JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2011, p. 607–612 Vol. 49, No. 2
0095-1137/11/$12.00 doi:10.1128/JCM.01216-09

Comparison of Conventional, Nested, and Real-Time Quantitative PCR for Diagnosis of Scrub Typhus▽

Dong-Min Kim,[1]† Geon Park,[2]† Hyong Sun Kim,[1] Joo Young Lee,[1] Ganesh Prasad Neupane,[1] Stephen Graves,[3] and John Stenos[3]*

Division of Infectious Disease, Department of Internal Medicine, Chosun University School of Medicine, Gwang-ju, Republic of Korea[1]; Department of Laboratory Medicine, Chosun University School of Medicine, Gwang-ju, Republic of Korea[2]; and Australian Rickettsial Reference Laboratory, Geelong Hospital, Australia[3]

Received 22 June 2009/Returned for modification 18 September 2009/Accepted 26 October 2010

Orientia tsutsugamushi **is the causative agent of scrub typhus. For the diagnosis of scrub typhus, we investigated the performances of conventional PCR (C-PCR), nested PCR (N-PCR), and real-time quantitative PCR (Q-PCR) targeting the *O. tsutsugamushi*-specific 47-kDa gene. To compare the detection sensitivities of the three techniques, we used two template systems that used plasmid DNA (plasmid detection sensitivity), including a partial region of the 47-kDa gene, and genomic DNA (genomic detection sensitivity) from a buffy coat sample of a single patient. The plasmid detection sensitivities of C-PCR, N-PCR, and Q-PCR were 5×10^4 copies/μl, 5 copies/μl, and 50 copies/μl, respectively. The results of C-PCR, N-PCR, and Q-PCR performed with undiluted genomic DNA were negative, positive, and positive, respectively. The genomic detection sensitivities of N-PCR and Q-PCR were 64-fold and 16-fold (crossing point [Cp], 37.7; 426 copies/μl), respectively. For relative quantification of *O. tsutsugamushi* bacteria per volume of whole blood, we performed real-time DNA PCR analysis of the human *GAPDH* gene, along with the *O. tsutsugamushi* 47-kDa gene. At a 16-fold dilution, the copy number and genomic equivalent (GE) of *GAPDH* were 1.1×10^5 copies/μl (Cp, 22.64) and 5.5×10^4 GEs/μl, respectively. Therefore, the relative concentration of *O. tsutsugamushi* at a 16-fold dilution was 0.0078 organism/one white blood cell (WBC) and 117 organisms/μl of whole blood, because the WBC count of the patient was 1.5×10^4 cells/μl of whole blood. The sensitivities of C-PCR, N-PCR, and Q-PCR performed with blood samples taken from patients within 4 weeks of onset of fever were 7.3% (95% confidence interval [CI], 1.6 to 19.9), 85.4% (95% CI, 70.8 to 94.4), and 82.9% (95% CI, 67.9 to 92.8), respectively. All evaluated assays were 100% specific for *O. tsutsugamushi*. In conclusion, given its combined sensitivity, specificity, and speed, Q-PCR is the preferred assay for the diagnosis of scrub typhus.**

Scrub typhus is an infectious disease caused by *Orientia tsutsugamushi* and is transmitted through the bite of trombiculid mites. It is a major acute febrile disease in the Asia-Pacific region (1). Fever, chills, headache, myalgia, and skin rashes occur 1 to 2 weeks after mite bites, and the occurrence of characteristic eschars is helpful for early diagnosis (18). Scrub typhus generally runs a mild clinical course and shows a good response to antibiotic therapy. However, if diagnosis is delayed, serious complications, such as interstitial pneumonia, acute renal failure, meningoencephalitis, gastrointestinal bleeding, and multiple organ failure, may develop, leading to death (10, 22–25). Thus, a method for rapid diagnosis is indispensable for successful treatment. Serologic tests such as the indirect immunofluorescence assay (IFA), immunoperoxidase test, enzyme-linked immunosorbent assay (ELISA), and passive hemagglutination test (PHA) are currently in widespread use. Since these serologic tests have low sensitivities in the early stage of scrub typhus due to insufficient production of antibodies, frequent follow-up tests are needed (2). Detection of specific *O. tsutsugamushi* genes has been used for the rapid diagnosis of scrub typhus, and nested PCR (N-PCR) is widely used to improve the sensitivity of conventional PCR (C-PCR) (3, 4, 13). In addition, real-time quantitative PCR (Q-PCR) targeting a specific gene permits the diagnosis of scrub typhus within 2 h and has high sensitivity and specificity (5). However, there have been few studies comparing the abilities of the three aforementioned PCR methods (C-PCR, N-PCR, and Q-PCR) to detect the same *O. tsutsugamushi*-specific target gene. Therefore, we have compared the results of the indirect IFA, the "gold standard" test for the diagnosis of scrub typhus, to those obtained by C-PCR, N-PCR, and Q-PCR of the *O. tsutsugamushi* 47-kDa gene. For comparative purposes, a set of primers for the 56-kDa gene was used. Additionally, for the relative quantification of *O. tsutsugamushi* per volume of a patient's whole blood, we also performed real-time DNA PCR analysis of the human *GAPDH* gene, along with the *O. tsutsugamushi* 47-kDa gene.

