Two-Step Polymerase Chain Reaction for Diagnosis of Scrub Typhus and Identification of Antigenic Variants of *Rickettsia tsutsugamushi*

Fumihiko KAWAMORI*, Masato AKIYAMA, Masaaki SUGIEDA, Takashi KANDA, Sousuke AKAHANE, Seigo YAMAMOTO¹⁾, Norio OHASHI²⁾, and Akira TAMURA²⁾

Department of Microbiology, Shizuoka Prefectural Institute of Public Health and Environmental Science, 4-27-2 Kitaando, Shizuoka 420, ¹⁾Miyazaki Prefectural Institute for Public Health and Environment, 2-3-2 Kibanadainishi, Gakuen, Miyazaki 889-21, and ²⁾Department of Microbiology, Niigata College of Pharmacy, 5-13-2 Kamishin'ei-cho, Niigata 950-21, Japan

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ABSTRACT. Two-step polymerase chain reaction (nested PCR) method was examined for the diagnosis of scrub typhus. Primers were derived from the type-specific antigen (TSA) gene DNA sequences of *Rickettsia tsutsugamushi*, Gilliam strain. These primers served to produce rickettsiae-specific products in the amplification of template DNA prepared from all serovariants, Gilliam, Karp, Kato, Kawasaki, Kuroki and Shimokoshi strains, and the fragments of product after digestion with several kinds of restriction endonuclease showed the respective patterns to strain in acrylamide or agarose gel electrophoresis. The rickettsiae-specific DNAs were also derived, by this nested PCR, by amplifying DNA from patients' bloods and mites from endemic areas, and the serotype of rickettsiae infected to these hosts could be identified from fragment patterns of the amplified products observed after endonuclease treatment. These results indicate that this PCR is sensitive and specific method not only for detection of rickettsial DNA in patient specimens and in mites, but also for the typing of rickettsiae infected to these hosts.—**KEY WORDS:** diagnosis, polymerase chain reaction, polymorphism, restriction endonuclease, *Rickettsia tsutsugamushi*.

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The scrub typhus, so called tsutsugamushi disease, is a febrile disease caused by the infection of *Rickettsia tsutsugamushi* which is transmitted by mites. The symptom appears suddenly after 1 to 2 week-latent period. Because the antibody titer against *R. tsutsugamushi* has not risen enough to be detected at the initial stage of hospitalization, the timely serodiagnosis of this disease is difficult sometimes. The recently introduced technique of polymerase chain reaction (PCR) [11] is expected to be excellent for the detection of rickettsial DNA in patient specimens of rickettsiemia stage which is seen at the acute phase of illness.

Several antigenic types are recognized in *R. tsutsugamushi*. In addition to Gilliam, Karp and Kato types which were found previously, the other serotypes, such as Shimokoshi, Kawasaki, and Kuroki were recently detected by us [9, 15, 18]. This antigenic variation of rickettsiae depends on the type-specific antigen (TSA) which is an immunodominant 56 kilodalton (kDa) major protein located on the rickettsial outer membrane [14]. The TSA genes were cloned and sequenced from Karp and Gilliam strains by Stover *et al.* [13] and us [7], respectively, and recently from the other 4 strains by us [8]. The TSA genes show 70 to 90% homologies in the nucleotide sequences among the strains of different serotypes, and have 4 variable domains [8].

We described previously an application of PCR method for the diagnosis of this disease [1]. In that case, the amplified 78-bp DNA corresponds to N-terminal side of

mature TSA molecule where the sequences are relatively well conserved among the strains. Since clinical specimens, such as patients' bloods, contain generally very low concentration of rickettsiae even in the rickettsiemia stage, the amplification of long chain DNA by one-step PCR did not result in the detectable amount of target DNA product. The nested PCR (two-step PCR) which was introduced recently [2], however, is expected to amplify a long chain DNA from a small number of the templates showing high specificity and sensitivity. Furthermore, if the long chain DNA product is obtained, the serotype of rickettsiae infected to the patients can be estimated from the comparison of nucleotide sequences with those of standard strains.

