFGR, 20 with TGR, 7 with *O. tsutsugamushi*, 6 with *E. chaffeensis*, 1 convalescent from *A. phagocytophilum* and 2 from *E. chaffeensis*, and 22 with malaria) were run in a singleplex and compared to results using the multiplex assay.

Blood volume and DNA preparation in analytical sensitivity using rickettsiae-spiked blood. To increase clinical sensitivity for vasculotropic rickettsiae (SFGR, TGR, and *O. tsutsugamushi*), the roles of starting blood volume, final DNA suspension volume, DNA preparation protocol, and pathogen DNA enrichment and isolation obtained with the MolYsis basic kit (Molzym GmbH & Co., Bremen, Germany) were studied using fresh human blood supplemented with spotted fever rickettsiae (*Rickettsia parkeri* Portsmouth strain)-infected human brain microvascular endothelial cells for which the quantity was determined by counting the proportion of infected cells among 200 cells and the average quantity of *R. parkeri* bacteria per infected cell in LeukoStat-stained cytofuged samples. Based on this calculation, an aliquot of 5×10^7 bacteria in endothelial cells was prepared by centrifugation and the pellet suspended in 5 ml of fresh EDTA-anticoagulated human blood. This 5-ml blood sample was then serially diluted (10-fold to 10^0 bacteria/ml) in human EDTA-anticoagulated blood, and 1 ml from each dilution was used to prepare buffy coat. The buffy coat and residual spiked blood were used for DNA preparation as below.

Effect of input volume of blood and output volume of resuspended DNA. To examine the role of input blood volume used for extraction of DNA and output volume of DNA (resuspension volume), different methods were employed as follows: (i) DiaSorin/Arrow DNA extraction kit on the DiaSorin (NorDiag) Arrow nucleic acid extraction instrument, (ii) the QIAamp DNA blood minikit (Qiagen, Germantown, MD, USA), and (iii) the MolYsis basic kit (Molzym, Bremen, Germany). For the Arrow method, 500 and 100 μl of rickettsia-spiked blood were resuspended into a final volume of 150 μl and 100 μl buffer, respectively. For the QIAamp DNA blood minikit, 100 μl blood was extracted into 200 μl buffer. For the MolYsis kit, the pellet from 1 ml of blood was extracted using the Arrow protocol and resuspended into 100 μl buffer. Each DNA preparation was then used in the SFGR qPCR protocol.

