

## Original Article

# Evaluation of Diagnostic Assay for Rickettsioses Using Duplex Real-Time PCR in Multiple Laboratories in Japan

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**SUMMARY:** Tsutsugamushi disease and Japanese spotted fever are representative rickettsioses in Japan, and are caused by infection with *Orientia tsutsugamushi* and *Rickettsia japonica*, respectively. For molecular-based diagnosis, conventional PCR assays, which independently amplify respective rickettsial DNA, are usually used; however, this approach is time-consuming. Here, we describe a new duplex real-time PCR assay for the simultaneous detection of *O. tsutsugamushi* and spotted fever group rickettsiae, and its evaluation using several PCR conditions in 6 public health laboratories. The detection limit of the assay was estimated to be 10<sup>2</sup> copies and the sensitivity was almost identical to that of 3 conventional PCR methods. A total of 317 febrile patients were selected as clinically suspected or confirmed cases of rickettsioses. The detection efficiency of this assay for *O. tsutsugamushi* from blood or skin (eschar) specimens appeared to be almost the same as that of the conventional PCR method, even when performed in different laboratories, whereas the efficiency for spotted fever group rickettsiae tended to be higher than that of the 2 traditional double PCR assays. Our duplex real-time PCR is thus a powerful tool for the rapid diagnosis of rickettsioses, especially at the acute stage of infection.

## INTRODUCTION

Tsutsugamushi disease (TD) and Japanese spotted fever (JSF) are rickettsioses and arthropod-borne infectious diseases; most of TD/JSF patients present with high fever with skin rash (1). TD is caused by infection with *Orientia tsutsugamushi*, and 6 *Orientia* serotypes (Gilliam, Karp, Kato, Irie/Kawasaki, Hirano/Kuroki, and Shimokoshi) are recognized as human pathogens in Japan (2). JSF, caused by infection with *Rickettsia japonica*, which was first reported in 1984, is the most prevalent spotted fever group (SFG) rickettsiosis; however, another SFG rickettsiosis caused by *R. heilongjiangensis* infection has recently been confirmed in Japan (3,4). *O. tsutsugamushi* is maintained in mites of the

*Leptotrombidium* genus in nature, whereas it is suspected that *R. japonica* and *R. heilongjiangensis* are transmitted by ticks of the *Haemaphysalis*, *Dermacentor*, or *Ixodes* genus (4–8). *O. tsutsugamushi* and SFG rickettsia infections result in severe symptoms with high fever, and can become fatal if suitable antibiotic treatment is delayed in patients. Usually, conventional PCR for molecular-based diagnosis are separately performed for the detection of *O. tsutsugamushi* and *R. japonica* (6,9–11), which is time-consuming and intricate. Additionally, it is sometimes difficult to distinguish positive and negative test results, based on the emergence of extra amplicons in conventional PCR tests. In this study, we developed a new diagnostic assay using duplex real-time TaqMan PCR for the simultaneous detection of *O. tsutsugamushi* and SFG rickettsiae (designated Ot-Rj-duplex real-time PCR), and its performance was evaluated using different PCR conditions in 6 public health laboratories of Japan.

## MATERIALS AND METHODS

**Primer and probe design:** The primers for Ot-Rj-duplex real-time PCR were designed based on the conserved regions of 16S rDNA sequences from *O. tsutsugamushi* strains and several SFG rickettsiae, and produced a 120-bp DNA fragment through amplification (Table 1). The TaqMan probes for the specific detection

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Table 1. Primers and TaqMan probes used in this study

PCR assay	Gene target	Amplicon size (bp)	Primer (probe)	Sequence (5' to 3')	Reference
Duplex TaqMan PCR for <i>O. tsutsugamushi</i> and <i>R. japonica</i> (Ot-Rj-duplex real-time PCR)	16S rDNA	120	OR-F OR-R (Ot-FAM) (Rj-VIC)	GGAGCATGCGGTTTAATTCG GCCATGCAACACCTGTGTGT FAM-AATGGAGACATTTTCTTC-MGB VIC-CGGATCGCAGAGATG-MGB	This study
<i>O. tsutsugamushi</i> nested PCR (Ot-nested PCR)	56 kDa	1,003–1,030 481–507	34' 55' 10' 11'	ATTGCTAGTGCAATGTCTGC CTGCTGCTGTGCTTGCTGCG CCTCAGCCTACTATAATGCC CGACAGATGCACTATTAGGC	9
Real-time PCR for <i>R. japonica</i> -related bacteria (Hanaoka Rj-FAM) <sup>1)</sup>	216 ORF (AB437281)	85	SpRija5' SpRija3' (SpRijaMGB)	GAACACGATGATACACCTCTGCAT GATTAGCCTCTGTCTTCAGTAGTATTTAACT FAM-TAGCGTCTATTCTAAGTAAAG-MGB	15
<i>R. japonica</i> double PCR (Rj-double PCR)	17 kDa	359	Rj5 Rj10	CGCCATTCTACGTTACTACC ATTCTAAAAACCATATACTG	10
Spotted fever group rickettsia double PCR (SFGR-double PCR)	17 kDa	537–540	R1 R2	TCAATTCACAACTTGCCATT TTTCAAAAATTCTAAAAACC	6

