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Detection of *Rickettsia tsutsugamushi* in Experimentally Infected Mice by PCR

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We developed a rapid procedure for the detection of *Rickettsia tsutsugamushi* DNA by the PCR technique. The primer pair used for the PCR was designed from the DNA sequence of the gene encoding a 120-kDa antigen, which was proven to be group specific by immunoblot analysis with mouse hyperimmune sera against various rickettsial strains. This PCR method was able to detect up to 10 ag of plasmid DNA (pKT12). Specific PCR products were obtained with DNAs from *R. tsutsugamushi* Kato, Karp, Gilliam, TA716, TA1817, and Boryong, but not with DNAs from other rickettsiae, such as *R. prowazekii*, *R. typhi*, *R. akari*, and strain TT118. In a study with experimentally infected mice, the PCR method could detect rickettsial DNA from 2 days after inoculation (DAI), whereas serum antibody against *R. tsutsugamushi* could be detected from 6 to 8 DAI by an immunofluorescence test. Although clinical manifestations subsided after 14 DAI, rickettsial DNA in blood samples could be detected by PCR for up to 64 DAI. These results suggest that this PCR method can be applied to the early diagnosis of scrub typhus and can also be used to detect the residual rickettsiae after clinical symptoms subside.

Scrub typhus is a major cause of febrile illness in Asia and the Pacific region (15). This disease is initially suspected by the presence of clinical manifestations such as high fever, febrile rash, and eschar formation. However, in order to differentiate scrub typhus from other febrile diseases such as leptospirosis, hemorrhagic fever with renal syndrome, and other rickettsial diseases, laboratory tests based on the serological responses of the host to *Rickettsia tsutsugamushi* are commonly used. The immunoperoxidase test, the immunofluorescence (IF) test, and the enzyme-linked immunosorbent assay are generally used for this purpose (2, 3, 9, 22, 25). However, the immunoperoxidase and IF tests require trained personnel and cell culture facilities for the laborious process of rickettsial antigen preparation. Recent advances in recombinant DNA technology solve these antigen preparation problems (10, 11). Nevertheless, diagnosis by these serological methods is sometimes difficult in the early stage of the illness, when the antibody titers are not yet high enough to be detected.

For the definite diagnosis of scrub typhus, detection of *R. tsutsugamushi* in biological materials from patients is required. One of these methods, the culture isolation method, can be used in the early stage of infection, but sometimes it does not yield results in time to influence patient management. The PCR method is an in vitro method for selectively amplifying specific target DNA sequences, and applications of PCR for use in the diagnosis of some infectious diseases, including rickettsial diseases, have been reported (7, 8). Furthermore, the PCR method is excellent in circumstances in which it is difficult to use serological tests for diagnosis.

We report here the development of a PCR assay for *R. tsutsugamushi* DNA detection that is based on amplification of the gene encoding the 120-kDa *R. tsutsugamushi* antigen. The diagnostic potential of this PCR assay was evaluated and compared with those of other diagnostic methods through experiments in animals.

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MATERIALS AND METHODS

Bacterial strains and cultivation. The strains *R. tsutsugamushi* Kato, Karp, Gilliam, and Boryong (6, 11), *R. typhi* ATCC VR-144, *R. akari* ATCC VR-148, and *R. prowazekii* ATCC VR-142 were used in the study. Strains TA716 and TA1817, which were provided by U.S. Army Medical Research Corp. (Kuala Lumpur, Malaysia), and strain TT118 (16) were also used. All rickettsiae were cultured in mouse fibroblasts (L929) as described previously (6), harvested, and purified by percoll density gradient centrifugation as described by Tamura et al. (24). For cloning experiments, *Escherichia coli* XL1-Blue was used as a host strain for bacteriophage lambda ZapII phagemid and the pBluescript plasmid (Stratagene).

Gene cloning and DNA sequencing. The genomic DNA of *R. tsutsugamushi* was isolated from purified rickettsiae. With the 1- to 5-kb DNA fragment from partially digested *Sau*3AI DNA (strain Kato), a genomic library was constructed by using the lambda ZapII phagemid system (Fig. 1). This library was screened with mouse hyperimmune sera against strain Kato, and then plasmid pKT12, which expressed the 120-kDa antigen, was obtained. Plasmid pKT12 was digested with *Hin*dIII, and then a 3.0-kb DNA fragment from digested products was subcloned and sequenced by the shotgun method (1, 18).