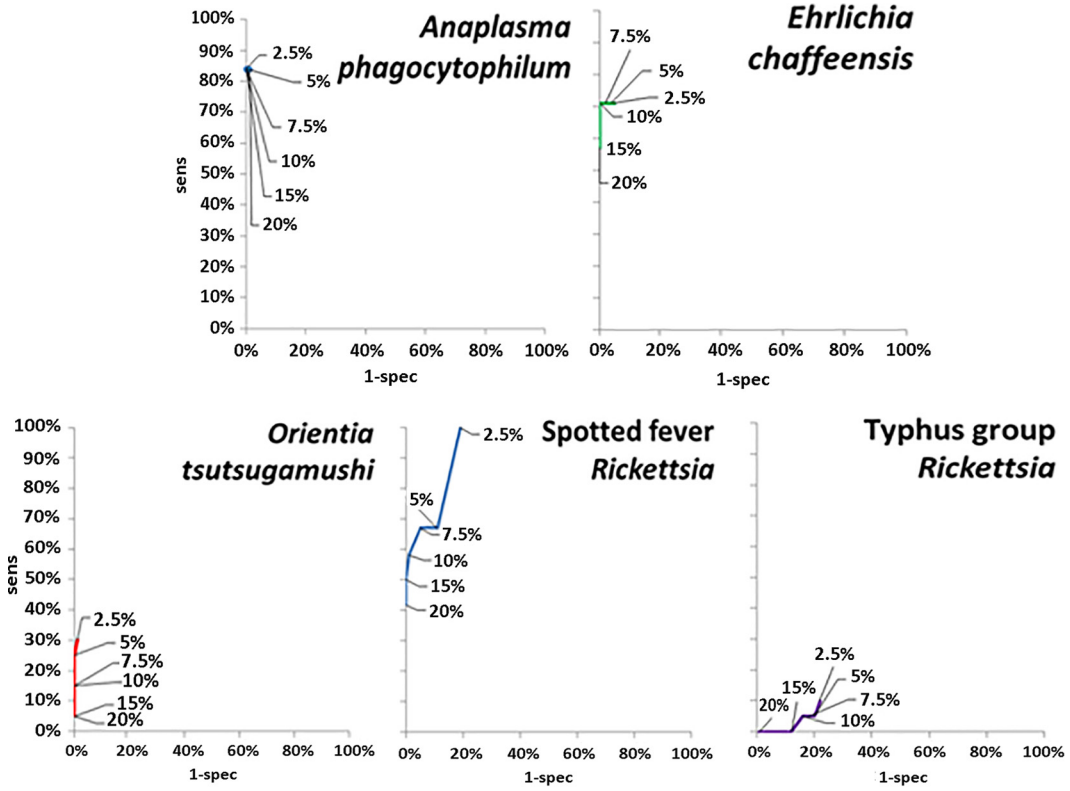

FIG 1 ROC curves used to establish positive cutoffs for multiplex qPCR assays for rickettsioses. The data labels represent the percent of the total RFU range per run added to the average of negative control RFUs in order to establish a cutoff as described in Materials and Methods.

Whole blood versus buffy coat. Since buffy coat should increase sensitivity by enriching for host cell-associated rickettsiae, the sensitivity of qPCR using whole blood versus buffy coat was also compared. Using preparations supplemented with *R. parkeri*-infected endothelial cells as above, blood (200 μl starting volume) or buffy coat from 1 ml of blood (~200 μl buffy coat after centrifugation) was used to prepare DNA (QIAamp DNA blood minikit), and both DNAs were resuspended in 200 μl DNA buffer. These preparations were then subjected to qPCR.

Ethics. The study was reviewed by the ethics committee of the Johns Hopkins School of Medicine. Institutional review board (IRB) approval was granted to use archived discarded deidentified samples since consent was deemed both impractical and unnecessary (JHM protocol NA_00021376).

RESULTS

Singleplex versus multiplex qPCR. Samples from 14 patients with *A. phagocytophilum* infection, 6 with *E. chaffeensis* infection, and 60 additional samples from patients with other rickettsial and nonrickettsial infections were tested using both the singleplex assay for *A. phagocytophilum* and our multiplex assay. We found no decrement in sensitivity with multiplexing. The singleplex assay for *A. phagocytophilum* detected 13 of 14 *A. phagocytophilum* infections (sensitivity, 93%) and detected 0 of 6 *E. chaffeensis* infections and 0 of 60 other infections (specificity, 100%). The multiplex assay correctly identified 12 of the 14 *A. phagocytophilum* infections (sensitivity, 86%) and 5 of 6 *E. chaffeensis* infections (sensitivity, 83%); specificity was 100% (66 and 74 other rickettsial and nonrickettsial samples, respectively, were negative). These findings confirmed multiple prior informal comparisons showing no differences in detection sensitivity between singleplex and multiplex assays as also described by others (25, 28).

Multiplex qPCR. For each run in the pilot studies, the sensitivity and specificity of the multiplex qPCR assay were determined at a range of cutoffs and plotted on ROC curves. As shown in Fig. 1, the point of inflection on the ROC curve at which the highest sensitivity and specificity was achieved (of plate RFU range above the average of negative controls) was at 7.5% for SFGR (sensitivity, 67%; specificity, 95%), 2.5% for TGR

