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Serotype-Specific Amplification of *Rickettsia tsutsugamushi* DNA by Nested Polymerase Chain Reaction

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Polymerase chain reaction (PCR) with nested primer pairs was used to diagnose scrub typhus and identify the *Rickettsia tsutsugamushi* serotype. The primer pairs used for PCR were designed on the basis of the nucleotide sequence of the gene that encodes the 56-kDa antigen. Serotype-specific primers were used in the second PCR amplification. Five serovariants, the Gilliam, Karp, Kato, Kawasaki, and Kuroki strains of *R. tsutsugamushi*, were identified by nested PCR. In addition, the serotype identified by PCR with DNA from blood clots was the same as that of the strain isolated from five patients with scrub typhus. These findings indicate that this method is useful for diagnosis and identification of the rickettsial serotype in infected patients.

Use of the polymerase chain reaction (PCR) in the diagnosis of some infectious diseases has been reported. This method is useful when immunological techniques or isolation of the causative agent is difficult. In Tsutsugamushi disease, PCR revealed rickettsial infection during the acute rickettsemia phase, which occurs before the antibody titer increases. Thus, diagnosis is possible in the early stage of the illness. We previously described the use of PCR for diagnosis of Tsutsugamushi disease (2), in which the amplified 78-bp DNA corresponds to the N-terminal side of mature type-specific antigens, the sequences of which are relatively well conserved among the strains.

Here, we describe the use of nested PCR for diagnosis and identification of rickettsial serotypes in infected patients.

Rickettsia tsutsugamushi Gilliam, Karp, Kato, Kawasaki, and Kuroki, used in our previous studies (4, 7–9), *R. rickettsii* Bitterroot (Denka Seiken Co., Tokyo, Japan), and *R. sibirica* (ATCC VR151, donated by N. Tachibana, Miyazaki Medical College, Miyazaki, Japan) were propagated in L929 cells as described previously (7, 9). 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 Tsutsugamushi disease were stored at −80°C until use. Rickettsiae from portions of the clots were isolated in mice or L929 cells, and the rickettsial serotype was identified with strain-specific monoclonal antibodies as described previously (1). DNAs were extracted from blood clots corresponding to about 0.5 ml of blood that were homogenized in 1.0 ml of distilled water with a mortar. DNAs extracted from these samples were suspended in 50 μl of TE buffer and used as templates for PCR.

The following oligonucleotide primers were obtained from Bex (Tokyo, Japan): 34 (5′-TCAAGCTTATTGCTAGTG CAATGTCTGC-3′), whose nucleotide sequence corresponds to that of a mature 56-kDa protein in *R. tsutsugamushi* Gilliam; 55 (5′-AGGGATCCCTGCTGCTGTGCTT GCTGCG-3′); 10 (5′-GATCAAGCTTCCTCAGCCTACTAT AATGCC-3′); 11 (5′-CTAGGGATCCCGACAGATGCACT ATTAGGC-3′); and 13 (5′-CTAGCTGCAGCGACAGATG CACTATTAGGC-3′).

PCR was performed with nested primer pairs. The PCR amplification mixture (total volume, 50 μl) contained 1.5 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; 0.2 μM primers 34 and 55, 1.25 U of AmpliTaq polymerase (Cetus, Norwalk, Conn.); and 5 μl of template DNA. The mixture was denatured at 94°C for 30 s, annealed at 57°C for 2 min, and then chain was extended at 70°C for 2 min in a thermal cycler (Perkin-Elmer Cetus). This cycle was repeated 30 times. To remove excess deoxyribonucleoside triphosphates and the primer pair, the PCR products were purified by extraction with an equal volume of phenol-chloroform (1:1) and then centrifuged with an Ultrafree-C3 unit incorporating a membrane with a molecular weight limit of 30,000 (Nippon Millipore Ltd., Tokyo, Japan). After centrifugation, the membrane was rinsed with 50 μl of TE buffer and purified first-PCR products were prepared. For the second PCR amplification, 5 μl of the purified first-PCR product was amplified as described above, except for substitution of primers 10 and 11, 10 and 13, or 34 and 11. The amplified sample (5 μl) was electrophoresed in a 1.5% agarose gel, and the DNA bands were stained with 0.5 μg of ethidium bromide per ml.