<sup>1)</sup> The assay can detect DNA specific for *R. japonica* and *R. heilongjiangensis* as described by Hanaoka et al. (15). The target gene used was 216-bp open reading frame (ORF, accession no. AB437281) specific for *R. japonica* and the closely-related rickettsia.

of *O. tsutsugamushi* and SFG rickettsial 16S rDNA were also designed and labeled with the fluorescent reporter FAM and VIC, respectively (Ot-FAM and Rj-VIC), at the 5' end; the nonfluorescent quenchers and minor groove binders (MGB) were labeled at the 3' end.

*Rickettsiales* bacteria were used for this study. *Rickettsiales* standards comprised 20 species including 31 strains as shown in Table 2. For DNA preparation, most rickettsiae were cultured with L929 cells according to the procedures of previous studies (2,6,9,10). For 2 *Rickettsia* strains, *R. prowazekii* and *R. rickettsia* antigen slides were obtained from Panbio Inc. (Sinnamon Park, Queensland, Australia) for DNA preparation. Additionally, *Anaplasma phagocytophilum* was propagated with HL60 cells (12), and *Ehrlichia chaffeensis* was cultured with DH82 cells (13). Spleen samples were obtained from mice infected with *E. muris* and *Ehrlichia* sp. HF (14). These infected cell-cultures and spleens were used for DNA preparation.

**Patients and clinical samples:** In total, 317 febrile patients (174 males and 143 females; average age, 63.2, range 1–90 years; average number of days after onset of fever 7.8, range 1–33) comprising suspected or confirmed cases of rickettsiosis in hospitals, from 1997 to 2016, were selected for this study. Clinical whole blood, blood clot, buffy coat, skin (eschar), and/or sera samples were obtained from these patients and used for DNA preparation or serological tests.

**DNA preparation:** Genomic DNA was extracted from *Rickettsiales* standards or clinical specimens using the QIAamp DNA mini kit (QIAGEN, Valencia, CA, USA) according to manufacturer's instructions. For blood clots, the specimens were homogenized using a BioMasher (Nippi Inc., Tokyo, Japan) prior to DNA extraction, and were then incubated with 100 units of streptokinase (WAKO Pure Chemical Industries Ltd., Osaka, Japan) at 37°C as described previously (12). For DNA preparation from antigen slides (15), briefly, we

added lysis buffer from the kit, supplemented with 0.1 mg/ml proteinase K, onto the slide surface coated with infected cells (300 µl/slide). After incubation at 37°C for 2 h, extraction steps were performed according to the manufacturer's instructions (Gentra Puregene kit, QIAGEN).

**Real-time and conventional PCR:** For Ot-Rj-duplex real-time PCR, the reaction mixture (25 µl) consisting of 0.3 µM of each primer, 0.2 µM of each MGB probe, 12.5 µl of Premix Ex *Taq*, 0.5 µl of ROX Reference Dye II (50 ×), and 2.5 µl of template DNA was prepared and amplified using an ABI 7500 real-time PCR system (Thermo Fisher Scientific Inc., Waltham, MA, USA). The thermal cycling protocol consisted of 1 cycle for 30 s at 95°C, followed by 45 cycles of 5 s at 95°C and 40 s at 62°C. The other real-time PCR conditions in respective public health laboratories are shown in Table 3. For assessing the detection limit of duplex real-time PCR, the assay was performed using the ABI 7500, Light Cycler 480 and Light Cycler Nano (Roche, Basel, Switzerland). The 140-bp target template DNA was synthesized in its entirety based on the 16S rDNA sequences of *O. tsutsugamushi* Gillam and *R. japonica* YH. Such synthetic templates included DNA regions that are amplified by real-time PCR, having additional 5'- and 3'-flanking sequences of CACAAGCGGT and TGTCGTCAGC, respectively, for *O. tsutsugamushi*, and having 5'- and 3'-flanking sequences of ACAAGCGGT and TGTCGTCAGCT, respectively, for *R. japonica*. The synthesized template DNA was quantified and serially diluted 10-fold ( $1.0 \times 10^{-2}$  –  $1.0 \times 10^4$  copies/well). Real-time PCR was conducted in triplicate 3 times, with these serial dilutions. Because the genomic 16S rDNA gene of *O. tsutsugamushi* and SFG rickettsiae exists as a single copy, the copy number of 16S rDNA genes corresponded to the bacterial genome copy number for each well. The number of positive wells was determined based on quantification cycle (Cq). When the target DNA was