MATERIALS AND METHODS

Bacterial strains and media. The standard bacterial strains used in this study were purchased from the American Type Culture Collection (ATCC), the Korea Culture Center of Microorganisms (KCCM), and the Korean Collection for Type Cultures (KCTC) (Table 1). All ordinary bacterial species used in this study were cultured on Luria-Bertani (LB) broth, brain heart infusion (BHI) broth (Difco, Lawrence, KS), or LB agar (Difco). The rickettsial strains were obtained from the Australian Rickettsial Reference Laboratory (ARRL).

Cloning of *O. tsutsugamushi* 47-kDa gene and human *GAPDH* gene. The 47-kDa gene was amplified using genomic DNA of the *O. tsutsugamushi* Karp

* Corresponding author. Mailing address: Australian Rickettsial Reference Laboratory, Geelong Hospital, Ryrie St., Geelong, VIC 3220, Australia. Phone: 61 3 5226 7552. Fax: 61 3 5260 3183. E-mail: johns@barwonhealth.org.au.

† Dong-Min Kim and Geon Park contributed equally to this work.

▽ Published ahead of print on 10 November 2010.

TABLE 1. Bacterial strains used in this study

Strain	Pathogen
1	*Aeromonas caviae* ATCC 15468
2	*Aeromonas hydrophila* subsp. *hydrophila* ATCC 7966
3	*Aeromonas hydrophila* subsp. *anaerogenes* ATCC 15467
4	*Vibrio alginolyticus* ATCC 17749
5	*Vibrio cholerae* ATCC14035
6	*Vibrio fluvialis* ATCC 33809
7	*Vibrio furnissii* ATCC 35016
8	*Vibrio hollisae* ATCC 33564
9	*Vibrio mimicus* ATCC 33653
10	*Vibrio parahaemolyticus* ATCC 17802
11	*Vibrio proteolyticus* ATCC 15338
12	*Vibrio vulnificus* ATCC 27562
13	*Streptococcus agalactiae* ATCC 13813
14	*Streptococcus mitis* ATCC 49456
15	*Streptococcus mutans* ATCC 15175
16	*Streptococcus pneumoniae* ATCC 33400
17	*Streptococcus pyogenes* ATCC 12344
18	*Streptococcus salivarius* ATCC 7073
19	*Streptococcus sanguinis* ATCC 10556
20	*Streptococcus sobrinus* ATCC 6715
21	*Staphylococcus aureus* subsp. *aureus* (MRSA[a]) ATCC 33591
22	*Staphylococcus aureus* (MRSA) ATCC 29213
23	*Staphylococcus epidermidis* ATCC 12228
24	*Staphylococcus saprophyticus* subsp. *saprophyticus* ATCC 15305
25	*Salmonella enterica* serovar Typhimurium KCTC 1925
26	*Klebsiella pneumoniae* ATCC 13883
27	*Shigella sonnei* ATCC 25931
28	*Pseudomonas aeruginosa* ATCC 27853
29	*Clostridium perfringens* ATCC 3624
30	*Aeromonas salmonicida* subsp. *salmonicida* ATCC 33658
31	*Clostridium difficile* ATCC 9689
32	*Rickettsia honei* RB
33	*Rickettsia rickettsii* Smith
34	*Rickettsia conorii* 7
35	*Rickettsia akari* MK (Kaplan)
36	*Rickettsia prowazekii* Breinl
37	*Rickettsia sibirica* 246
38	*Rickettsia australis* JC
39	*Rickettsia typhi* Wilmington
40	*Leptospira interrogans*
41	*Orientia tsutsugamushi* Kato
42	*Orientia tsutsugamushi* Karp
43	*Orientia tsutsugamushi* Gilliam

[a] MRSA, methicillin-resistant S. *aureus*.

strain as the template. The PCR conditions consisted of an initial denaturation at 94°C for 5 min and 39 cycles of 30 s at 94°C, 30 s at 56°C, and 1 min at 72°C, with a final extension of 10 min at 72°C. The amplified 622-bp product was cloned into the pGEM-T Easy vector using T/A cloning methods. The protocol for cloning of the human *GAPDH* gene was the same as the protocol for cloning of the 47-kDa gene, except different primers (Gint11 and Gint12; see below) were used and human genomic DNA was used as the template. The plasmid DNA was sent to Daejeon SolGent Co., Ltd., for sequencing.

Primers and probe. The primers and probes used in this study are summarized in Table 2. A diagram of the locations of the primers and probes is shown in Fig. 1. The probe OtsuPR665 and primers OtsuFP630 and OtsuRP747 were designed as described by Jiang et al. (5). The probe was labeled at the 5′ end with 6-carboxyfluorescein (FAM) and at the 3′ end with black hole quencher 1 (BHQ-1). All primers except the Q-PCR primers and probe were designed with the Basic Local Alignment Search Tool (BLAST) database search program and the Primer 3 program from the National Center for Biotechnology Information (NCBI) (8). The 47-kDa gene assay was compared with an assay based on the nucleotide sequences of the 56-kDa antigen of a Gilliam strain of *O. tsutsugamushi* (3). For molecular quantification of patients' white blood cells (WBCs), we used primers Gint21 and Gint22, from intron 2 of the GAPDH gene. The Gint23 probe was labeled at the 5′ end with FAM and at the 3′ end with BHQ-1.