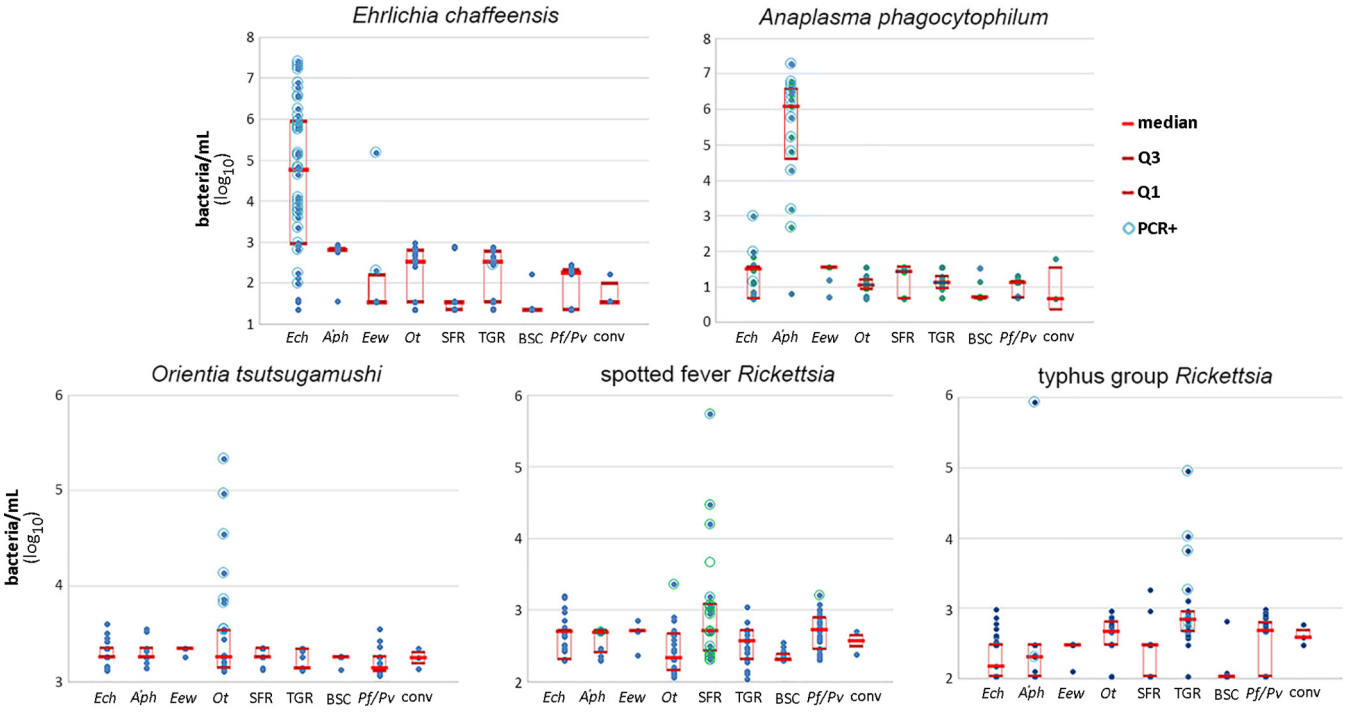

FIG 2 Quantitative multiplex results for the entire cohort by pathogen analyte (graph title). The *x* axis denotes the true identity of the individual sample tested (*Ech*, *E. chaffeensis*; *Aph*, *A. phagocytophilum*; *Eew*, *E. ewingii*; *Ot*, *O. tsutsugamushi*; SFR, spotted fever *Rickettsia*; TGR, typhus group *Rickettsia*; BSC, blood culture-positive sample controls; *Pf*/*Pv*, *P. falciparum*/*P. vivax*; conv, convalescent from *E. chaffeensis* or *A. phagocytophilum* infection), and the *y* axis is the bacterial quantity as discerned from the specific standard curve applied to the plate on which the sample was tested. Each individual solid circle denotes a single sample; open circles denote samples that were PCR positive for that analyte; the median for each group is denoted by the central red bar within the 1st and 3rd quartiles that are denoted by the top and bottom of the red box.

(sensitivity, 10%; specificity, 78%), 5% for *O. tsutsugamushi* (sensitivity, 25%; specificity, 100%), 5% for *A. phagocytophilum* (sensitivity, 84%; specificity, 100%), and 10% for *E. chaffeensis* (sensitivity, 71%; specificity, 100%). These values were used to calculate the specific cutoffs for each 96- or 384-well multiplex plate assay for that specific analyte.

The established cutoffs were used in the full optimization study, from which final clinical sensitivity and specificity for each analyte could be estimated. Using the quantitation derived by comparing average final RFUs for each sample with the standard curve for that pathogen analyte/fluor and adjusting for DNA preparation dilution/concentration and DNA qPCR assay input volume, quantitative multiplex results were obtained (Fig. 2). The clinical diagnostic performance of the multiplex qPCR for the detection of rickettsial agents in the blood of humans with acute febrile illness is shown in Table 3.

In light of limited clinical sensitivity but high analytical sensitivity of the multiplex for vasculotropic rickettsioses (spotted fever and typhus group *Rickettsia* and *Orientia tsutsugamushi*), we examined the preanalytical role of input blood volume for DNA preparation versus final output of DNA suspension. We hypothesized that using a larger

TABLE 3 Performance of multiplex quantitative PCR for detection of acute rickettsial infections in patients with acute febrile illness

Bacterium	Gene target	No. true positives	No. test positives	Sensitivity[a]	True negatives	Test negatives	Specificity[a]	PPV[b]	NPV[b]
Anaplasma phagocytophilum	*msp2* (*p44*)	14	14	0.93	184	182	0.99	0.88	0.99
Ehrlichia chaffeensis	*vlpt*	45	38	0.84	154	151	0.98	0.93	0.96
Orientia tsutsugamushi	*tsa56* (56-kDa surface antigen)	30	8	0.27	169	169	1.0	1.0	0.88
Spotted fever *Rickettsia*	*ompA*	20	5	0.25	176	173	0.98	0.63	0.92
Typhus group *Rickettsia*	17-kDa surface antigen[a]	30	6	0.2	167	165	0.99	0.75	0.87

[a]Sensitivity is the proportion of true positives with a positive test result (test positive/all with disease = test positives plus false negatives); specificity is the proportion of true negatives (nondiseased) with a negative test result (test negative/all without disease = test negatives plus false positives).
[b]PPV, positive predictive value; NPV, negative predictive value.

