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Specific Amplification of *Rickettsia japonica* DNA from Clinical Specimens by PCR

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The gene encoding the 17,000-molecular-weight genus-common antigen (17K genus-common antigen) has been cloned and sequenced from *Rickettsia japonica*. The primer pair used for PCR was designed from this sequence. A 357-bp fragment was observed by amplifying the genomic DNA from *R. japonica* and also the DNA from blood clots of patients with spotted fever group rickettsiosis. The results indicated that this method is suitable for the diagnosis of spotted fever group rickettsiosis in Japan.

Rickettsiae have been detected by PCR in clinical specimens of patients infected with Rocky Mountain spotted fever (10). The primers were derived from the *Rickettsia rickettsii* 17,000-molecular-weight genus-common antigen (17K genus-common antigen) gene sequence.

In 1984, the first Japanese patients infected with spotted fever group (SFG) rickettsiae were identified (6). Uchida et al. proposed the taxonomic name *Rickettsia japonica* for the new species that differed from other known pathogenic rickettsiae of the SFG (11).

The characteristics of the *R. japonica* gene encoding the 17K genus-common antigen seemed to be similar to those of 17K antigen genes of other SFG rickettsiae, such as *R. rickettsii* and *Rickettsia conorii*. To determine the degree of genetic conservation among these regions, we cloned and sequenced this common antigen from *R. japonica*. We designed primers based upon these different sequences and performed PCR. Here, we describe the use of PCR for detecting *R. japonica* DNA in infected patients.

R. japonica YH (provided by T. Uchida, School of Medicine, The University of Tokushima) (11), *R. rickettsii* Smith (ATCC VR-149), *R. conorii* Moroccan (ATCC VR-141), Thai tick typhus strain TT-118 (ATCC VR-599), *Rickettsia sibirica*, and *Rickettsia montana* (provided by Y. Tsuboi, National Institute of Health) were propagated in Vero cells. *Rickettsia akari* MK (ATCC VR-148) and *Rickettsia typhi* Wilmington (provided by Y. Tsuboi, National Institute of Health) were propagated in BSC40 cells. *Rickettsia prowazekii* Breinl strain and the Katayama strain (provided by Y. Tsuboi, National Institute of Health) (5) were propagated in L929 cells. *Rickettsia tsutsugamushi* Gilliam, Karp, Kato, Kawasaki, and Kuroki, used in our previous studies (7, 9, 12, 13), were propagated in L929 cells as described previously (9, 13).

Infected cells were homogenized with a Dounce homogenizer (Kontes Glaso Co.) in 5 ml of 10 mM Tris-HCl buffer, pH 8.0, containing 0.1 mM EDTA (TE buffer), and the DNA was extracted from homogenate supernatants (2). Blood clots obtained from patients with SFG rickettsiosis were stored at −80°C until used. DNA was extracted from blood clots (corresponding to about 0.5 ml of blood) that were homogenized with a mortar in 0.25 ml of distilled water. To extract DNA from these samples, the rickettsial suspension or blood clot homogenates were mixed with a 1/10 volume of 10% sodium dodecyl sulfate (SDS) (final concentration of SDS, 1%) and then digested with proteinase K (Merck & Co., Inc., Rahway, N.J.) at a final concentration of 0.2 mg/ml for 1 h at 55°C. The DNA in this lysate was purified by three extractions with an equal volume of phenol-chloroform-isomyl alcohol (25:24:1), precipitated with 2 volumes of ethanol, suspended in 50 μl of TE buffer, and used as templates for PCR.

A number of primers homologous to various regions of the coding and flanking sequences of the 17K antigen gene from *R. rickettsii* were selected to PCR amplify *R. japonica* DNA. The PCR amplification mixture (total volume, 50 μl) contained 1.5