# Diagnostic Assay for Rickettsioses Using Duplex Real-Time PCR

Table 2. Specificity of Ot-Rj-duplex real-time PCR in *Rickettsiales* bacteria

Species	Strain (serotype)	Source	Ot-Rj-duplex real-time PCR		Hanaoka Rj-FAM <sup>1)</sup>	Laboratory tested <sup>2)</sup>
			Ot-FAM	Rj-VIC		
<i>(Orientia)</i>						
<i>O. tsutsugamushi</i>	Gilliam	Human	Ot-FAM positive	Negative	Negative	SIEHS
	Karp	Human	Ot-FAM positive	Negative	Negative	SIEHS
	Kato	Human	Ot-FAM positive	Negative	Negative	SIEHS
	Irie/Kawasaki	Human	Ot-FAM positive	Negative	Negative	SIEHS
	Hirano/Kuroki	Human	Ot-FAM positive	Negative	Negative	SIEHS
	Shimokoshi	Human	Ot-FAM positive	Negative	Negative	SIEHS
	JP-2: Sato (Karp)	Human	Ot-FAM positive	Negative	Negative	SIEHS
	JG: Kasei (Gilliam)	<i>Apodemus speciosus</i>	Ot-FAM positive	Negative	Negative	SIEHS
<i>(Rickettsia)</i>						
<i>R. japonica</i>	YH	Human	Negative	Rj-VIC positive	Rj-FAM positive	SIEHS
	FT	Human	Negative	Rj-VIC positive	Rj-FAM positive	SIEHS
<i>R. heilongjiangensis</i>	CH8-1	<i>Haemaphysalis concinna</i>	Negative	Rj-VIC positive	Rj-FAM positive	NIID
<i>R. asiatica</i>	IO-1	<i>Ixodes ovatus</i>	Negative	Rj-VIC positive	Negative	SIEHS
	IO-46	<i>Ixodes ovatus</i>	Negative	Rj-VIC positive	Negative	NIID
<i>R. conorii</i>	Malish 7	Human	Negative	Rj-VIC positive	Negative	SIEHS
<i>R. helvetica</i>	IP-1	<i>Ixodes persulcatus</i>	Negative	Rj-VIC positive	Negative	SIEHS
	IC-1	<i>Ixodes columnae</i>	Negative	Rj-VIC positive	Negative	NIID
<i>R. honei</i>	TT-118	<i>Ixodes</i> sp.	Negative	Rj-VIC positive	Negative	SIEHS
<i>R. rickettsii</i>	Sheila Smith	Human	Negative	Rj-VIC positive	Negative	NIID
<i>R. sibirica</i>	246	Human	Negative	Rj-VIC positive	Negative	SIEHS
<i>R. tamurae</i>	AT-1	<i>Amblyomma testudinarium</i>	Negative	Rj-VIC positive	Negative	SIEHS
	HM-1	<i>Haemaphysalis megaspinosa</i>	Negative	Rj-VIC positive	Negative	NIID
<i>R. australis</i>			Negative	Rj-VIC positive	Negative	NIID
<i>Rickettsia</i> sp. LON	LON-2	<i>Haemaphysalis longicornis</i>	Negative	Rj-VIC positive	Negative	NIID
<i>R. monacensis</i> (formaly In-56)	IN-1	<i>Ixodes nipponensis</i>	Negative	Rj-VIC positive	Negative	NIID
<i>R. prowazekii</i>	brein1	Human	Negative	Negative	Negative	NIID
<i>R. typhi</i>	Wilmington	Human	Negative	Negative	Negative	SIEHS
<i>R. canadensis</i>	FLA-2	<i>Haemaphysalis flava</i>	Negative	Negative	Negative	NIID
<i>Anaplasma phagocytophilum</i>	HZ	Human	Negative	Negative	Negative	SIEHS
<i>(Ehrlichia)</i>						
<i>E. chaffensis</i>	Arkansas	Human	Negative	Negative	Negative	SIEHS
<i>E. muris</i>	AS145 <sup>T</sup>	<i>Eothenomys kageus</i> (synonym of <i>E. simithii</i> )	Negative	Negative	Negative	SIEHS
<i>Ehrlichia</i> sp. HF ( <i>E. ovatus</i> )	HF565	<i>Ixodes ovatus</i>	Negative	Negative	Negative	SIEHS

<sup>1)</sup>: Real-time PCR specific for *R. japonica* and *R. heilongjiangensis* was previously reported by Hanaoka et al. (15).