PCR with primer pairs 34 and 55, 10 and 11, and 34 and 11, with *Hin*dIII and *Bam*HI sites, and that with the primer pair 10 and 13, with *Hin*dIII and *Pst*I sites, produced 1,003- to 1,030-, 481- to 507-, 868- to 892-, and 481- to 507-bp polynucleotides, respectively, from the base sequences of the gene determined by Stover et al. (6) and Ohashi et al. (3).

After nested PCR, the products were digested with restriction endonucleases *Hin*dIII and *Bam*HI or *Hin*dIII and *Pst*I. The fragments were ligated with T4 DNA ligase into dephosphorylated, *Hin*dIII-*Bam*HI-cleaved bacteriophage M13 vectors M13mp18 and M13mp19 (10) or *Hin*dIII-*Pst*I-cleaved M13mp18 and M13mp19 and then transformed into *Escherichia coli* TG1 by standard procedures (5).

DNA inserts were sequenced with the Bac Best dideoxy sequencing kit (Takara Shuzo Co., Ltd., Kyoto, Japan).

The Gilliam, Karp, Kato, Kawasaki, and Kuroki strains

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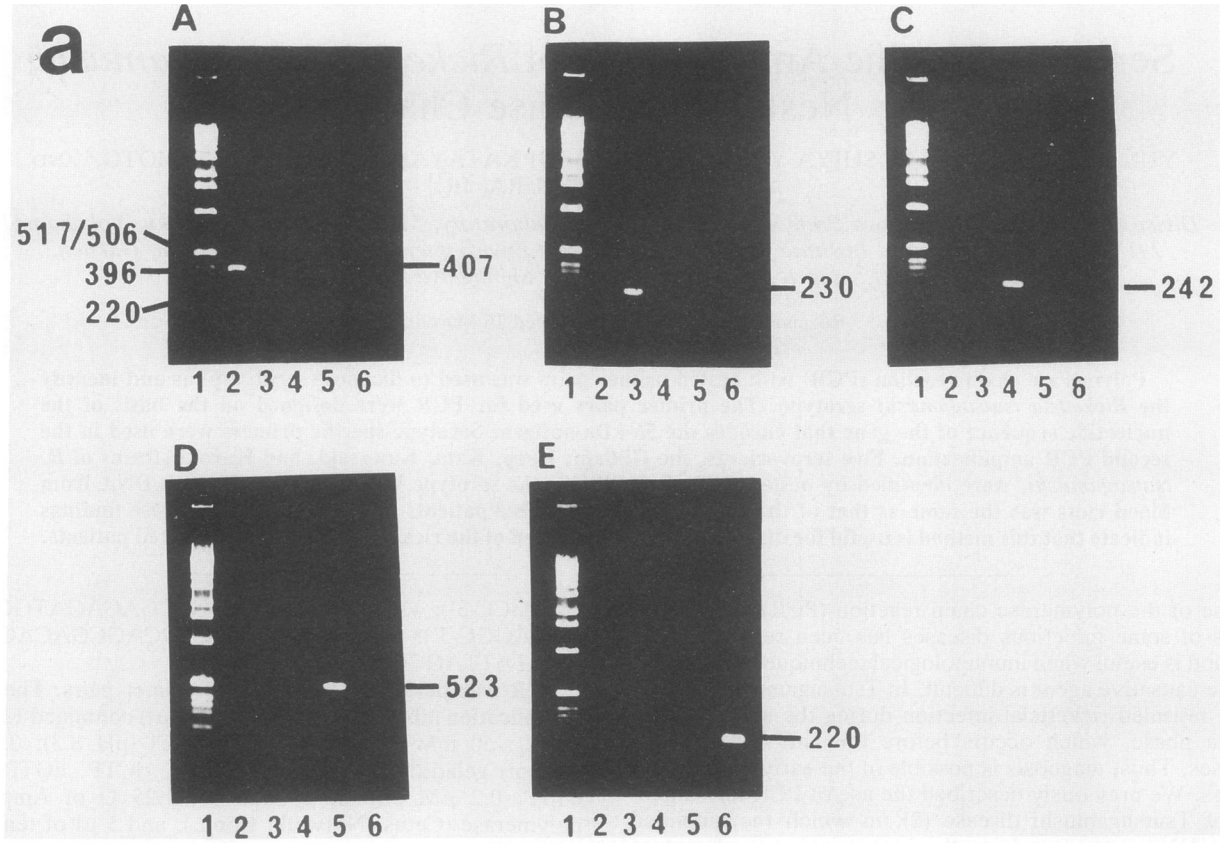
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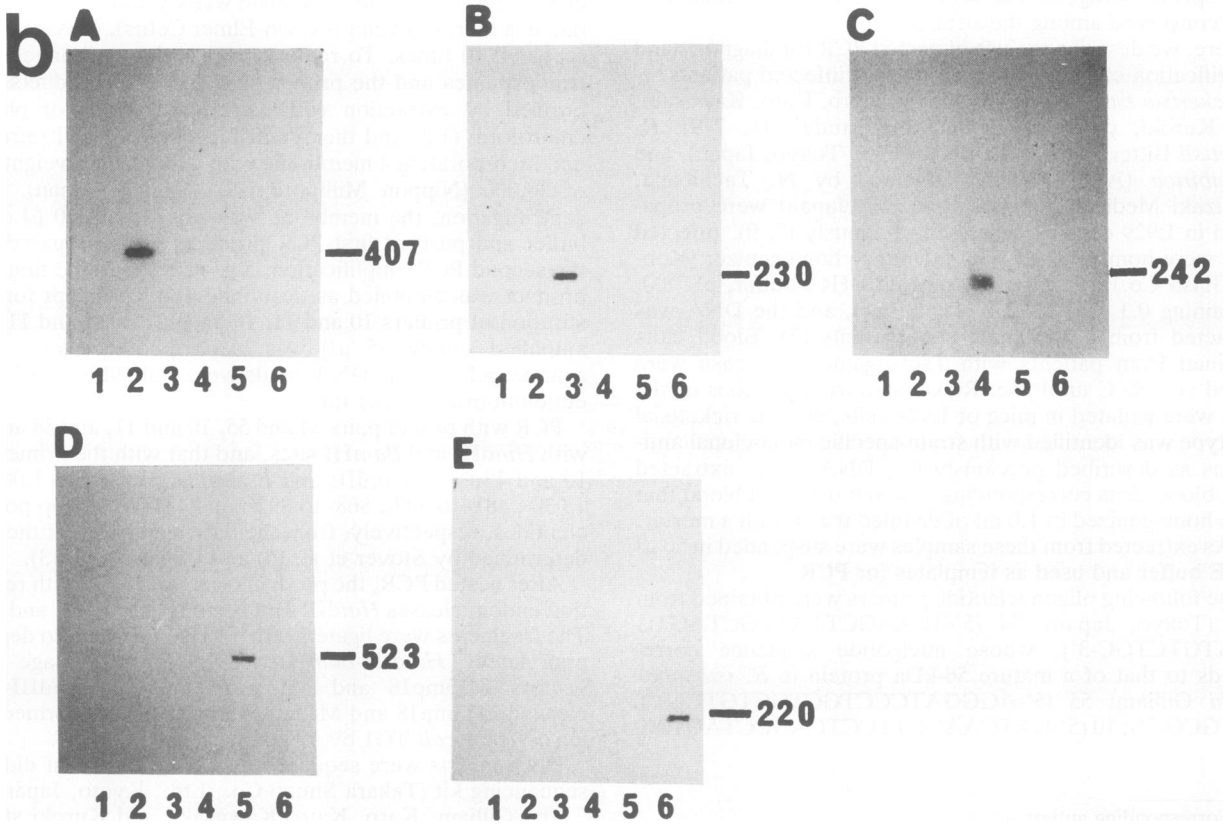
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FIG. 1. (a) Agarose gel electrophoresis of amplified DNAs by nested PCR with serotype-specific primers (for the Gilliam [A], Karp [B], Kato [C], Kawasaki [D], and Kuroki [E] serotypes) with template DNAs from strains Gilliam (lanes 2), Karp (lanes 3), Kato (lanes 4), Kawasaki (lanes 5), and Kuroki (lanes 6). Lane 1 contained a 1-kb DNA ladder 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. The numbers beside the panels are sizes in base pairs. (b) Southern blot of DNAs from panel a with an amplified, digoxigenin-labeled, serotype-specific probe (for the Gilliam [A], Karp [B], Kato [C], Kawasaki [D], and Kuroki [E] serotypes). The numbers beside the panels are sizes in base pairs.