Recently, Murai *et al.* reported a nested PCR assay for diagnosis of scrub typhus [5]. In their case, however, the target DNA is a part of gene for the 58-kDa group-specific antigen of *R. tsutsugamushi*. Here we describe the nested PCR as the target DNA corresponding to 56-kDa type-specific antigen for diagnosis of scrub typhus, and the method for identification of rickettsial serotype infected to patients by the electrophoretic analysis of restriction fragments obtained after digestion of PCR-products with endonucleases.

MATERIALS AND METHODS

Rickettsiae: Rickettsiae used were *R. tsutsugamushi*, Gilliam, Karp, Kato, Kawasaki, Kuroki and Shimokoshi strains, and *Rickettsia sibirica* ATCC No. VR151. These rickettsiae were propagated in L-929 cells as described previously [16].

Isolation of rickettsiae from patients and mites, and the

* PRESENT ADDRESS: KAWAMORI, F., The Western Meat Inspection Center, Prefectural of Shizuoka, 93 Kaneshiro, Kakegawa, Shizuoka 436, Japan.

serotyping of the isolates: Bloods of scrub typhus patients were obtained in Shizuoka and Miyazaki Prefectures in 1990 to 1991. Unfed larvae of *Leptotrombidium scutellare* were collected in Shizuoka Prefecture by black plate-method [12] in October in 1991. These samples were used both for the DNA preparation of PCR and for the isolation of rickettsiae. The rickettsiae were isolated in both cyclophosphamide (CY)-treated and CY-untreated ddY mice as described previously [3]. Serotype of the isolated rickettsiae was determined by immunofluorescence (IF) test with strain-specific monoclonal antibodies, each against Gilliam, Karp, Kato, Kawasaki or Kuroki strain as described previously [3].

Assay of IF titers in patient sera: Indirect IF test was performed by the method mentioned previously [3]. Five antigens, Gilliam, Karp, Kato, Kawasaki and Kuroki strains, were used. Fluorescein isothiocyanate labeled anti-human IgG (γ chain specific) and -IgM (μ chain specific) were purchased from Tago Co. (Burlingame, Calif., U.S.A.) and 30-fold dilution of antibodies were used as secondary antibodies.

Preparation of DNA for PCR: Approximately 2×10^6 L-929 cells which were infected with the laboratory strains of *R. tsutsugamushi* at 2 to 5 multiplicity and incubated at 37°C for 4 days, were collected by centrifugation at $11,600 \times g$ for 5 min. The pellet obtained was suspended in 50 μ l of 10 mM Tris-HCl buffer, pH 8.3, containing 0.1 mg proteinase K, 0.5% Nonidet P-40, 0.5% Tween 20 (proteinase-containing buffer). After incubation at 56°C for 1 hr, the mixture was heated at 95°C for 10 min to inactivate proteinase. The preparation obtained was used as DNA sample for PCR without further purification.

To prepare DNA from patients blood, 0.2 to 0.3 g of blood clot, or blood cells collected from heparinized blood by centrifugation, were mixed with 2.5 ml of 1% Triton X-100 in SPG (3.0 mM KH_2PO_4 , 7.2 mM K_2HPO_4 , 40 mM L-glutamic acid and 218 mM sucrose in distilled water, pH 7.0). The blood clot was homogenized with a Dounce homogenizer at 30 strokes, and the cells from heparinized blood were mixed well with a vortex mixer. After incubation for 5 min at room temperature, the preparation was clarified at $200 \times g$ for 5 min and the supernatant was centrifuged at $11,600 \times g$ for 5 min. The pellet obtained was washed twice with SPG by repeating centrifugation at $11,600 \times g$ for 5 min, and then incubated with proteinase-containing buffer as described above.

For the preparation of DNA from chigger mites, 90 mites were pooled and homogenized lightly with teflon potter homogenizer in 1.5 ml SPG by hand-grinding. The homogenate was centrifuged at $11,600 \times g$ for 5 min and the pellet was incubated with proteinase-containing buffer as described above.

Oligonucleotide primers: Oligonucleotide primers were obtained from a commercial source (Bex, Tokyo) and used without further purification. Primers used were: primer a (5'-TACATTAGCTGCGGGTATGACA-3': corresponding to the sequence at 225 to 246 bp downstream from the start codon of TSA gene of Gilliam strain

Fig. 1. Schematic representation of the TSA gene with the primers for primary and secondary PCRs. Primers are indicated by arrows. Signal and VD1 to 4 show the positions corresponding to signal peptide and variable domains 1 to 4 in TSA molecule.