<sup>2)</sup>: PCR conditions in respective laboratories are shown in Table 3.

SIEHS, Shizuoka Institute of Environment and Hygiene; NIID, National Institute of Infectious Diseases.

detected in all 3 independent wells, the lowest copy number (bacterial number) was considered the detection limit. The sensitivity of the duplex real-time PCR was further compared to that of 3 conventional PCR assays using the following procedure. Genomic DNA was prepared from *O. tsutsugamushi*-infected and *R. japonica*-infected L929 cells, 10-fold serially diluted, and used as templates. The duplex real-time PCR with serial dilutions was conducted using the ABI7500 real-time PCR system. The Ot-nested PCR for *O. tsutsugamushi*, and the 2 conventional PCR assays (Rj-double and SFGR-double PCRs) for SFG rickettsiae, with the same serial dilutions, were performed using respective primer pairs as shown in Table 1, using previously described procedures (6,9–11). The highest dilution (representing the lowest bacterial numbers) that was detected by real-time

PCR was compared to that obtained using 3 conventional PCR assays. The specificity of the real-time PCR was examined using 20 *Rickettsiales* spp. including 31 strains in respective laboratories, as shown in Table 2.

**Serological tests:** Indirect immunofluorescence assays (IFAs) or indirect immunoperoxidase assay (IPAs) using *O. tsutsugamushi*-infected and *R. japonica*-infected cells as antigens were conducted using patient sera as described previously (6,16). The serum samples were considered positive when antibody titers were greater than 40 for IFA and 80 for IPA, or when titers increased by 4-fold or more between paired sera based on seroconversion.

**Statistical analysis:** All statistical tests including sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy, Cohen's



Table 3. PCR conditions and instruments for Ot-Rj-duplex real-time PCR in 6 public health laboratories in Japan

Assay condition	Laboratory tested <sup>1)</sup>				
	NIID	SIEHS	FIPH	ARCPHE	HPTRI
Instrument	ABI7500 (Thermo Fisher)	ABI7500 (Thermo Fisher)	ABI StepOnePlus (Thermo Fisher)	LightCycler 480 (Roche)	LightCycler Nano (Roche)
Reagent	Premix Ex Taq (Perfect Real Time) (TAKARA BIO)	Premix Ex Taq (Perfect Real Time) (TAKARA BIO)	Premix Ex Taq (Perfect Real Time) (TAKARA BIO)	LightCycler 480 Probes Master (Roche)	Premix Ex Taq (Perfect Real Time) (TAKARA BIO)
PCR condition					
(1) Initial denaturation	(1) 95°C 30 s	(1) 95°C 30 s	(1) 95°C 30 s	(1) 95°C 1 min	(1) 95°C 30 s
(2) Denaturation	(2) 95°C 5 s	(2) 95°C 5 s	(2) 95°C 5 s	(2) 95°C 10 s	(2) 95°C 10 s
(3) Annealing/extension	(3) 62°C 40 s	(3) 62°C 40 s	(3) 62°C 40 s	(3) 62°C 30 s	(3) 62°C 35 s
(4) Cycles	(4) 45 cycles Manual	(4) 45 cycles	(4) 45 cycles Manual	(4) 45 cycles	(4) 45 cycles
Analysis setting	Threshold 0.2 Autobaseline	Autobaseline	Threshold 0.3 Autobaseline	Autobaseline	Automatic quantification

<sup>1)</sup>: NIID, National Institute of Infectious Diseases; SIEHS, Shizuoka Institute of Environment and Hygiene; FIPH, Fukushima Institute for Public Health; ARCPHE, Akita Research Center for Public Health and Environment; HPTRI, Hiroshima Prefectural Technology Research Institute.

kappa coefficient ( $\kappa$ ), and Fisher's exact test were performed using R, version 3.2.4 (R Foundation for Statistical Computing, Vienna, Austria).

**Ethics statement:** Experiments were performed in compliance with relevant laws and guidelines and in accordance with the ethical standards established by the Declaration of Helsinki. The study was approved by the Ethics Committee of the National Institute of Infectious Diseases, the University of Shizuoka, and respective Public Health Institutes.