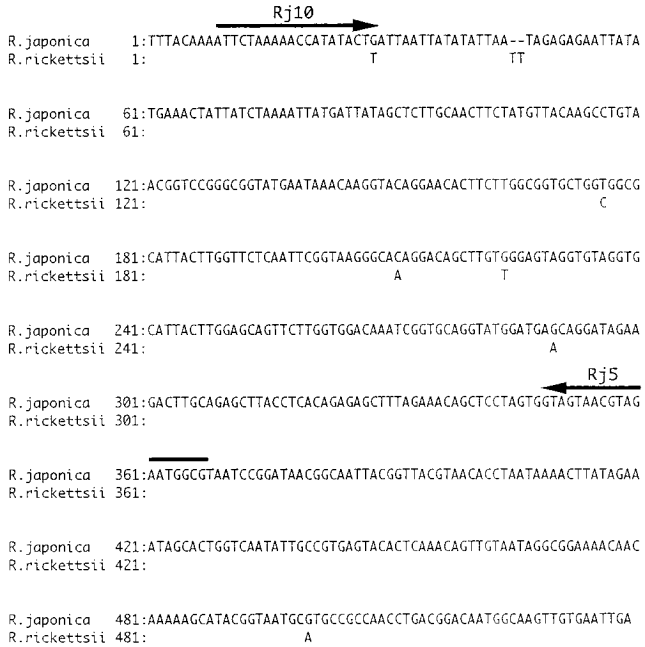

FIG. 1. Nucleotide sequence alignment for *R. japonica* and *R. rickettsii* (1) 17K genus-common antigen genes. Only nucleotides differing from those in the *R. japonica* sequence are shown in the *R. rickettsii* sequence. The arrows indicate the primer positions, and dashes indicate deletions.

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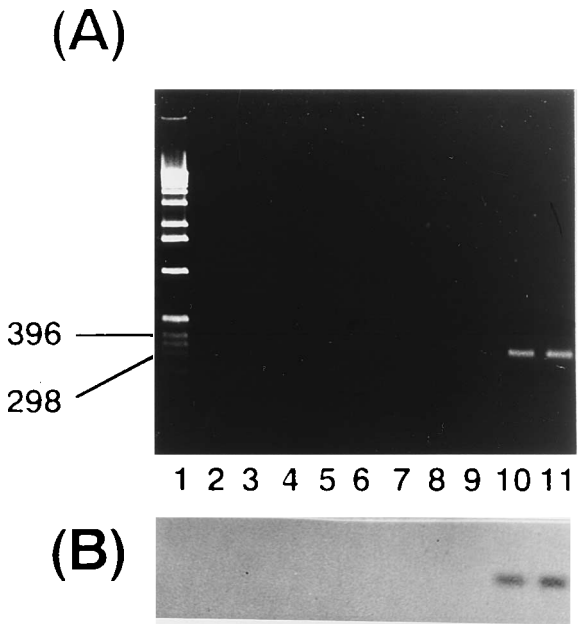

FIG. 2. (A) Agarose gel electrophoresis of DNAs amplified by PCR with primers Rj5 and Rj10 with template DNAs from *R. prowazekii* (lane 2), *R. typhi* (lane 3), *R. rickettsii* (lane 4), *R. sibirica* (lane 5), *R. conorii* (lane 6), *R. akari* (lane 7), *R. montana* (lane 8), Thai tick typhus strain TT-118 (lane 9), the Katayama strain (lane 10), and *R. japonica* YH (lane 11). Lane 1 contained a 1-kb DNA ladder used as size markers (Bethesda Research Laboratories Life Technologies, Inc.). The sizes of the fragments were 12,216, 11,198, 10,180, 9,162, 8,144, 6,108, 5,090, 4,072, 3,054, 2,036, 1,636, 1,018, 517, 506, 396, 344, 298, 220, 201, 154, 134, and 75 bp. (B) Southern blot of DNAs shown in panel A with a digoxigenin-labeled probe. The numbers on the left are sizes in base pairs.

mM $MgCl_2$; 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 0.001% (wt/vol) gelatin; 200 μM (each) dATP, dCTP, dGTP, and dTTP; 1 μM each primer; 1.25 U of AmpliTaq polymerase (Perkin-Elmer Cetus, Norwalk, Conn.); and 5 μl of template DNA. The mixture was placed in a thermal cycler (Perkin-Elmer Cetus) and denatured at 94°C for 30 s, annealed at 57°C for 2 min, and then chain extended at 70°C for 2 min (2, 3). This cycle was repeated 30 times.