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis were done as described by Tamura et al. (23). The immunoaffinity-purified antibody for the 120-kDa antigen was made as described by Oaks et al. (13).

PCR and Southern blot analysis. A primer pair (pc4, 5′-ACTATCTAGCATCGGATCTG-3′; pc5, 5′-TTGGTTAT AATGAGCAGTTA-3′) was selected from the DNA sequence in the presumed open reading frame (Fig. 1) and was synthesized by Oligo's Etc., Inc. (Wilsonville, Oreg.). Target DNA was amplified in a mixture of 100 μM deoxynucleotide triphos-

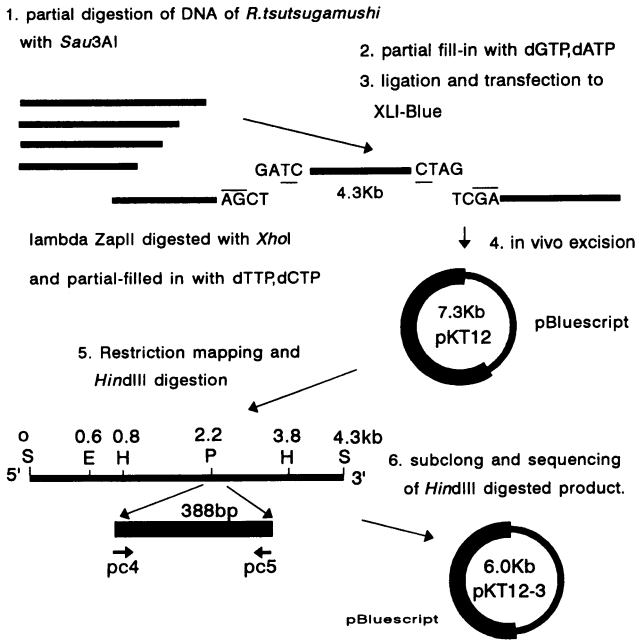

FIG. 1. Strategy for cloning pKT12 and selecting the oligonucleotide primers (pc4 and pc5) from the sequence of pKT12-3. S, *Sau*3AI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I. The sequence is deposited in GenBank under accession number L23765.

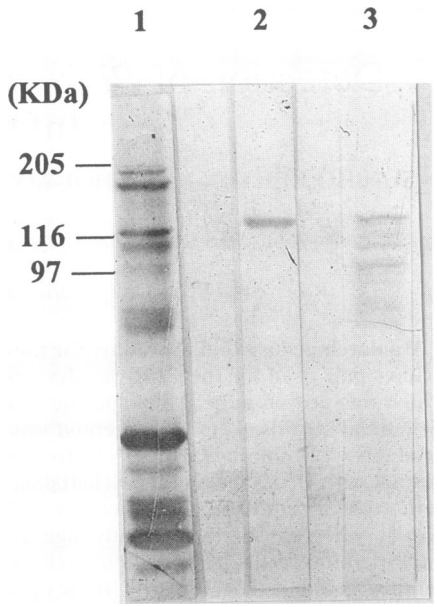

FIG. 2. Expression of the 120-kDa antigen in *R. tsutsugamushi* Kato and *E. coli*. Whole-cell extracts of strain Kato were immunologically stained after SDS-PAGE and Western blotting (immunoblotting) with mouse hyperimmune sera against strain Kato (lane 1). Staining of whole-cell extracts of strain Kato (lane 2) and *E. coli* containing pKT12 (lane 3) with affinity-purified antibody to the recombinant clone (KT12) was also done.

phates, 1 μM (each) primer, 1.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris (pH 8.3), and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer Cetus) with an automatic PCR processor (Intelligent Heat Block; Hybaid, Middlesex, United Kingdom). The reaction was performed in 35 cycles of 94°C for 15 s, 55°C for 15 s, and 72°C for 30 s. The final cycle was followed by an extension step at 72°C for 5 min. For the Southern blot analysis of amplified products, ^{32}P labeling of the probe, capillary blotting, and hybridization were done as described by Sambrook et al. (17).