## RESULTS

**Sensitivity and specificity:** Using 3 real-time PCR instruments, the detection limit of Ot-Rj-duplex real-time PCR was estimated to be  $1.0 \times 10^2$  copies/well for *O. tsutsugamushi* and *R. japonica*. Upon comparing the sensitivity to conventional PCR methods using the cultured rickettsial genome, real-time PCR and conventional PCR were able to detect approximately the same copy numbers ( $10^2$  copies/test). Regarding specificity, 8 strains of *O. tsutsugamushi* and 16 strains of SFG rickettsiae were detected by Ot-FAM and Rj-VIC, respectively, by real-time PCR, whereas other *Rickettsiales* bacteria of the typhus group (*R. prowazekii* and *R. typhi*), ancestral group (*R. canadensis*), and *A. phagocytophilum*, as well as 3 *Ehrlichia* spp., were not (Table 2). Another TaqMan PCR assay proposed by Hanaoka et al. (15) could also detect *R. japonica* and *R. heilongjiangensis*, but not *O. tsutsugamushi* and SFG rickettsiae other than those 2 species.

**Assay of clinical specimens:** A total of 317 febrile patients with rickettsiosis-like symptoms including 4 fatal infections were examined by Ot-Rj-duplex real-time PCR with blood samples and/or skin (eschar) specimens in respective laboratories (Table 4). In Akita and Fukushima prefectures, specimens from patients previously diagnosed with TD by conventional PCR and/or serological testing were designated confirmed cases for the evaluation of our duplex real-time PCR. In these 2 prefectures, only TD occurs, whereas JSF does not. By

duplex real-time PCR, *O. tsutsugamushi* 16S rDNA was detected from all or most patient samples tested (33/33, 100% in Akita and 64/67, 96% in Fukushima). In Shizuoka, samples from patients previously diagnosed with TD were also tested as confirmed cases, whereas samples from patients testing positive for JSF were examined as clinically suspected cases. In Wakayama and Miyazaki, specimens from all patients previously diagnosed with TD/JSF were tested as confirmed cases. Patients in Hiroshima were all examined as clinically suspected cases; therefore, duplex real-time PCR itself was used for TD/JSF diagnosis. As shown in Table 4, our real-time PCR could detect either *O. tsutsugamushi* or SFG rickettsiae from those patients in respective prefectures. We further compared the detection efficiency of our duplex real-time PCR method with that of conventional PCR assays. In 5 prefectures (Akita, Fukushima, Shizuoka, Wakayama, and Miyazaki), the detection efficiency of our real-time PCR for *O. tsutsugamushi* (129/178, 72%) appeared to be almost the same as that of Ot-nested PCR (132/178, 74%). In contrast, the efficiency of our assay for SFG rickettsiae in Wakayama and Miyazaki (16/50, 32%) tended to be higher than that of Rj-double PCR (7/50, 14%) and SFGR-double PCR (7/50, 14%; see subtotal in Table 4), suggesting that duplex real-time PCR has higher sensitivity for clinical samples. In Hiroshima, where examined samples included suspected rickettsiosis cases, the detection efficiency was approximately similar to that in other prefectures. We next compared the detection efficiency between blood and skin (eschar) samples. We selected 43 (TD) and 41 (JSF) patients from whom both blood and skin (eschar) samples were obtained; for these individuals, blood, skin (eschar), or both samples were Ot-FAM-positive or Rj-VIC-positive. For TD, 35/43 patients (81%) and 43/43 patients (100%) were positive in blood and skin (eschar) samples, respectively. For JSF, 27/41 patients (66%) and 39/41 patients (95%) were positive in blood and skin (eschar) specimens, respectively. Thus, *O. tsutsugamushi* (Fisher's exact test,  $p = 0.0055$ ) and SFG rickettsiae ( $p = 0.0015$ ) appeared

# Diagnostic Assay for Rickettsioses Using Duplex Real-Time PCR

Table 4. Detection assays of *O. tsutsugamushi* (Ot) and *R. japonica* (Rj) DNAs from febrile patients by Ot-Rj-duplex real-time PCR, conventional PCRs, and serological tests in 6-endemic prefectures of Japan, 1997–2016