PCRs. (i) C-PCR. Bacterial DNA was extracted using a QIAamp DNA minikit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and was used as the template for the PCR. The C-PCR targeting the 47-kDa gene was performed in 20-μl reaction volumes containing 10 μl of 2× Excel *Taq* premix (Corebiosystems, Seoul, South Korea), 2 μl of the template DNA, and 1 μl each (5 pmol) of the OtsuFP630 and OtsuRP747 primers. The PCR conditions consisted of denaturation at 94°C for 10 min, followed by 30 cycles, each consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. The final elongation step was prolonged for 7 min at 72°C. Additionally, the 56-kDa gene C-PCR was performed in 20-μl reaction volumes containing 2 μl of template DNA, 5 pmol of each primer (P10 and P11), and 10 μl of 2× Excel *Taq* premix (Corebiosystems). The PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 63°C for 1 min, and extension at 72°C for 1 min. The final elongation step was prolonged for 7 min at 72°C.

(ii) N-PCR. The first round of N-PCR for the amplification of the 47-kDa gene was performed under the same conditions used for the C-PCRs, except that the annealing step was at 56°C for 1 min and 10 pmol of each primer (OtsuFP555 and OtsuRP771) was used. The second round of N-PCR for the 47-kDa gene was performed using the first PCR product as the template DNA and 10 pmol/μl of each primer (OtsuFP630 and OtsuRP747). The second-round PCR conditions involved an initial denaturation at 94°C for 10 min, followed by 25 cycles, each consisting of denaturation at 94°C for 30s, annealing at 60°C for 30s, and extension at 72°C for 1 min, with a final extension for 7 min at 72°C.

The first round of N-PCR for the 56-kDa gene was performed under the same conditions used for the C-PCRs, except the annealing step was performed at 61°C and 5 pmol/μl of each primer (P34 and P55) was used. The second round of N-PCR for the 56-kDa gene was performed using the first-round PCR product as the template DNA and 5 pmol/μl of each primer (P10 and P11). The second-round PCR conditions involved an initial denaturation at 94°C for 5 min and 30 cycles of 30 s at 94°C, 30 s at 63°C, and 1 min at 72°C, with a final extension for 7 min at 72°C.

(iii) Q-PCR. We performed the same protocols for Q-PCR of the *O. tsutsugamushi* 47-kDa and human *GAPDH* genes. Plasmid DNA of *O. tsutsugamushi* or human *GAPDH* was quantified with a spectrophotometer (DU 530 Life Science UV/visible spectrometer; Beckman Coulter). A standard curve was generated on the basis of serial dilutions of a net suspension of 1 μl of 10^8 copies/μl of plasmid DNA. Q-PCRs were run in 20-μl reaction volumes containing 5 μl of DNA, 1 μl (5 pmol/μl) of each primer, 1 μl of probe at 2 pmol/μl, 4 μl of 5× master mix (reaction buffer, Fast Start *Taq* DNA polymerase, $MgCl_2$ and deoxynucleoside triphosphates [with dUTP instead of dTTP]), and distilled water (D/W). The PCR conditions consisted of an initial activation at 95°C for 10 min, followed by 45 cycles of 10 s at 95°C and 30 s at 60°C. The second derivative (crossing point [Cp]), which calculates the fractional cycle where the Cp of the Q-PCR fluorescence intensity curve reaches its maximum value, was utilized for the assay analysis. The data for the Q-PCR were analyzed using the LightCycler software program (version 4.0). The *O. tsutsugamushi* data in ng/μl were then converted to numbers of copies/μl.

Detection sensitivity. We compared the detection sensitivities of the three techniques using plasmid DNA (plasmid detection sensitivity) and genomic DNA (genomic detection sensitivity). For plasmid detection sensitivity, we performed Q-PCR, N-PCR, and C-PCR with serial dilutions of plasmid DNA from 5×10^8 copies/μl to 5×10^{-3} copies/μl using sterile D/W. For genomic detection sensitivity, we performed Q-PCR, N-PCR, and C-PCR with serial dilutions of genomic DNA from undiluted to a 64-fold dilution. For serial dilutions of genomic DNA from the buffy coat of an *O. tsutsugamushi*-infected patient, we used genomic DNA from a buffy coat sample of whole blood from an individual not infected with *O. tsutsugamushi*.

Relative quantification of *O. tsutsugamushi* in whole blood. For relative quantification of *O. tsutsugamushi* bacteria per volume of whole blood, we performed real-time DNA PCR analysis of the human *GAPDH* gene, along with the *O. tsutsugamushi* 47-kDa gene. Each of the diluted genomic DNA samples (undiluted to 64-fold dilution) with different concentrations of *O. tsutsugamushi* was used for Q-PCR to quantify the dosages of the *O. tsutsugamushi* 47-kDa gene and human *GAPDH* gene in two separate tubes. We calculated the relative quantity of *O. tsutsugamushi* in patients' whole blood using the genomic equivalent (GE) of *GAPDH* and WBC counts.