[7]); primer b (5'-CCAGCATAATTCTTCAACCAAG-3': at 536 to 557 bp); primer a' (5'-GAGCAGAGCTAGGTGTTATGTA-3': at 263 to 284 bp); primer b' (5'-TAGGCATTATAGTAGGCTGAGG-3': at 409 to 430 bp). A scheme of the 56-kDa gene and the primer-positions are depicted in Fig. 1.

PCR amplification: The first amplification for nested PCR was carried out in 50 μ l-reaction mixture containing 5 μ l of DNA sample, 200 μ M (each) deoxynucleoside triphosphate (dNTPs), 100 nM (each) primers a and b, and 2 units of *Taq* DNA polymerase in reaction buffer (Promega, Madison, WI, U.S.A.; consisting of 1.5 mM MgCl_2 , 50 mM KCl, 0.01% gelatin in 10 mM Tris-HCl, pH 8.3). The second PCR reaction mixture consisted of the same as above except that 5 μ l of the first PCR reaction mixture was used as DNA sample, and the primers were replaced to 400 nM (each) primers a' and b'. The mixtures were overlaid with mineral oil to prevent evaporation and were subjected to thermal cycles of amplification in an automated DNA thermal cycler (ASTEC PC-700, Astec, Fukuoka). For the first PCR, the cycle (40 cycles) involved heating to 95°C for 30 sec (DNA denaturation), cooling to 50°C for 2 min (primer annealing), and again heating to 72°C for 1 min with an extension of 2 sec per cycle (primer extension). The cycle for the second PCR (25 cycles) involved 95°C for 30 sec, 55°C for 2 min and 72°C for 1 min with an elongation of 3 sec per cycle.

For the single PCR experiment used in some cases, the reaction mixture was the same with the second PCR and the thermal cycles (40 cycles) involved 95°C for 30 sec, 55°C for 2 min and 72°C for 1 min with an extension of 2 sec per cycle.

Analysis of DNA product: The samples (5 or 10 μ l) obtained after PCR or after further treatment with restriction endonuclease were mixed with one tenth amount of 10-fold concentrated loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 50% glycerol in water) for electrophoresis, and fractionated by 2.0% agarose or 12% polyacrylamide gel-electrophoresis. The DNA was visualized by ethidium bromide staining and UV fluorescence. Restriction endonuclease (*Hha*I, *Sfa*NI, *Mn*II, *Alw*NI, *Bgl*II or *Mbo*II) digestion was performed at 37°C for 3 hr in 10 μ l mixture containing 2 μ l of the reaction mixture obtained after PCR, and 1 to 5 units of restriction enzymes in digestion buffer. Southern blotting was performed by enhanced chemiluminescence

(ECL) method [10] using commercially prepared reagents (Amersham, Buckinghamshire, England). Briefly, PCR-amplified DNA was electrophoresed, transferred to nylon membrane and hybridized to a probe prepared as follows. The nested PCR-product obtained by the amplification of Gilliam-infected cell DNA was separated in 2% low-melting-point agarose gel electrophoresis, isolated from the gel, purified by standard procedures [4] and conjugated to horseradish peroxidase (HRP) with glutaraldehyde. After hybridization at 42°C, ECL reaction was catalyzed by the probe conjugated HRP and the target bands were detected by exposure on blue sensitive film (Hyperfilm-ECL, Amersham).

RESULTS

Specificity of the PCR: The PCRs with the pair-primers a/b and a'/b', is expected to produce 306 to 339 bp and 150 to 168 bp polynucleotides, respectively, from the base-sequences of the genes determined by Stover *et al.* [13] and us [7, 8]. Indeed, "single" PCR-products with these sets of primers and the templates of DNAs prepared from L cells infected with individual strains of *R. tsutsugamushi* formed bands in electrophoresis at the position expected in size (Fig. 2A). These bands were hybridized with Gilliam DNA probe prepared by PCR (Fig. 2B). The PCR with DNA from *R. sibirica*-infected cells as template did not produce any bands of product with both sets of primers in the electrophoresis and in the hybridization test, indicating that the amplified products are specific for *R. tsutsugamushi*.