The amplified fragments were cloned into bacteriophage M13 vectors M13mp18 and M13mp19 (14) and transformed into *Escherichia coli* TG1 by standard procedures (8). DNA inserts were sequenced by using the Bca Best dideoxy sequencing kit (Takara Shuzo Co., Ltd., Kyoto, Japan). The nucleotide sequence of the 17K antigen gene from *R. japonica* was determined and is shown in Fig. 1.

The 17K antigen gene of *R. japonica* had a high degree of sequence homology with the 17K-antigen genes of *R. rickettsii*, *R. conorii*, *R. prowazekii*, and *R. typhi* (1). Homology to the *R. japonica* 17K antigen gene was 98.5% for *R. rickettsii*, 98.3% for *R. conorii*, 87.9% for *R. prowazekii*, and 88.8% for *R. typhi*. We designed the primers, Rj5 (5′-CGCCATTCTACGTTAC TACC-3′) and Rj10 (5′-ATTCTAAAAACCATATACTG-3′), containing a unique 3′-end nucleotide, to specifically amplify *R. japonica* DNA by PCR. These primers yielded 357-bp polynucleotides. For Southern blotting, the probe was selected from the 357-bp polynucleotides amplified with primers Rj5 and Rj10. The 3′ end of the probe (5′-GGCGCATTACTTG GTTCTCA-3′) was labeled with digoxigenin by using the DIG oligonucleotide 3′-end labeling kit (Boehringer Mannheim, Mannheim, Germany). Amplified *R. japonica* DNA was resolved by electrophoresis, transferred to a nylon membrane, and hybridized with digoxigenin-labeled DNA probes in a solution containing 0.02% SDS and 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 60°C for 10 h. After hybridization, the membrane was washed twice with 0.1× SSC containing 0.1% SDS at 60°C for 30 min and reacted with alkaline phosphatase-conjugated anti-digoxigenin antibody. DNA was labeled with digoxigenin and detected in accordance with the manufacturer's instructions for use of the Genius DNA Labeling and Detection Kit (Boehringer Mannheim).

TABLE 1. IF titers of patients' sera

Patient no.	No. of days after onset	Ig class	IF titer against[a]:					
			R. rickettsii	*R. sibirica*	*R. conorii*	*R. akari*	TT-118[b]	*R. japonica* YH
1	5	M	<10	<10	<10	20	<10	20
		G	<10	<10	<10	<10	<10	<10
	14	M	<10	<10	<10	20	<10	20
		G	<10	<10	<10	<10	<10	<10
	30	M	20	20	<10	20	20	20
		G	320	160	160	320	1,280	1,280
2	1	M	20	20	20	20	20	20
		G	<10	<10	<10	<10	<10	<10
	14	M	40	40	40	40	40	40
		G	320	320	160	320	640	1,280
3	4	M	<10	<10	NT[c]	<10	<10	<10
		G	<10	<10	NT	<10	<10	<10
	15	M	20	20	NT	20	20	20
		G	1,280	1,280	NT	1,280	2,560	10,240
4	4	M	<10	40	NT	20	<10	20
		G	<10	<10	NT	<10	<10	<10
	15	M	20	40	NT	40	20	80
		G	160	160	NT	320	160	320

[a] Titers are expressed as the reciprocal of the highest dilution of the sera which gave positive reactions.
[b] Thai tick typhus strain TT-118.
[c] NT, not tested.

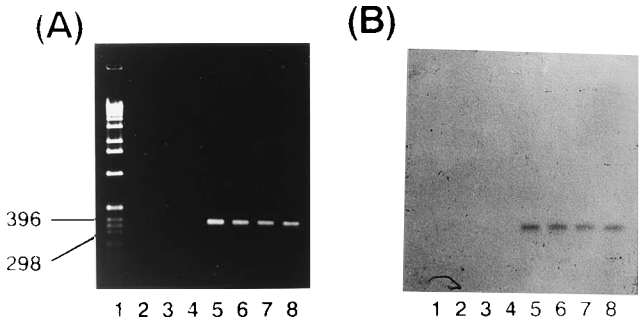

FIG. 3. (A) Agarose gel electrophoresis of DNAs amplified by PCR with primers Rj5 and Rj10, using template DNAs from healthy donors (lanes 2 through 4) and patients 1 to 4 (lanes 5 through 8, respectively). Lane 1 contained molecular size markers as described in the legend to Fig. 2. (B) Southern blot of DNAs shown in panel A with a digoxigenin-labeled probe. The numbers on the left are sizes in base pairs.