were amplified by nested PCR with primer pairs 34 and 55, 10 and 11, 10 and 13, and 34 and 11. The products obtained with primer pairs 10 and 11, 10 and 13, and 34 and 11 were sequenced, and individual strain-specific sequences were identified, from which specific primers for PCR were designed. PCR with Gilliam-specific primers 10 and G (5′-CTTTATATCACTATATATCTT-3′) was expected to yield 407-bp polynucleotides. PCR with Karp-specific primers 10 and KP (5′-ACAATATCGGATTTATAACC-3′) was expected to yield 230-bp polynucleotides. PCR with Kato-specific primers 10 and KT (5′-GAATATTTAATAGCAC TGGA-3′) was expected to yield 242-bp polynucleotides. PCR with Kawasaki-specific primers 11 and KW (5′-ATGC TGCTATTGATACAGGC-3′) was expected to yield 523-bp polynucleotides. PCR with Kuroki-specific primers 10 and KR (5′-CACCGGATTTACCATCATAT-3′) was expected to yield 220-bp polynucleotides.

For Southern blotting, probes labeled with digoxigenin were prepared by incorporating digoxigenin-11-dUTP during amplification of rickettsial DNA (purified Gilliam, Karp, Kato, Kawasaki, and Kuroki strains) with the primers. DNAs obtained by amplification of the five strains were electrophoresed, transferred to a nylon membrane by electrophoresis, and hybridized with the digoxigenin-labeled DNA probes under highly stringent conditions in a solution containing 0.02% sodium dodecyl sulfate and 5× SSC (1× SSC is 0.15 M sodium chloride and 0.015 M sodium citrate) at 68°C for 10 h. After hybridization, the membrane was washed twice with 0.1× SSC containing 0.1% sodium dodecyl sulfate at 68°C for 30 min and allowed to react with alkaline phosphatase-conjugated anti-digoxigenin antibody. DNA labeling with digoxigenin and detection of DNA bands containing the drug were performed in accordance with the manufacturer's instructions for use of the Genius DNA Labeling and Detection Kit (Boehringer, Mannheim, Germany).

After the first PCR with primers 34 and 55, purified PCR products were used as templates in the second PCR with