In the case of infected cultured cell, "single" PCR is enough to detect the amplified products because many rickettsial target DNAs were present in the sample. In many cases of patient specimens, however, the "single" PCR with the primers did not produce enough amount of detectable products (data not shown). Therefore nested PCR was examined. Samples used were 10 patients' bloods and 14 pools of *L. scutellare* (90 mites in each pool). Records of 10 patients are shown in Table 1. In the cases of paired bloods samples, the bloods on the earlier day of onset were used for the PCR and for the isolation of rickettsiae. Rickettsiae were isolated from the bloods of nos. 1 to 7 patients only in CY-treated mice, and the serotypes of rickettsiae were defined as Kuroki or Kawasaki type. From the patients nos. 8 and 9, rickettsiae were isolated both in CY-treated and -untreated mice, and the serotype was Karp. The earlier day-sera of patients nos. 1 to 4 showed less than IF titers of 1:20 but rickettsiae were isolated from the bloods. The patient no. 10 showed high IF-titer in IgG but less than 1:20 in IgM, suggesting that this patient received re-infection.

When the DNA from these samples were amplified in the condition of first PCR with the set of primers a/b, clear bands of products corresponding to 300 to 340 bp in length were not always seen in the electrophoresis (data not shown). However, when the reaction products of the first PCR were amplified by the second PCR with the set of

Fig. 2. Agarose gel electrophoresis of single PCR-products with the sets of primer a'/b', and a/b by using template DNAs which were extracted from L cells infected with individual strains of *R. tsutsugamushi*. Panel A: Electrophoretic patterns observed by staining with ethidium bromide. Panel B: Hybridization profile of bands in panel A with PCR-amplified Gilliam-DNA probe. M: Molecular marker prepared by *TaqI* + *DraI* digestion of ϕ X 174 RFI DNA (Nippon Gene Co., Ltd.), G: Gilliam, KP: Karp, KT: Kato, KW: Kawasaki, KR: Kuroki, SH: Shimokoshi, Rs: *R. sibirica*. The numbers at the left side show the sizes in bp.

primers a'/b', the products were clearly observed in the electrophoresis (Fig. 3A, lanes 1 to 10), even from the samples of patient nos. 1 to 4, of which the sera showed no detectable IF-titers. On the other hand, Kawasaki-type rickettsiae were isolated from only 2 of 14 pools of mites in CY-treated mice, and these 2 pools showed positive in PCR test (Fig. 3C, lanes 11 to 24). These positive bands formed in the electrophoresis were ascertained to hybridize with the amplified DNA probe of Gilliam strain (Fig. 3B and D), indicating that these products have sequences specific to *R. tsutsugamushi*.

As a negative control, the DNA from bloods of convalescent patients who received the chemotherapeutic treatments (the later day blood of paired samples of nos. 3, 4 and 6 patients in Table 1) and from 20 bloods obtained from the Blood Bank in Japanese Red Cross Society were examined by this nested PCR method. In all cases, no

Table 1. Records on the patients from whom the blood samples were obtained for the present study^{a)}

Sample no.	Place (year)	Bloods collected: Day after onset of illness	State of blood obtained	IF titers in sera ^{b)}										Isolation of rickettsiae in mice treated with:		Typing of isolated rickettsia
				IgG					IgM					CY	w/oCY	
1	Miyakonojo in Miyazaki (1990)	8	Blood clot	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	+	-	Kuroki
		23		<20	40	80	<20	1280	20	80	40	20	160			
2	Oyama in Shizuoka (1991)	9	Heparinized	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	+	-	Kawasaki
3	Gotemba in Shizuoka (1991)	7	Heparinized	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	+	-	Kawasaki
		14		5120	2560	1280	10240	2560	160	<20	<20	160	<20			
4	Gotemba in Shizuoka (1991)	8	Heparinized	<20	<20	<20	<20	<20	<20	<20	<20	<20	<20	+	-	Kawasaki
		17		640	160	160	1280	160	320	20	20	640	80			
5	Oyama in Shizuoka (1991)	11	Heparinized	640	320	320	1280	320	640	<20	<20	640	<20	+	-	Kawasaki
		16		640	80	80	5120	160	1280	20	20	640	80			
6	Oyama in Shizuoka (1991)	8	Heparinized	80	40	40	80	80	<20	<20	<20	80	<20	+	-	Kawasaki
		16		640	320	320	2560	640	640	160	160	1280	160			
7	Gotemba in Shizuoka (1991)	10	Heparinized	160	20	<20	640	40	<20	<20	<20	320	<20	+	-	Kawasaki
8	Oyama in Shizuoka (1991)	8	Heparinized	1280	10240	1280	1280	5120	1280	5120	2560	160	2560	+	+	Karp
9	Ito in Shizuoka (1991)	11	Blood clot	160	640	160	160	640	160	640	160	160	320	+	+	Karp
10	Gotemba in Shizuoka (1991)	6	Heparinized	320	640	160	320	640	<20	<20	<20	<20	<20	ND	ND	
		25		5120	5120	640	1280	2560	<20	<20	<20	<20	<20			