TABLE 2. Oligonucleotide primers and a probe used in this study and PCR conditions

Primer or probe name	Sequence	Amplicon size (bp)	Reference	PCR assay/function
OtsuFPS622	5′-GAAGTGTTCTTAGGTTCTGGGGTTATC-3′	622		Cloning primer
OtsuRPS622	5′-CTTTTATAACTTCAGTTATTAGAACTCC-3′			
OtsuFP555	5′-TCCTTTCGGTTTAAGAGGAACA-3′	238		47-kDa N-PCR external primer
OtsuRP771	5′-GCATTCAACTGCTTCAAGTACA-3′			
OtsuPR665 (probe)	5′-FAM-TGGGTAGCTTTGGTGGACCGATGTTTAATCT-BHQ1-3′		5	47-kDa Q-PCR probe
OtsuFP630	5′-AACTGATTTTATTCAACTAATGCTGCT-3′	118	5	47-kDa Q-PCR, C-PCR, and N-PCR internal primer
OtsuRP747	5′-TATGCCTGAGTAAGATACRTGAATRGAATT-3′		5	
P34	5′-TCAAGCTTATTGCTAGATCTGC-3′	1,003	25	56-kDa N-PCR external primer
P55	5′-AGGGATCCCTGCTGCTGTGCTTGCTGCG-3′		25	
P10	5′-GATCAAGCTTCCTCAGCCTACTATAATGCC-3′	483	25	56 kDa
P11	5′-CTAGGGATCCCGACAGATGCACTATTAGGC-3′	455	25	
Gint11	5′-TAAGTGCATGTGTGTGGGGAGA-3′			C-PCR
Gint12	5′-CCGGGTGATGCTTTTCCTAGAT-3′			N-PCR internal primer
Gint21	5′-GTTTATGGAGGTCCTCTTGTGTC-3′	90		*GAPDH* cloning primer
Gint22	5′-ACTACCCATGACTCAGCTTCTC-3′			*GAPDH* Q-PCR
Gint23 (probe)	5′-FAM-ACCATGCCACAGCCACCACACCT-BHQ1-3′			

Specificity test. For the specificity test, bacterial cells grown on brain heart infusion broth and LB agar were suspended in phosphate-buffered saline (PBS; pH 7.4) to 1×10^8 CFU/ml. Bacterial DNA was extracted from 200 μl of the suspension, using the QIAamp DNA minikit (Qiagen), following the manufacturer's instructions.

Patient selection. Specimens from patients aged 18 years or older who visited Chosun University Hospital within 4 weeks of fever onset had been collected between 2005 and 2007 and stored (6, 7). Scrub typhus was defined as a 4-fold or greater increase in antibody titers by IFA. Non-scrub typhus specimens were selected on the basis of negative results by culture and peripheral blood smear and the absence of IFA antibodies against *O. tsutsugamushi*. Infectious disease specialists randomly selected 41 patients with scrub typhus and 52 patients without scrub typhus and sent blood samples to our laboratory. The blood buffy coat specimens were processed and tested blindly by the PCR assays for the 56-kDa and 47-kDa genes of *O. tsutsugamushi*.

Statistical analysis. The sensitivities and specificities of the PCR assays were analyzed using the MedCalc software program (Mariakerke, Belgium) (20). Statistical significance was bestowed on data with P values of <0.05. A 4-fold or more increase in antibody titer against *O. tsutsugamushi*, as measured by IFA, was used to evaluate sensitivity and specificity under the receiver operator characteristic (ROC) curve. The diagnostic accuracies of the three PCR assays were compared using the MedCalc software program (20).

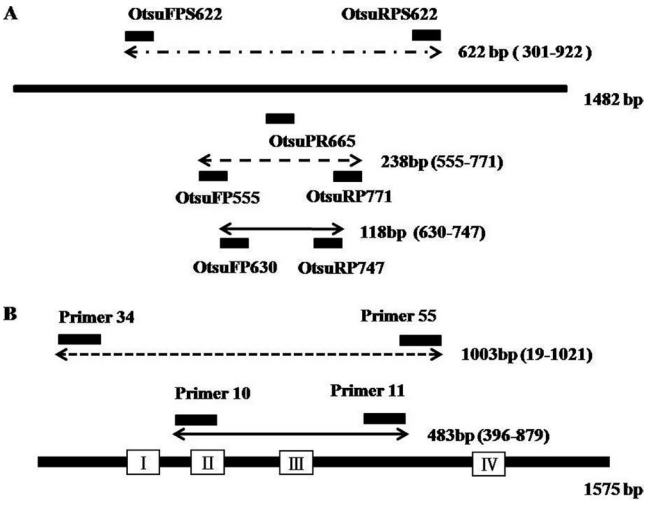

FIG. 1. (A) Locations of the selected primers and probes in the 47-kDa outer membrane protein gene (strain Kato, GenBank accession number L11697). External primers OtsuFR555 and OtsuRP771 were used to amplify a 238-bp segment of the *O. tsutsugamushi*-specific 47-kDa gene, and internal primers OtsuFP630 and OtsuRP747 were used to amplify a 118-bp segment. The probe OtsuPR665 was used for Q-PCR. (B) Locations of the selected primers in the 56-kDa outer membrane protein gene (strain Gilliam, GenBank accession number L31933). External primers p34 and p55 were used to amplify a 1,003-bp segment of the *O. tsutsugamushi*-specific 56-kDa gene, and internal primers p10 and p11 were used to amplify an internal 483-bp segment. The open reading frame of the 56-kDa gene is represented by a heavy line, and boxes I, II, III, and IV indicate the variable domains.