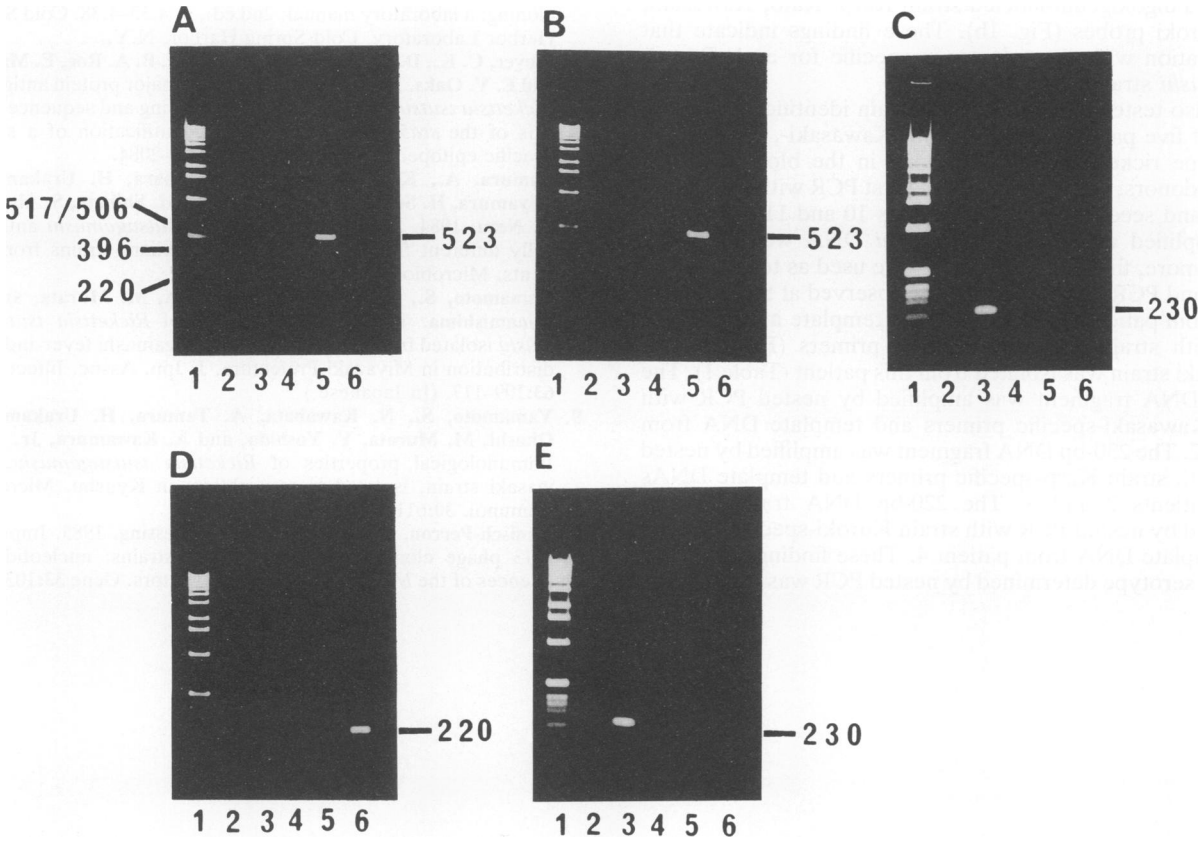

FIG. 2. Agarose gel electrophoresis of amplified DNA by nested PCR with serotype-specific primers for strains Gilliam (lane 2), Karp (lane 3), Kato (lane 4), Kawasaki, (lane 5), and Kuroki (lane 6) and template DNAs from patients. Panels: A, patient 1; B, patient 2; C, patient 3; D, patient 4; E, patient 5. Lane 1 contained molecular size markers as described in the legend to Fig. 1.

TABLE 1. Patient blood used for PCR

Patient no.	Days after onset	Serotype of isolated rickettsia[a]
1	1	Kawasaki
2	2	Kawasaki
3	7	Karp
4	8	Kuroki
5	9	Karp

[a] Serotypes of rickettsiae isolated were identified by immunofluorescence with strain-specific antibodies as described previously (8).

strain-specific primers. With Gilliam-specific primers, the predicted 407-bp DNA fragment was identified as a band by agarose gel electrophoresis with template DNA from the Gilliam strain. However, no amplified band was seen when DNAs from the Karp, Kato, Kawasaki, and Kuroki strains were used (Fig. 1). Also, after PCR with strain Karp-, Kato-, Kawasaki-, and Kuroki-specific primers, the predicted 230-, 242-, 523-, and 220-bp DNA fragments were identified as bands when template DNAs from only the respective strains were used (Fig. 1a). The enzymatic amplification was not seen in tests with DNAs of *R. rickettsii*, *R. sibirica*, and *E. coli* TG1 (data not shown) or with that of the L929 host cells. In the hybridization test with the amplified digoxigenin-labeled strain Gilliam probe, only the 407-bp band from the Gilliam strain hybridized. Only the 230-, 242-, 523-, and 220-bp bands from the respective strains hybridized with amplified digoxigenin-labeled strain Karp, Kato, Kawasaki, and Kuroki probes (Fig. 1b). These findings indicate that amplification with the primers is specific for each *R. tsutsugamushi* strain.

We also tested nested PCR for strain identification in the blood of five patients infected with Kawasaki-, Kuroki-, or Karp-type rickettsiae (Table 1) and in the blood of three healthy donors. In the nested PCR (first PCR with primers 34 and 55 and second PCR with primers 10 and 11), the DNA was amplified and *R. tsutsugamushi* DNA was detected. Furthermore, first-PCR products were used as templates for the second PCR. A clear band was observed at 523 bp when DNA from patient 1 was used as the template in the second PCR with strain Kawasaki-specific primers (Fig. 2). The Kawasaki strain was isolated from this patient (Table 1). The 523-bp DNA fragment was amplified by nested PCR with strain Kawasaki-specific primers and template DNA from patient 2. The 230-bp DNA fragment was amplified by nested PCR with strain Karp-specific primers and template DNAs from patients 3 and 5. The 220-bp DNA fragment was amplified by nested PCR with strain Kuroki-specific primers and template DNA from patient 4. These findings indicated that the serotype determined by nested PCR was the same as that of the isolated *R. tsutsugamushi* strain. There were no detectable bands in amplification tests of DNAs from the blood of three healthy donors. Thus, the serotypes of patients were determined by nested PCR (Fig. 2).

The nested PCR described here amplified the rickettsial DNA, and the serotypes were determined. Nested PCR is a very rapid and sensitive means of detecting *R. tsutsugamushi* DNA.

Application of this method to clinical specimens from acute-phase patients suggests its usefulness for diagnosis of Tsutsugamushi disease and identification of *R. tsutsugamushi* serotypes.

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