We performed PCR with the primers Tz-15-19 and Tz-16-20 described by Tzianabos et al. (10), and 246-bp bands were amplified in DNAs from *R. prowazekii*, *R. typhi*, *R. rickettsii*, *R. sibirica*, *R. conorii*, *R. akari*, *R. montana*, Thai tick typhus strain TT-118, *R. japonica*, and the Katayama strain. Using these strains as templates, we then performed PCR using the primers Rj5 and Rj10. The 357-bp DNA fragment was identified by agarose gel electrophoresis with template DNA from *R. japonica* and the Katayama strain. However, no amplified band was evident in DNAs from *R. prowazekii*, *R. typhi*, *R. rickettsii*, *R. sibirica*, *R. conorii*, *R. akari*, *R. montana*, and Thai tick typhus strain TT-118 (Fig. 2A). The enzymatic amplification was not seen in tests with DNAs of *R. tsutsugamushi* Gilliam, Karp, Kato, Kawasaki, and Kuroki or with those of *E. coli* TG1 or L929, Vero, or BSC40 cells.

With the digoxigenin-labeled probe, only the 357-bp band from *R. japonica* and the Katayama strain hybridized (Fig. 2B). This finding indicated that amplification with these primers was specific for the SFG rickettsiae isolated in Japan.

R. japonica DNA was diluted to determine the sensitivity of the primers. At a 5×10^2 dilution, the 357-bp band was amplified with the primers Rj5 and Rj10. With the primers Tz-15-19 and Tz-16-20, a 10^3 dilution of *R. japonica* DNA was amplified. The primer pair Rj5 and Rj10 was twofold less sensitive than the primer pair Tz-15-19 and Tz-16-20, which may be because the latter pair was composed of conserved nucleotides.

We also tested the blood of three healthy donors and four patients with SFG rickettsiosis by PCR. The four patients were not severely infected, and they recovered after antibiotic therapy.

An indirect immunofluorescence (IF) test was performed with SFG rickettsiae (4) and *R. tsutsugamushi* as described previously (12). The secondary antibodies were fluorescein isothiocyanate-conjugated goat immunoglobulin Gs (IgGs) against human IgM (μ-chain specific) and the Fc fragment of human IgG (γ-chain specific), all purchased from Tago, Inc. (Burlingame, Calif.). The antibodies in the four patients' sera were titrated by indirect IF with *R. rickettsii*, *R. sibirica*, *R. conorii*, *R. akari*, Thai tick typhus strain TT-118, and *R. japonica* (Table 1). The convalescent sera of patients had high titers against SFG rickettsiae and did not react with *R. tsutsugamushi* Gilliam, Karp, Kato, Kawasaki, and Kuroki (data not shown). By serological tests, these four patients were diagnosed as having SFG rickettsiosis.

With primers Tz-15-19 and Tz-16-20, 246-bp bands were amplified from blood of the four patients in a second round of PCR. In the first PCR using primers Rj5 and Rj10, DNA was not detected. Therefore, 5 μl of the first-round PCR products was added to the PCR amplification mixture (total volume, 50 μl). In the second round of PCR, the DNA was amplified and *R. japonica* DNAs were detected in samples from the four patients. There were no detectable bands in amplification tests of DNAs from the blood of the three healthy donors. In hybridization tests using the digoxigenin-labeled probe, 357-bp bands from patient specimens hybridized, indicating that these bands were DNA fragments specific to *R. japonica* DNA. The 357-bp bands amplified from the blood of patients 1 to 4 are shown in Fig. 3. Thus, only *R. japonica* DNA was detected by PCR with primers Rj5 and Rj10.

The causative agent of SFG rickettsiosis in these four patients was *R. japonica*. We directly detected *R. japonica* DNA using PCR. This method can distinguish *R. japonica* DNA from that of other SFG rickettsiae. The application of this method to clinical specimens from acute-phase patients will be useful for diagnosing SFG rickettsiosis and for identifying *R. japonica.*

The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under the accession number D16515.

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