RESULTS

Detection sensitivity. The plasmid detection sensitivities of C-PCR, N-PCR, and Q-PCR were 5×10^4 copies/μl, 5 copies/μl, and 50 copies/μl, respectively (data not shown). However, in the detection sensitivity system using genomic DNA, the detection sensitivities of N-PCR and Q-PCR were 64-fold and 16-fold, respectively (Cp, 37.7; 426 copies/μl of genomic DNA from a buffy coat sample), but C-PCR could not detect the 47-kDa gene of *O. tsutsugamushi* in undiluted genomic DNA from a buffy coat sample (Fig. 2).

Relative quantification of *O. tsutsugamushi* in whole blood. At the 16-fold dilution, the copy number and GE of *GAPDH* were 1.1×10^5 copies/μl (Cp, 22.64) and 5.5×10^4 GEs/μl, respectively. Therefore, the relative concentration of *O. tsutsugamushi* at a 16-fold dilution was 0.0078 organism/one WBC cell and 117 organisms/μl of whole blood, because the WBC count of the patient was 1.5×10^4 cells/μl of whole blood.

Detection specificity. The detection specificities of the 47-kDa and 56-kDa PCR assays were assessed using various rickettsial DNAs and various other bacterial DNAs. C-PCR and N-PCR of the type strains did not give rise to any bands other than those for *O. tsutsugamushi* (data not shown). Q-PCR showed high Cp values (>30) for all strains except the *O. tsutsugamushi* Gilliam (Cp, 20.09), Karp (Cp, 23.22), and Kato (Cp, 23.02) strains (data not shown).

Sensitivity and specificity using patient blood samples. In scrub typhus patients, C-PCR targeting the 47-kDa gene gave a positivity rate of 7% (3/41 samples). In contrast, N-PCR

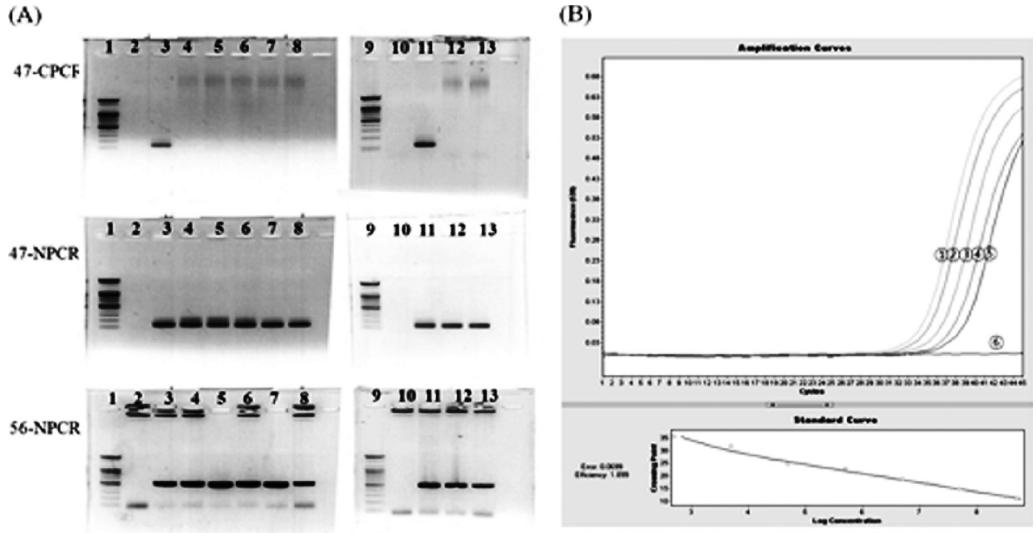

FIG. 2. (A) Detection sensitivities of C-PCR and N-PCR. Lane 1, 100-bp ladder marker (Bioneer); lane 2, negative control (sterile distilled water); lane 3, positive-control strain Karp; lane 4, undiluted genomic DNA; lane 5 to lane 8, from 2-fold-diluted genomic DNA to 16-fold-diluted genomic DNA, respectively; lane 9, 100-bp ladder marker (Bioneer); lane 10, sterile D/W; lane 11, positive-control strain Karp; lane 12 and lane 13, from 32-fold-diluted genomic DNA and 64-fold-diluted genomic DNA, respectively; 47-kDa C-PCR (47-CPCR) and 47-kDa N-PCR (47-NPCR) target size, 118 bp; 56-kDa N-PCR (56-NPCR) target size, 483 bp. (B) Detection sensitivities of Q-PCR. Circle 1, undiluted genomic DNA (Cp, 33.30); circle 2, 2-fold-diluted genomic DNA (Cp, 34.01); circle 3, 4-fold-diluted genomic DNA (Cp, 35.21); circle 4, 8-fold diluted genomic DNA (Cp, 36.19); circle 5, 16-fold-diluted genomic DNA (Cp, 37.47); circle 6, D/W. The results of Q-PCR using 32-fold- and 64-fold-diluted genomic DNA were negative (Cp, 39.36 and >40.00, respectively) due to use of a cutoff value of >38 (data not shown).