Animal experiment. ICR mice were inoculated intraperitoneally with *R. tsutsugamushi* Kato at a sublethal dose (5×10^4 infected cell units [ICUs]). For the determination of the ICUs, a confluent culture of L929 cells on a spot slide was infected with *R. tsutsugamushi*, cultured for 3 to 4 days, and stained by the indirect IF method, and the counts for cells showing fluorescence in each spot were calculated. After inoculation, the blood and spleens were serially harvested from three mice, and rickettsiae were isolated from the spleens (5), the IF test was done to determine the antibody titer in serum (2), and the DNAs in blood and spleen cells were amplified by PCR. In order to isolate DNA from spleen and blood cells, the spleen cells and heparinized blood were collected from mice, washed two times with phosphate-buffered saline (0.1 M; pH 7.2), reacted with 145 mM ammonium chloride for erythrocyte lysis, and mixed with *Taq* DNA polymerase buffer containing Tween 20 (0.01% [vol/vol]), Nonidet P-50 (0.01% [vol/vol]), and proteinase K (50 μg/ml); the mixture was then incubated at 56°C for 3 h. The DNA in the lysate was purified by extraction two times with a mixture of phenol-chloroform, which were present in equal volumes. This was followed by precipitation with 2 volumes of ethanol, and the precipitate was resuspended in 20 μl TE buffer.

Nucleotide sequence accession number. The nucleotide sequence of the 120-kDa fragment described here has been deposited in GenBank under accession number L23765.

RESULTS

Cloning of the 120-kDa antigen. Screening of recombinant bacteriophage lambda ZapII plaques from an *R. tsutsugamushi* genomic library with mouse hyperimmune sera resulted in the identification of a recombinant phagemid KT12 clone, and the KT12 phagemid was converted to pKT12 plasmid through in vivo excision. Plasmid pKT12 contained a 4.3-kb insert DNA and was found to express a 120-kDa antigen by SDS-PAGE and immunoblot analysis. Identical results of testing with the 120-kDa antigen with mouse hyperimmune sera against strains Gilliam, Karp, Kato, and Boryong implied that this antigen contains group-specific epitopes. An antigen of identical size was also found in purified rickettsiae by immunoblot analysis by using immunoaffinity-purified antibody to the 120-kDa antigen (Fig. 2).

Nucleotide sequence and oligonucleotide primers. The 3.0-kb DNA fragment from the *Hin*dIII digestion product of pKT12 was subcloned (pKT12-3) and sequenced. Plasmid pKT12-3 contained a 3,083-bp insert DNA with a 33.54% G+C ratio. In this DNA sequence, a 2,397-bp protein-coding region was found, and 799 amino acids from this region was deduced to be the carboxy-terminal portion of the 120-kDa antigen. An analysis of the deduced amino acids indicated that 55.4% of the amino acids were hydrophobic and 44.6% were neutral. In the middle of this gene, the sequence of the oligonucleotide primers which amplified the 388-bp target DNA was chosen and synthesized.

PCR amplification of purified DNA. PCR was used to detect rickettsial DNA by using the gene encoding the 120-kDa antigen as a target sequence. By this PCR, various purified

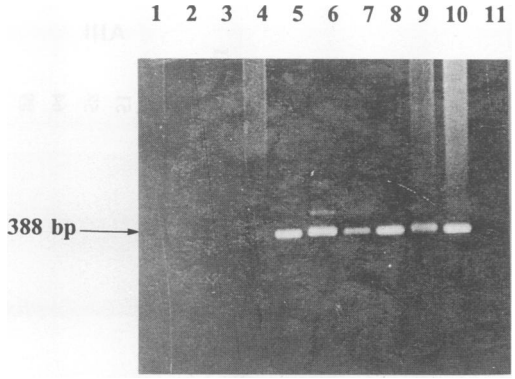

FIG. 3. Agarose gel electrophoresis of PCR-amplified DNAs from various rickettsial species. Lane 1, *R. akari*; lane 2, TT118; lane 3, *R. prowazekii*; lane 4, *R. typhi*; lane 5, TA1817; lane 6, TA716; lane 7, Boryong; lane 8, Gilliam; lane 9, Karp; lane 10, Kato; lane 11, L929 cells.

rickettsial DNAs were analyzed. The predicted 388-bp DNA fragments were identified by agarose gel electrophoresis with template DNAs from *R. tsutsugamushi* Gilliam, Karp, Kato, Boryong, TA1817, and TA716, while no amplification product was detected with template DNAs from typhus and spotted group rickettsiae such as *R. prowazekii*, *R. typhi*, *R. akari*, or strain TT118 or with the host cell (L929) (Fig. 3). The sensitivity of the reaction was determined by using plasmid pKT12 DNAs ranging in size from 1 pg to 1 ag. The lower limit of detection of pKT12 DNA in the assay was 10 fg by agarose gel electrophoresis and 10 ag by Southern blot hybridization (Fig. 4A and B). The DNAs of other pathogenic organisms such as *Leptospira interrogans*, *E. coli*, *Salmonella typhimurium*, and *Shigella flexneri* were not amplified (data not shown).