Location (year)	Total nos. of febrile patients tested	Laboratory tested <sup>1)</sup>	Ot				Rj				Ot-Rj- duplex real-time PCR	Range of Cq value
			Ot-Rj- duplex real-time PCR	Conven- tional PCRs <sup>2)</sup>	Sero- logical test	Nos. of patients diag- nosed/ total patients tested (%) <sup>3)</sup>	Ot-Rj- duplex real-time PCR	Conventional PCRs <sup>2)</sup>	Sero- logical test	Nos. of patients diag- nosed/ total patients tested (%) <sup>3)</sup>		
			Nos. of positive/ patients tested (%)	Nos. of nested PCR positive/ patients tested (%)	Nos. of sero- positive/ patients tested (%)		Nos. of positive/ patients tested (%)	Nos. of double PCR positive/ patients tested (%)	Nos. of SFGR- double PCR positive/ patients tested (%)		Nos. of sero- positive/ patients tested (%)	
			(A) <sup>8)</sup>	(B)	(C)		(D)	(E)	(F)		(G)	
Akita (1997–2015)	33	ARCPHE	33/33 (100)	33/33 (100) <sup>4)</sup>	18/33 (55)	33/33 (100)	0/33	NT <sup>9)</sup>	NT	NT	0/33	24.4–37.7
Fukushima (2010–2013)	67	FIPH	64/67 (96)	67/67 (100) <sup>4)</sup>	67/67 (100)	67/67 (100)	0/67	NT	NT	NT	0/67	21.6–41.0
Shizuoka (2010–2016)	28	SIEHS	18/28 (64)	18/28 (64)	21/28 (75)	21/28 (75)	3/28 (11) <sup>7)</sup>	NT	NT	3/28 (11)	4/28 (14)	27.9–40.0
Wakayama (2008–2012)	22	SIEHS	3/22 (14)	3/22 (14)	3/22 (14)	3/22 (14)	9/22 (41)	5/22 (23)	5/22 (23)	12/22 (55)	12/22 (55)	21.3–40.1
Miyazaki (2008–2009)	28	SIEHS	11/28 (39)	11/28 (39)	11/28 (39)	11/28 (39)	7/28 (25)	2/28 (7)	2/28 (7)	9/28 (32)	9/28 (32)	22.6–41.0
Subtotal <sup>6)</sup>	178		129/178 (72)	132/178 (74)	120/178 (67)	135/178 (76)	16/50 (32)	7/50 (14)	7/50 (14)	21/50 (42)	21/50 (42)	
Hiroshima (2013–2015)	139	HPTRI	6/139 (4) <sup>7)</sup>	6/33 (18)	2/13 (15)	6/139 (4)	55/139 (40) <sup>7)</sup>	12/31 (39) <sup>5)</sup>	54/102 (53) <sup>5)</sup>	4/13 (31)	56/139 (40)	22.0–37.9
Total	317		135/317 (43)			141/317 (44)	74/317 (23)				81/317 (26)	

<sup>1)</sup>: PCR condition in each laboratory is shown in Table 3.

<sup>2)</sup>: Conventional PCRs were performed by the procedures of previous studies: Ot-nested PCR (9, 11), Rj-double PCR (10), and SFGR-double PCR (6).

<sup>3)</sup>: Final results of rickettsiosis determined by a combination of Ot-Rj-duplex real-time PCR, conventional PCRs, and/or serological tests (IFA or IPA).

<sup>4)</sup>: Ot-nested PCR was done by some modifications to improve the low detection efficiency of *O. tsutsugamushi* Shimokoshi strain as described previously (11). An additional designed primer (SH6: 5'-TAGTATCTGACTGCTTCTTATCCTTAGAG-3') was added in the reaction mixture for first-step PCR and the PCR was run with the 3 primers. An alternative degenerate primer (10 m2: 5'-CCDCCT CARCCTAMTATRATGCC-3') instead of 11' primer in Table 1 was used for second-step PCR.

<sup>5)</sup>: Rj and SFGR conventional PCRs in Hiroshima were modified and performed as follows. Outer primers were R1 and R2 in both PCRs. Inner primers were Rr17.61p (GCTCTTGCAACTTCTATGTT) and Rr17.492n (CATTGTTTCGTCAGGTTGGCG) instead of Rj5 and Rj10 under the same PCR condition. *R. japonica* infection was finally confirmed by direct sequencing of these amplicons.

<sup>6)</sup>: The subtotal for *O. tsutsugamushi* detection was obtained from confirmed cases in Akita, Fukushima, Shizuoka, Wakayama, and Miyazaki, while the subtotal for *R. japonica* detection was from confirmed cases in Wakayama and Miyazaki. These subtotals were used for statistical analysis in Table 5.

<sup>7)</sup>: Ot-Rj-duplex real-time PCR was used for diagnosis in clinically suspected cases of rickettsioses.

<sup>8)</sup>: (A) to (G) were designated for Table 5.

<sup>9)</sup>: Not tested.

Table 5. Comparison of Ot-Rj-duplex real-time PCR and other diagnostic tests for rickettsioses

Statistics	Ot ( <i>n</i> = 178)			Rj ( <i>n</i> = 50)		
	(A)/(B) <sup>1)</sup>	(A)/(B) + (C)	(A) + (C)/(B) + (C)	(D)/(E or F)	(D)/(E or F) + (G)	(D) + (G)/(E or F) + (G)
Sensitivity	0.977	0.956	1.000	1.000	0.762	1.000
Specificity	0.939	0.878	1.000	1.000	0.850	1.000
PPV <sup>2)</sup>	1.000	1.000	1.000	0.438	1.000	1.000
NPV <sup>3)</sup>	1.000	1.000	1.000	0.790	1.000	1.000
Accuracy	0.983	0.966	1.000	0.820	0.900	1.000
$\kappa$ <sup>4)</sup>	0.957	0.912	1.000	0.514	0.788	1.000

<sup>1)</sup>: (A) to (G) correspond to those in Table 4.