a) Abbreviation: G: Gilliam, KP: Karp, KT: Kato, KW: Kawasaki, KR: Kuroki, CY: cyclophosphamide, w/o: without, ND: not done. b) Titers are expressed as the reciprocal of the antibody dilution which shows positive fluorescence.

detectable bands were observed after electrophoresis, indicating that this nested PCR can be used as a specific diagnostic method for scrub typhus.

Sensitivity of the nested PCR: The average number of rickettsiae to an L cell in a culture infected with *R. tsutsugamushi* was determined in a smear stained by IF-method, and then DNA was prepared from the known amount of cells. In an experiment, DNA was prepared from 2.57×10^6 infected cell culture in which the ratio of rickettsiae to cell was 1.83. The DNA in 50 μ l were 10-fold serially diluted, and an aliquot of 5 μ l at each dilution, was tested with nested PCR. Positive bands were observed by 10^{-5} dilution, indicating that the 4.7 rickettsiae in 5 μ l sample gave positive results.

Digestion of the product of nested PCR with restriction enzymes: When the nested PCR-products obtained from respective standard strains were digested with restriction enzymes, *Hha*I, *Sfa*NI, *Mn*II, *Alw*NI, *Bgl*II and *Mbo*II, patterns specific to individual strain were observed in the electrophoresis (Fig. 4). *Hha*I digested the amplified

DNA of Gilliam, Kawasaki and Kuroki. The fragments from Gilliam and Kuroki strains showed the same pattern, but the Kawasaki strain showed a distinguishable pattern. *Sfa*NI digested the DNA from Karp, Kato, Kuroki and Shimokoshi strains, while the Karp and Kuroki strains showed similar patterns. In *Mn*II digestion, the fragments from Kawasaki and Shimokoshi strains showed the same patterns, but each of the other strains showed a unique pattern. *Alw*NI digested the DNA from Gilliam, Karp, Kawasaki and Kuroki strains, and the Gilliam, Kawasaki and Kuroki strains showed similar patterns. On the other hand, the treatments with *Bgl*II and *Mbo*II resulted in digestion of amplified DNAs from Kato and Karp strains, respectively. These results indicate that the amplified products obtained from each strain can be distinguished from the digestion patterns with several restriction enzymes.

This method was applied to the amplified DNAs in the nested PCR of patients' bloods and mites' samples. The products of Fig. 3 were digested with *Hha*I and *Sfa*NI (in

Fig. 3. Agarose gel electrophoresis of nested PCR-products with the DNAs obtained from patient bloods and pools of mites. Panels A and C: Ethidium bromide staining. Panels B and D: Hybridization profiles of bands in panels A and C, respectively, with PCR-amplified Gilliam-DNA probe. The numbers on the top of each lane indicate the sample nos. Lanes 1 to 10 correspond to the patient nos. in Table 1, and lanes 11 to 24 are the samples of mites pools.

Fig. 5). The product of patient no. 1 showed the same digestion patterns with that of Kuroki strain in *Hha*I digestion, and with those of both Kuroki and Karp strains in *Sfa*NI digestion. The PCR product of Gilliam strain was digested with *Hha*I showing same pattern with that of

Fig. 4. Restriction fragments of nested PCR products with endonuclease *Hha*I (A), *Sfa*NI (B), *Mnl*I (C), *Alw*NI (D), *Bgl*II (E) and *Mbo*II (F) in polyacrylamide gel electrophoresis. The letters on the top of columns of (A) to (F) are the same as those in Fig. 2.