Animal experiments. All of the mice that were experimentally infected with 5×10^4 ICUs of *R. tsutsugamushi* Kato showed signs of illness, such as loss of activity and fur coarseness from 5 days after inoculation (DAI), and began to recover from the illness after 15 DAI. Spleen and blood samples from three mice were obtained at regular intervals after infection until 106 DAI, and samples were also harvested from preinfected mice as negative controls. By PCR with DNAs from blood cells, positive amplification products (388-bp band) were detected from 2 to 64 DAI. These positive signals could be detected by ethidium bromide-stained agarose gel electrophoresis and Southern blot hybridization (Fig. 5). By using spleen cells, positive signals could be detected at up to 32 DAI by agarose gel electrophoresis and at up to 44 DAI by Southern blot hybridization (Fig. 5). By culture isolation, *R. tsutsugamushi* could be isolated from 2 to 16 DAI (Table 1). The immunoglobulin M (IgM) titer against *R. tsutsugamushi* began to be detected from 6 DAI and reached the peak level (1:320 to 1:640) at 14 to 16 DAI (Table 2). In the case of the IgG fraction, the antibody titer began to rise from 8 DAI and reached a maximum titer (1:1,280 to 1:2,560) at 16 DAI. These antibody titers (IgM, IgG, and total immunoglobulin) decreased after 60 DAI (Table 1).

DISCUSSION

Our choice of the 388-bp genomic sequence in the gene encoding the 120-kDa antigen as a target for PCR amplification was based on the fact that the 120-kDA antigen is group specific. The strain diversity within the species *R. tsutsugamushi* is well established (12, 20) and concomitant infection with multiple *R. tsutsugamushi* serotypes could occur in endemic areas (6, 20). Thus, for the diagnosis of scrub typhus in an endemic area, multiple strains of *R. tsutsugamushi* must be detected; therefore, it was more appropriate to use the conserved sequence in the gene of group-specific antigen for the PCR than a strain-specific sequence or a sequence of limited specificity in *R. tsutsugamushi*. For this reason, we cloned the gene encoding the 120-kDa group-specific antigen. In the cloning procedures, a bacteriophage lambda ZapII phagemid plaque which reacted with all the mouse hyperimmune sera against *R. tsutsugamushi* was selected. By using affinity-purified antibody to this phagemid, cloned protein was proven to be expressed in purified rickettsiae by SDS-PAGE and immunoblot analysis (Fig. 2). Consequently, this 120-kDa antigen was thought to be a group-specific antigen of *R. tsutsugamushi*.

After consideration of the G+C ratio, the secondary structure of the DNA (such as polypurine and polypyrimidine and

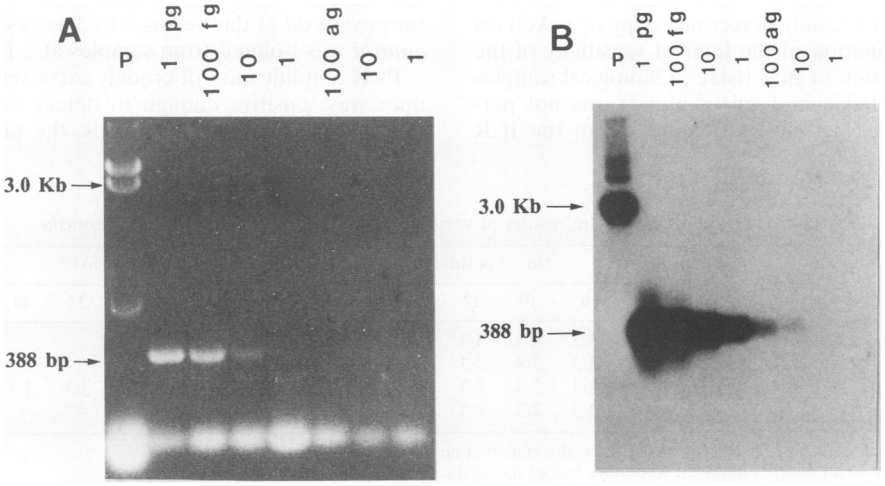

FIG. 4. Comparison of sensitivity of the PCR assay for plasmid pKT12 after agarose gel electrophoresis (A) and Southern blot hybridization (B). The ^{32}P-labeled 388-bp probe was prepared from the gel-eluted amplified product of pKT12. pKT12 cleaved with *Hin*dIII, and hybridization of the 3.0-kb fragment of digestion products is shown in lane P. The amounts of DNA used in each PCR mixture are given above the lanes.