targeting the same gene gave a positivity rate of 85% (35/41). However, none of the non-scrub typhus patients showed positivity by either C-PCR or N-PCR assay. If we adopted a negative-cutoff value of 38 Cp for Q-PCR using patients' blood (8, 15–17), the Q-PCR yielded positivity rates of 83% (34/41) in patients with scrub typhus and 0% (0/52) in those without scrub typhus. Although C-PCR targeting the 56-kDa gene was not positive for any of the 41 patients with scrub typhus, N-PCR yielded a positivity rate of 87.8% (36/41). Neither C-PCR nor N-PCR was positive for patients without scrub typhus.

Comparison of the diagnostic accuracies of the three assays in patients with scrub typhus. In the scrub typhus patients, the C-PCR for the 47-kDa gene had a sensitivity of 7.3% (95% confidence interval [CI], 1.6 to 19.9) and a specificity of 100% (95% CI, 87.9 to 100). In contrast, the N-PCR had a sensitivity of 85.4% (95% CI, 70.8 to 94.4) and a specificity of 100% (95% CI, 93.1 to 100). If we adopted a negative-cutoff value of 38 Cp for the Q-PCR, it had a sensitivity of 82.9% (95% CI, 67.9 to 92.8) and a specificity of 100% (95% CI, 93.1 to 100).

The areas under the curve (AUCs) for the 47-kDa N-PCR and Q-PCR were 0.93 (95% CI, 0.84 to 0.98; $P = 0.743$) and 0.92 (95% CI, 0.82 to 0.97), respectively. However, the AUC of C-PCR for the 47-kDa gene was 0.54 (95% CI, 0.41 to 0.66), which was significantly different from the AUCs of Q-PCR and N-PCR (Q-PCR versus C-PCR, $P < 0.001$; N-PCR versus C-PCR, $P < 0.001$). On the other hand, the 56-kDa C-PCR had a sensitivity of 0% (95% CI, 0 to 8.7), a specificity of 100% (95% CI, 93.1 to 100), and an AUC of 0.55 (95% CI, 0.44 to 0.65), which were not significantly different from those of the 47-kDa C-PCR (47-kDa C-PCR versus 56-kDa C-PCR, $P = 0.67$). The 56-kDa N-PCR had a sensitivity of 87.8% (95% CI, 73.8 to 95.9), a specificity of 100% (95% CI, 93.1 to 100), and an AUC of 0.94 (95% CI, 0.87 to 0.98), which, again, were not statistically different from those of the 47-kDa N-PCR (47-kDa N-PCR versus 56-kDa N-PCR, $P = 0.67$).

DISCUSSION

Scrub typhus runs a mild clinical course and responds well to proper antibiotic therapy. However, in patients with a delayed diagnosis, it may cause fatal complications (21). Thus, a rapid diagnosis of scrub typhus is essential for successful treatment. PCR assays have been widely used for rapid identification of fastidious organisms or rickettsiae that are difficult to cultivate. Murai et al. (13) have reported that C-PCR requires 20 ng of DNA for detection. As N-PCR requires only 200 pg of DNA, N-PCR is 100 times more sensitive than C-PCR. Q-PCR is useful for rapid diagnosis of scrub typhus because it takes only 2 h and it is helpful in evaluating the response to treatment and clinical outcome. However, Q-PCR has the disadvantage of high financial cost. N-PCR also has some disadvantages: it requires more time than Q-PCR or C-PCR, and there is a higher risk of spurious results due to DNA contamination (12). There have been few studies comparing the diagnostic accuracies of C-PCR, N-PCR, and Q-PCR against the same target gene specific for *O. tsutsugamushi*. We selected the 47-kDa gene, which encodes an outer membrane protein/antigen also known as the high-temperature-requirement A protein (HtrA), as the target DNA, since this was the only gene for which well-verified real-time PCR results were available (5). The *htrA* gene is induced by environmental stress, such as high temperature, and proteins of this family from *Escherichia coli*, *Rickettsia prowazekii*, *Haemophilus influenzae*,

Brucella abortus, and even eukaryotic organisms, including humans, are known (9).