TABLE 4 The effect of starting blood volume and DNA concentration methods on detection sensitivity in *Rickettsia*-spiked whole-blood and buffy coat samples

Method	Sample type	Starting blood vol (μl)	Elution vol (μl)	Concn factor	LOD[a] *Rickettsia parkeri*/ml in blood	LOD *Rickettsia parkeri*/μl in eluate
Qiagen blood minikit	Blood	100	200	0.5	5,000	5.0
Arrow v2	Blood	100	100	1.0	1,000	1.0
Arrow v1	Blood	500	150	3.3	333	0.3
MolYsis basic/Arrow v1	Blood	1,000	100	10.0	500	5.0
QIAsymphony	Blood	1,000	100	10.0	100	1.0
Qiagen blood minikit	Blood	200	200	1.0	5,000	0.2
Qiagen blood minikit	Buffy coat	1,000	200	5.0	100	0.1

[a]LOD, limit of detection in *Rickettsia parkeri* Portsmouth strain.

input volume of blood and smaller elution volume of extracted DNA would improve clinical sensitivity of qPCR. As shown in Table 4, input blood volume and elution DNA resuspension volume were correlated with sensitivity of qPCR; the limit of detection was 5×10^3 bacteria/ml when 100 μl of blood was resuspended into 200 μl final buffer (1:2 dilution from blood) but 1×10^2 bacteria/ml when using a method to obtain a 10:1 concentration of DNA from blood (1 ml of blood resuspended into 100 μl buffer). Regardless of method used to prepare DNA from blood, the final concentration of bacteria per microliter of eluted DNA at the limit of detection varied by 10-fold or less (from 0.3 bacteria/μl to 5/μl), which confirmed the excellent analytical sensitivity of the assay and suggested that concentration of blood DNA could further advance clinical sensitivity. Since larger starting blood volumes and smaller final DNA resuspension buffer volumes improved sensitivity, we finally employed the Molzym MolYsis basic kit to enable an input volume of 1 ml of blood with output volume of 100 μl buffer (theoretical 10:1 concentration from blood), which resulted in analytical sensitivity (5×10^2 bacteria/ml) comparable to that obtained with other methods that yield similar blood DNA concentration. Therefore, we next compared using the standard 200-μl input volume of blood versus 200 μl of buffy coat prepared from 1 ml blood with DNA in both cases eluted into 200 μl buffer. The use of buffy coat blood resulted in a 50-fold improvement in analytical sensitivity from 5×10^3 to 10^2 bacteria/ml blood.

DISCUSSION

New diagnostic approaches are essential to reduce morbidity and mortality from rickettsioses, scrub typhus, and ehrlichiosis worldwide and to support large epidemiologic studies that define the global burden of these infections, including emerging species and ecologic niches of SFGR. Detection of bacteremia due to SFGR, TGR, and *O. tsutsugamushi* is inherently difficult compared with that due to *A. phagocytophilum* and *E. chaffeensis* because endothelial cells are infected rather than circulating leukocytes, which results in very low rickettsemia (29–32). The few molecular assays described to date, which include multiplexing and optimization using highly pedigreed samples, are difficult to compare because of lack of a uniform diagnostic standard comparator and different sample types and storage conditions (33, 34). In general, analytical sensitivity is lower for conventional PCR than for nested PCR and real-time PCR (1,000 to 10,000 and <100 to 5,000 genome equivalents/ml of blood DNA, respectively) (33) but heavily dependent on stage and severity of illness (30). A real-time multiplex qPCR assay to detect SFGR, TGR, *O. tsutsugamushi*, *A. phagocytophilum*, and *E. chaffeensis* would be ideal since there is great clinical, epidemiologic, and geographic overlap among them, and well-designed multiplex qPCR assays have similar sensitivity to singleplex assays (28). Finally, any assay with adequate analytical sensitivity requires clinical validation.