<sup>2)</sup>: Positive predictive value.

<sup>3)</sup>: Negative predictive value.

<sup>4)</sup>: Cohen's kappa coefficient:  $0 < \kappa \leq 0.2$  slight,  $-0.2 < \kappa \leq 0.4$  fair,  $-0.4 < \kappa \leq 0.6$  moderate,  $0.6 < \kappa \leq 0.8$  substantial,  $0.8 < \kappa \leq 1.0$  almost perfect agreement.

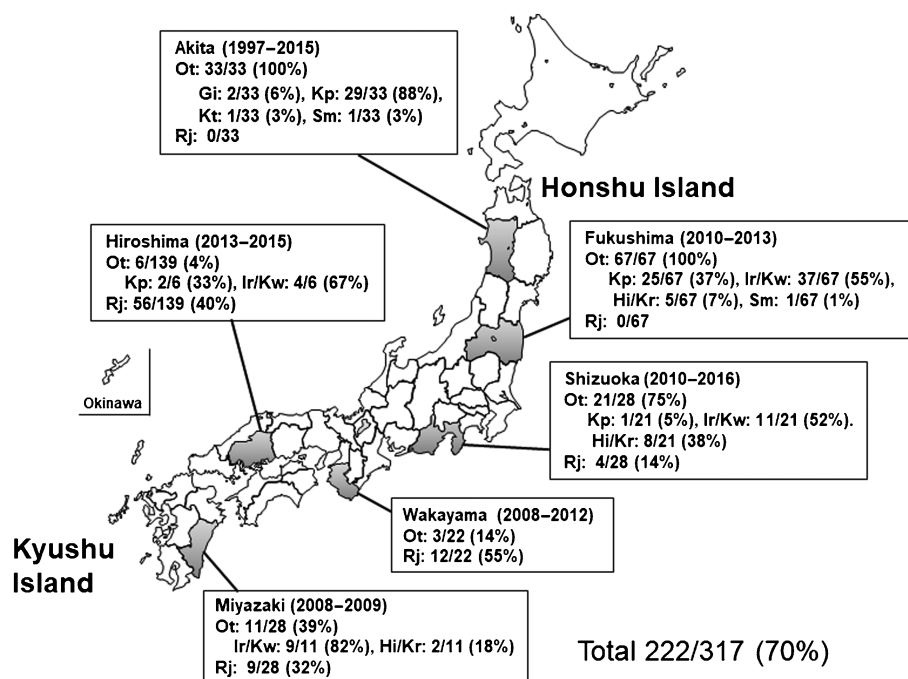


Fig. 1. Geographic representation of locations where febrile patients with rickettsiosis-like symptoms were genetically and serologically tested in 1997–2016. Ot, *O. tsutsugamushi* infection; Rj, *R. japonica* infection. Numbers indicate nos. of Ot-patients or Rj-patients diagnosed by the combination test per nos. of total febrile patients examined. Serotypes of *O. tsutsugamushi* were determined by serological tests or direct-sequencing analysis of Ot-nested PCR products (9); Gi, Gilliam; Kp, Karp; Kt, Kato; Ir/Kw, Irie/Kawasaki; Hi/Kr, Hirano/Kuroki; Sm, Shimokoshi. The ratio of respective rickettsiosis and years tested in each area are shown in parenthesis.

to be more detectable in skin (eschar) than in blood samples.

**Evaluation of the duplex real-time PCR:** Statistical comparisons between our duplex real-time PCR and other diagnostic tests for rickettsioses are shown in Table 5. For *O. tsutsugamushi* detection, the comparison between Ot-real-time PCR (A) and Ot-nested PCR (B), between (A) and (B) + serological test (C), and between (A) + (C) and (B) + (C) showed nearly perfect agreement. The concordance of Rj-real-time PCR (D), Rj-double PCR (E), SFGR-double PCR (F), and/or serological tests (G) were mixed, especially regarding the comparison between (D) and conventional PCR (E or F). These approaches were poorly concordant in terms of PPV (0.438) and were in moderate agreement based on Cohen's  $\kappa$  (0.514), which was probably due to the poorer performance of the 2 conventional PCR assays for clinical samples. However, the combination of (D) + (G) showed almost perfect agreement with (E or F) + (G) (Table 5).