Primers OtsuFP630 and OtsuRP747 were designed on the basis of the 47-kDa gene sequence, which is conserved in the Karp, Kato, Gilliam, Boryong, and TH187 strains of *O. tsutsugamushi*. The sequence is not detected in the *htrA* genes of other genera related to *Rickettsia* or its human homologues. For Q-PCR, the Cp values adopted for cultured organisms are usually lower than those adopted for clinical specimens, such as blood, tissue, fluid, and biopsy samples (15–17). Generally, a Cp value of >30 or >28 is regarded as a negative result for Q-PCR of cultured organisms, whereas a Cp value of >40 or >38 is regarded as a negative result for Q-PCR of clinical specimens, such as blood, stool, and biopsy samples (8, 19). We adopted a Cp value of >38 as a negative outcome for Q-PCR of blood and a cutoff Cp value of >30 for Q-PCR of cultured bacterial and rickettsial isolates. The 47-kDa gene cloned into plasmid DNA was detected at up to a dilution of 5×10^4 copies/μl by C-PCR and 5 copies/μl by N-PCR. Plasmid DNA could also be detected down to 50 copies/μl under the same conditions as the Q-PCR. However, plasmid gene copies are of little value, as these do not represent true sensitivity generalized to clinical samples. Therefore, we selected buffy coat samples from a scrub typhus patient to determine true detection sensitivities. The genomic detection sensitivity of Q-PCR targeting the 47-kDa gene was 16-fold (Cp, 37.7; 426 copies/μl of genomic DNA from a buffy coat using plasmid standard curves). N-PCR targeting the 47-kDa and 56-kDa genes showed similar sensitivities, with detection at up to a 64-fold dilution (Fig. 2A and B).

For relative quantification of *O. tsutsugamushi* bacteria per volume of whole blood, we also performed real-time DNA PCR analysis of the human *GAPDH* gene, along with the *O. tsutsugamushi* 47-kDa gene. The general idea of this analysis was induced from enumeration of the malaria parasite density in peripheral blood smears using the WBC count as a reference (24). The genomic detection sensitivity for relative quantification using the WBC count (1.5×10^4 cells/μl) as a reference was 117 organisms/μl of whole blood. In clinical practice, for patients with scrub typhus, C-PCR and N-PCR targeting the 47-kDa gene gave positivity rates of 7% (3/41) and 85% (35/41), respectively. According to our detection sensitivity data and comparison of the results of molecular assays using patient blood samples, this study showed that N-PCR is much more sensitive than C-PCR for the diagnosis of scrub typhus. If we adopted a negative-cutoff value of >38 Cp for Q-PCR using patients' blood, Q-PCR had 83% (34/41) positivity for scrub typhus patients and 0% (0/52) for non-scrub typhus patients. N-PCR and Q-PCR targeting the 47-kDa gene gave false-negative results for 6 and 7 out of the 41 patients with scrub typhus, respectively (data not shown). If we exclude one patient who was positive for N-PCR but negative for Q-PCR, the results of N-PCR were concordant with those of Q-PCR in all patients (data not shown). It is generally recognized that the 56-kDa protein is the major cell membrane antigen of *O. tsutsugamushi* and the major immunodominant antigen. It has been widely used to diagnose scrub typhus because it contains both group-specific and type-specific epitopes (14).

Since the 56-kDa gene is used much more frequently in clinical practice than the 47-kDa gene, we also compared the C-PCR and N-PCR results for the 47-kDa gene with those for the 56-kDa gene. C-PCR targeting the 56-kDa gene was negative for all 41 patients with scrub typhus; however, N-PCR for the same target gene showed a positivity rate of 87.8%. The results of the C-PCR and N-PCR assays for the 56-kDa gene were not significantly different from those for the 47-kDa gene.

In conclusion, Q-PCR and N-PCR targeting the *O. tsutsugamushi*-specific 47-kDa gene are more sensitive than C-PCR for detection of the organism in patients with suspected scrub typhus. In particular, Q-PCR is the assay of choice, as amplicon containment is easier to achieve and the rapid turnover of the assay gives it the edge over the other assays.

ACKNOWLEDGMENT

We do not have any commercial interest or other association that might pose a conflict of interest.