Paris et al. (35) described a multiplex qPCR assay for SFGR rickettsiae, TGR, and *Orientia* using *ompB*, *gltA*, and 47-kDa (unique to *Orientia*) gene targets. The limit of detection by multiplex qPCR was 1 copy/μl for SFGR and TGR and 24 copies/μl for *O. tsutsugamushi*. Clinical samples evaluated included 12 buffy coat samples from patients with suspected acute rickettsial infections on the basis of IgM- and IgG-based rapid immunochromatographic tests with or without IgM or IgG detection by IFA using

paired serum samples. Of the 12 (3 SFGR, 2 TGR, and 7 *O. tsutsugamushi* samples), 6 were PCR positive (1 for TGR and 5 for *O. tsutsugamushi*); however, only 2 had a 4-fold rise in IgM and/or IgG antibody titer (1 TGR sample and 1 *O. tsutsugamushi* sample). A recent review (33), which included studies of >10 patients published since 2013 evaluated with serology and PCR, found that the median clinical sensitivity of real-time PCR for the detection of SFGR and TGR in blood was 18% overall, with SFGR improved (42%) versus TGR (3%). Tshokey et al. (37) evaluated 1,004 febrile patients in Bhutan for acute rickettsial infections, defining acute infection as a single high IgM titer or positive qPCR. Of 1,044 patients, 46 (4.4%), 4 (0.4%), and 70 (6.7%) patients had acute SFGR, TGR, and *O. tsutsugamushi* infection, respectively; however, only 7 were positive by qPCR for *O. tsutsugamushi* (4 PCR positive only and 3 qPCR and single-serum sample IFA positive).

To address the unmet need for improved detection of globally-distributed rickettsioses, we previously described development of a multiplex triplex qPCR to detect SFGR, TGR, and *O. tsutsugamushi* (25), in which analytical sensitivity and specificity were similar to that of Paris et al. (35), as well as development and limited clinical validation of a real-time duplex assay for *Ehrlichia* and *Anaplasma* (26). The primary strength of the current study is clinical validation of a 5-target real-time multiplex qPCR assay to detect and distinguish all 5 major rickettsioses and related infections worldwide using a large panel of specimens for which rigorous reference standard testing was completed. We found excellent clinical sensitivity for *A. phagocytophilum* and *E. chaffeensis* and clinical sensitivity for SFGR, TGR, and *O. tsutsugamushi* comparable to that of other reports with many fewer clinical samples and/or unclear confirmatory testing. We do not think that the low sensitivity of our assay for *O. tsutsugamushi* is due to a choice of antigen gene target since the original primers used in this study were established via identification of highly conserved regions of the 56-kDa antigen gene from 101 sequences deposited into GenBank using the AlleleID algorithms. Pilot studies examining amplification efficacy across the Kato, Karp, and Gilliam strains showed equivalence. Furthermore, the combination of primers and probes, when subjected to a BLAST search against the NCBI RefSeq Genome Database (*Orientia* [taxid 69474]), identified appropriate targets for amplification in a range of geographically distinct whole genomes, including those from Korea, Japan, Thailand, and even *Orientia chuto* from Dubai. The sensitivity of our assay is indeed very similar to that observed in other published studies, including those that target the *O. tsutsugamushi* 47-kDa gene (32–34, 38). It is recognized that the limited clinical sensitivity results from low-level bacteremia (38). Moreover, the 56-kDa antigen gene is a preferred target owing to its specificity for *O. tsutsugamushi* (31, 33); PCR positivity for this target provides strong evidence of pathogen DNA (33). Although the clinical sensitivities for SFGR, TGR, and *O. tsutsugamushi* were low (25%, 20%, and 27%, respectively), specificity was excellent; therefore, a positive result confirms acute infection when treatment decisions must be made (need for doxycycline) and when a convalescent-phase serum sample is not available (typical case and fatal cases). Furthermore, the high-throughput (384-well plate) platform of our multiplex PCR assay supports large clinical and epidemiological studies. We found no decrement in sensitivity with multiplexing and experimentally showed that increasing effective input blood volume and decreasing elution volume increased analytical sensitivity for detecting rickettsial DNA under experimental circumstances. Removing host DNA and concentrating microbial DNA from blood, an approach used to increase sensitivity of PCR for *Mycobacterium tuberculosis* (39), did not increase sensitivity of PCR for the spotted fever group rickettsia *R. parkeri* beyond that already obtained with other DNA concentration methods that lacked removal of host cell DNA.

In summary, our real-time multiplex qPCR assay showed high clinical specificity for all 5 rickettsial targets but higher clinical sensitivity for leukocytic rickettsiae versus vasculotropic rickettsiae. Further clinical validation of the assay, optimally using buffy coat and/or another method to concentrate nucleic acids (DNA ± RNA) from a larger volume of blood, is needed to yield a limit of detection of 10^1 to 10^3 bacteria/ml, the median rickettsemia observed *in vivo* during vasculotropic rickettsial infections in humans (29, 30, 40).

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We declare no conflict of interest.

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