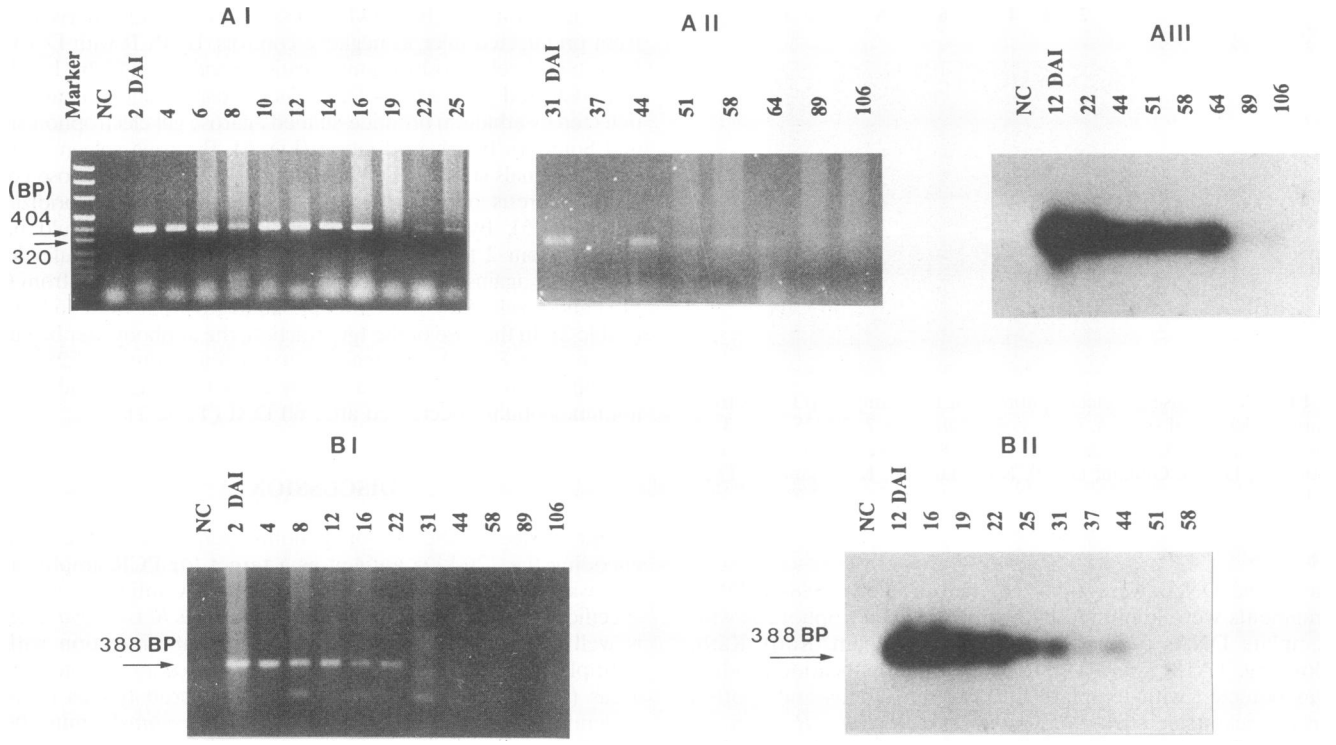

FIG. 5. Agarose gel electrophoresis of PCR-amplified products from the extracts of mouse blood cells (AI and AII) and spleen cells (BI), and the results of Southern blot hybridization of those products with the 388-bp probe are also shown (AIII, blood cells; BII, spleen cells). The DAI that the samples were harvested from mice are given above the lanes. Lane NC, samples obtained from mice before infection (negative controls). Molecular weight markers (marker VIII; Boehringer, Mannheim, Germany) are shown in the lane labeled Marker in panel AI.

the inverted structure of the DNA), the sequence of a primer pair (pc4 and pc5) which could amplify the 388-bp target DNA in the protein coding region was finally selected.