REFERENCES

1. **Blacksell, S. D., N. J. Bryant, D. H. Paris, J. A. Doust, Y. Sakoda, and N. P. Day.** 2007. Scrub typhus serologic testing with the indirect immunofluorescence method as a diagnostic gold standard: a lack of consensus leads to a lot of confusion. Clin. Infect. Dis. **44:**391–401.
2. **Bozeman, G. W., and B. L. Elisberg.** 1963. Serological diagnosis of scrub typhus by indirect immunofluorescence. Proc. Soc. Exp. Biol. Med. **112:**568–573.
3. **Furuya, Y., Y. Yoshida, T. Katayama, F. Kawamori, S. Yamamoto, N. Ohashi, A. Tamura, and A. Kawamura.** 1991. Specific amplification of *Rickettsia tsutsugamushi* DNA from clinical specimens by polymerase chain reaction. J. Clin. Microbiol. **29:**2628–2630.
4. **Horinouchi, H., K. Murai, A. Okayama, Y. Nagatomo, N. Tachibana, and H. Tsubouchi.** 1996. Genotypic identification of *Rickettsia tsutsugamushi* by restriction fragment length polymorphism analysis of DNA amplified by the polymerase chain reaction. Am. J. Trop. Med. Hyg. **54:**647–651.
5. **Jiang, J., T. C. Chan, J. J. Temenak, G. A. Dasch, W. M. Ching, and A. L. Richards.** 2004. Development of a quantitative real-time polymerase chain reaction assay specific for Orientia tsutsugamushi. Am. J. Trop. Med. Hyg. **70:**351–356.
6. **Kim, D.-M., and J. N. Byun.** 2008. Effects of antibiotic treatment on the results of nested PCRs for scrub typhus. J. Clin. Microbiol. **46:**3465–3466.
7. **Kim, D. M., K. D. Yu, J. H. Lee, H. K. Kim, and S. H. Lee.** 2007. Controlled trial of a 5-day course of telithromycin versus doxycycline for treatment of mild to moderate scrub typhus. Antimicrob. Agents Chemother. **51:**2011–2015.
8. **Kim, H. S., D. M. Kim, G. P. Neupane, Y. M. Lee, N. W. Yang, S. J. Jang, S. I. Jung, K. H. Park, H. R. Park, C. S. Lee, and S. H. Lee.** 2008. Comparison of conventional, nested, and real-time PCR assays for a rapid and accurate detection of Vibrio vulnificus. J. Clin. Microbiol. **46:**2992–2998.
9. **Kim, J. H., and M. J. Hahn.** 2000. Cloning and sequencing of the gene encoding the candidate HtrA of *Rickettsia typhi*. Microb. Immunol. **44:**275–278.
10. **Kim, S. J., I. K. Chung, I. S. Chung, D. H. Song, S. H. Park, H. S. Kim, and M. H. Lee.** 2000. The clinical significance of upper gastrointestinal endoscopy in gastrointestinal vasculitis related to scrub typhus. Endoscopy **32:**950–955.
11. Reference deleted.
12. **Lee, S. E., S. Y. Kim, S. J. Kim, H. S. Kim, J. H. Shin, S. H. Choi, S. S. Chung, and J. H. Rhee.** 1998. Direct identification of *Vibrio vulnificus* in clinical specimens by nested PCR. J. Clin. Microbiol. **36:**2887–2892.
13. **Murai, K., N. Tachibana, A. Okayama, E. Shishime, K. Tsuda, and T. Oshikawa.** 1992. Sensitivity of polymerase chain reaction assay for *Rickettsia tsutsugamushi* in patients' blood samples. Microbiol. Immunol. **36:**1145–1153.
14. **Ohashi, N., H. Nashimoto, H. Ikeda, and A. Tamura.** 1992. Diversity of immunodominant 56-kDa type-specific antigen (TSA) of Rickettsia tsutsugamushi. Sequence and comparative analyses of the genes encoding TSA homologues from four antigenic variants. J. Biol. Chem. **267:**12728–12735.
15. **Perellea, S., M. Josefsenb, J. Hoorfarb, F. Dilassera, J. Grouta, and P. Fach.** 2004. A LightCycler real-time PCR hybridization probe assay for detecting food-borne thermophilic Campylobacter. Mol. Cell. Probes **18:**321–327.
16. **Poppert, S., A. Essig, B. Stoehr, A. Steingruber, B. Wirths, S. Juretschko, U. Reischl, and N. Wellinghausen.** 2005. Rapid diagnosis of bacterial meningitis by real-time PCR and fluorescence in situ hybridization. J. Clin. Microbiol. **43:**3390–3397.
17. **Rodríguez-Lázaroa, D., M. Hernándezb, T. Esteveb, J. Hoorfarc, and M. Pla.** 2003. A rapid and direct real time PCR-based method for identification of Salmonella spp. J. Microbiol. Methods **54:**381–390.

18. **Saini, R., J. C. Pui, and S. Burgin.** 2004. Rickettsialpox: report of three cases and a review. J. Am. Acad. Dermatol. **51:**S137–S142.
19. **Schabereiter-Gurtner, C., A. M. Hirschl, B. Dragosics, P. Hufnagl, S. Puz, Z. Kovách, M. Rotter, and A. Makristathis.** 2004. Novel real-time PCR assay for detection of Helicobacter pylori infection and simultaneous clarithromycin susceptibility testing of stool and biopsy specimens. J. Clin. Microbiol. **42:**4512–4518.
20. **Shidham, V., D. Gupta, L. M. Galindo, M. Haber, C. Grotkowski, P. Edmonds, S. J. Subichin, V. George, and J. England.** 2000. Intraoperative scrape cytology: comparison with frozen sections, using receiver operating characteristic (ROC) curve. Diagn. Cytopathol. **23:**134–139.
21. **Silpapojakul, K., S. Chupuppakarn, S. Yuthasompob, B. Varachit, D. Chaipak, T. Borkerd, and K. Silpapojakul.** 1991. Scrub and murine typhus in children with obscure fever in the tropics. Pediatr. Infect. Dis. J. **10:**200–203.
22. **Thap, L. C., W. Supanaranond, S. Treeprasertsuk, S. Kitvatanachai, S. Chinprasatsak, and B. Phonrat.** 2002. Septic shock secondary to scrub typhus: characteristics and complications. Southeast Asian J. Trop. Med. Public Health **33:**780–786.
23. **Tsay, R. W., and F. Y. Chang.** 1998. Serious complications in scrub typhus. J. Microbiol. Immunol. Infect. **31:**240–244.
24. **Warhurst, D. C., and J. E. Williams.** 1996. ACP broadsheet no 148. July 1996. Laboratory diagnosis of malaria. J. Clin. Pathol. **49:**533–538.
25. **Yen, T. H., C. T. Chang, J. L. Lin, J. R. Jiang, and K. F. Lee.** 2003. Scrub typhus: a frequently overlooked cause of acute renal failure. Ren. Fail. **25:**397–410.