**Geographical distribution of rickettsioses-patients:** The location of 222 (of 317) patients with TD and JSF is summarized in Fig. 1. TD occurred in the northern parts of Honshu Island (Akita and Fukushima) of Japan, whereas both TD/JSF were confirmed frequently in the central and western parts of Japan (Shizuoka, Wakayama, Hiroshima, and Miyazaki).

## DISCUSSION

In Japan, the prevalence of the rickettsioses TD and JSF, arthropod-vector-borne infectious diseases, are

considered significant threats to public health. Three traditional conventional PCR assays including Ot-nested PCR, Rj-double PCR, and SFGR-double PCR (6,9–11) have been used for the molecular-based diagnosis of TD and JSF. However, these methods require 2 separate reactions for the detection of *Orientia* and *Rickettsia*. Hence, they are time-consuming, intricate, and associated with a potential risk of contamination. The duplex real-time PCR assay developed in this study can examine both *O. tsutsugamushi* and SFG rickettsia infection simultaneously by performing 1 real-time PCR. Therefore, the biggest advantage associated with this assay is rapid diagnosis. When a blood sample is sent to the laboratory as a diagnostic specimen, the examination could be completed within 3 h, including DNA extraction, PCR, and diagnostic decision.

Previously, 2 multiplex real-time PCR assays have been reported for the detection of both *Orientia* and *Rickettsia* (17,18). Paris et al. described a multiplex real-time PCR method using a SYBR green PCR system (17). Because these types of systems require further melting curve analysis, it takes additional time to obtain the results compared to that using TaqMan PCR; moreover, the specificity of SYBR green assays is usually lower than that of the TaqMan method (19). Prakash et al. reported another multiplex real-time PCR using multiple primer pairs and TaqMan probes (18). However, because of the use of multiple primer pairs, it is hard to determine the optimal reaction conditions for PCR, and the sensitivity of the assay with multiple primer pairs become lower (20). In this study, we designed 1 set of primer pairs using 2 different TaqMan probes for Ot-Rj-duplex



real-time PCR to enhance sensitivity and specificity. The simple and easy preparation of the PCR master mixture will ultimately improve laboratory diagnosis. This assay was also designed to detect not only *R. japonica* or *R. heilongjiangensis*, but also other human pathogenic SFG rickettsiae such as *R. helvetica* (5) and *R. tamurae* (21), which could potentially exist in Japan.

For the detection of *R. japonica* DNA from clinical samples, there was a difference in detection efficiency between our duplex real-time PCR and double PCR assays. This difference might be due by the size of the formed eschar being smaller than that of *O. tsutsugamushi*, the low DNA copy number of rickettsia in clinical samples, or the different sizes of amplicons. In addition, the inhibitors and flora of skin might also have an effect, especially in eschar samples.

Our duplex real-time PCR assay showed higher detection efficiency for skin (eschar) samples than blood samples for both *Orientia* and *Rickettsia*. Indeed, the Cq values from the real-time PCR assays of positive skin (eschar) samples tended to be lower than those from positive blood samples, supporting the higher sensitivity for skin (eschar) specimens. Accordingly, we recommend that a skin (eschar) sample, if obtained at the acute stage of illness, should be used preferentially for this assay. Kim et al. and Kurokawa et al. also reported that areas of skin bitten by arthropod vectors (eschar) are available as sample specimens for the PCR detection of *O. tsutsugamushi* and *R. japonica*, respectively (22,23).

Although our duplex real-time PCR seems to have the same performance as 3 conventional PCR assays during the acute stage of infection, it is likely to be less performance than serological tests at the convalescent stage of infection. As shown in Table 5, the use of only duplex real-time PCR might be insufficient for TD and JSF diagnosis when compared to combined molecular-based analysis and serological testing. Thus, the serological test will also be required for TD/JSF diagnosis; especially, the seroconversion in sera obtained from acute and convalescent stages of infection should be confirmed, because there were some seropositive/PCR-negative samples identified in this study. In addition, in some cases, samples appropriate for PCR might not be provided, as eschar specimens could be lacking. In Japan, severe and fatal infections of TD or JSF still occur, therefore methods for rapid and simultaneous detection, such as Ot-Rj-duplex real-time PCR, are essential for the diagnosis of rickettsioses at the acute stage of infection, especially in central and western parts of Japan where both TD and JSF are endemic. In conclusion, although we recommend the use of a combination of our duplex real-time PCR and serological testing for TD/JSF diagnosis, it is anticipated that our system will improve the efficiency of laboratory diagnosis of rickettsiosis.

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**Conflict of interest** None to declare.

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