Before PCR amplification was attempted for the detection of rickettsiae in biological samples, we showed that PCR amplification of the 388-bp fragment in the gene encoding the 120-kDa antigen was a highly sensitive method. By Southern blot analysis, 10 ag of pKT12 plasmid (7.4 kb) was detected, and this amount was equivalent to one copy of plasmid (Fig. 4). This result meant that Southern blot analysis after PCR amplification could theoretically detect one copy of rickettsial DNA. Further determination of the limit of sensitivity of the PCR method for detection of rickettsiae in biological samples (for example, 10 rickettsiae in 1 ml of blood) was not performed. Instead, DNAs in samples obtained from the ICR mice experimentally infected with *R. tsutsugamushi* were amplified, and the results were analyzed and compared with those of other diagnostic tests for the verification of the diagnostic potential of this PCR method. In mice inoculated with 5×10^4 ICU of rickettsiae, positive signals were detected by PCR from 2 DAI. Compared with the serological method (IF test), which could detect antibody to *R. tsutsugamushi* only after clinical manifestations developed (7 to 10 DAI), the PCR method was more powerful in the diagnosis of early infection. Our results showed that the PCR is faster than culture for isolating *R. tsutsugamushi* (1 day versus 2 to 3 weeks), although *R. tsutsugamushi* was isolated from samples at 2 DAI (Table 1).

PCR amplification of crudely extracted DNA from infected mice was sensitive enough to detect the residual rickettsiae (Table 1). By using spleen cells, the target sequence in the

TABLE 1. Comparison of the results of various methods for detecting *R. tsutsugamushi*

Detection method	No. of positive samples/total no. of samples tested on DAI[a]:																		
	0[b]	2	4	6	8	10	12	14	16	19	22	25	31	37	44	51	58	64	106
Clinical manifestation[c]				3/3	3/3	3/3	3/3	3/3											
Rickettsial isolation		3/3	2/3	3/3	3/3	3/3	3/3	3/3	2/3										
PCR amplification (blood)[d]		3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	3/3	2/3	2/3	3/3	
PCR amplification (spleen)[d]		3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	+[e]	+				

[a] Blank spaces indicate negative results. * Negative results were also obtained on 89 and 106 DAI.
[b] Samples obtained at 0 day mean samples obtained from mice before inoculation.
[c] Clinical manifestation actually developed from 5 DAI.
[d] The samples which were negative by ethidium bromide staining were confirmed to be negative by Southern blot hybridization. Southern blot hybridization was performed with one sample randomly selected from three samples.
[e] Positive results obtained by only Southern blot hybridization.

TABLE 2. Immunoglobulin titers determined on various DAI

DAI	Titer[a]		
	IgM	IgG	Total immunoglobulin
6	20		30
8	50	10	160
10	210	430	640
12	270	850	1,710
14	320	1,710	2,560
16	430	2,560	2,560
19	320	2,560	2,560
22	420	2,560	2,560
25	640	2,560	2,560
31	530	2,560	2,560
37	430	2,560	2,560
44	320	2,130	2,560
51	430	1,280	1,280
58	320	1,280	1,280
64	210	1,280	1,280
89	80	1,280	1,280
106	80	1,280	1,280

[a] Titers are expressed as the reciprocals of the serum dilution. Antibody titers are means for three samples harvested on the same day.

rickettsial DNA was less efficiently amplified than when blood cells were used, and some nonspecific bands were observed in ethidium bromide-stained gels. It was thought that DNA extracted from the spleens contained a greater proportion of eukaryotic DNA in comparison with that of rickettsial DNA, and this contaminated DNA might interfere with the appropriate amplification by the PCR assay. This effect was true of blood cells as well, but in the case of spleen cells it was more serious, because a large number of cells amassed in the spleen.

We could not demonstrate the viability of the residual rickettsiae detected by PCR, although isolation of rickettsiae was attempted (Table 1). The low rate of isolation of rickettsiae from mice that recovered may have resulted from the low sensitivity of the isolation method. This might be due to the presence of antibodies or other substances in samples from mice that recovered, and these antibodies or substances may interfere with the growth of rickettsiae (4, 14). However, our results agreed with those presented in reports of other studies which demonstrated rickettsial persistence in tissues (19, 21). We thought that the PCR assay could be one of the possible methods for detecting persistent rickettsiae.

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