Kuroki strain, but not digested with *Sfa*NI (see Fig. 4). Therefore, the rickettsia infected to patient no. 1 was defined as Kuroki type. The PCR products of patient nos. 2 to 7 and of mites nos. 13 and 23 were not digested with *Sfa*NI, but the rickettsial serotype infected to these hosts were identified as Kawasaki type from the digestion pattern with *Hha*I. The rickettsiae infected to patient nos. 8 to 10 were defined as Karp type from the behaviors after the treatment with both enzymes. Thus, the serotypes of rickettsiae from patients and mites were identified from the combination of digestion patterns with several kinds of endonucleases, and the results coincided to the serotype determined by the serological analyses of isolated rickettsiae, except the case of patient no. 10 from whom the rickettsia was not isolated.

DISCUSSIONS

Symptom of scrub typhus appears suddenly and, if timely diagnosis and quick adequate treatment delay, pneumonia, encephalitis and, in some cases, disseminated vasculature coagulation occur. The present study demonstrated that this PCR method has high sensitivity and specificity and is useful for diagnosis at the early phase of this disease.

R. tsutsugamushi includes several antigenic variants, and recent reports described that the distribution of these

Fig. 5. Polyacrylamide gel electrophoresis of *Hha*I (A) and *Sfa*NI (B) restriction fragments of nested PCR products obtained in the experiments of Fig. 3. The letters and numbers on the top of each column are the same as those in Figs. 2 and 3.

serotypes is different in each endemic areas. For example, main types of rickettsiae isolated from patients in Niigata Prefecture, the northern part along Japan Sea side of Honshu, are Gilliam and Karp [6], but all rickettsiae isolated from patients in Miyazaki and Kagoshima Prefectures, the southern area of Kyushu, are classified into either Kawasaki or Kuroki type [17]. In Shizuoka Prefecture, the middle southern area of Honshu along the Pacific Sea side, 3 serotypes of rickettsiae, Karp, Kawasaki and Kuroki are isolated [3]. The serotype of rickettsiae infected to patients is not identified clearly from the cross-reactivity of patient sera with the different serotypes of antigens, because the sera cross-react with several kinds of antigens without showing clear specificity. Therefore the serotyping is performed finally after the isolation of rickettsiae from the patients. The nested PCR described here amplified directly the rickettsial DNA of TSA gene from patient blood and the serotype of the rickettsiae was determined from the digestion patterns with restriction endonucleases. This is a simple and easy method for serotyping of rickettsiae infected to patients, and more

precise distribution of rickettsiae in each endemic area is expected to be clarified by this method.

TSA genes of six serotype rickettsiae were cloned and sequenced by us and Stover *et al.* [7, 8, 13]. Each primer designed from the sequence of Gilliam strains in the present study has 1 to 4 unpaired bases to the DNA of TSA genes from the other serotype strains. Nevertheless, the primers produced PCR-products from all strains, although the PCR-method of our previous study [1] failed to amplify the DNA of Shimokoshi strain.

The sequencing results of TSA genes [8] indicated that the genes contain 4 variable domains where the sequences differ greatly among strains. The set of primers a/b for the first PCR amplifies a part of the TSA gene containing variable domains 1 and 2, and the pair of primers a'/b' for the second PCR amplifies the area including variable domain 1. The divergences of the base sequences among the different serotypes in the amplified area with the primers a'/b' (124 bp from 3'-end of primer a' to 5'-end of primer b') are as follows: Gilliam and Kuroki strains have only 3-bp replacements; 15- to 45-bp replacements exist in the other strains in the comparison with the sequence of Gilliam strain; the length of amplified polynucleotide sequences of Kato, Kawasaki and Shimokoshi strains are 9- to 18-bp-shorter by base-deletions than those of Gilliam, Karp and Kuroki strains. This variation of sequences in each strain is recognized as the difference of fragment patterns in the digestion with restriction endonucleases. Amplified DNAs from Gilliam and Kuroki strains have high homology but both are distinguished by *Sfa*NI and *Mn*II digestions (Fig. 4B and 4C). In spite of these variations in the sequences of amplified products, products obtained from all strains hybridized with the probe originated from Gilliam strain. The degree of hybridization depended on the homology, such as the probe showed high affinities to the products of Gilliam and Kuroki strains but low to those of Kato and Shimokoshi strains (Fig. 2B).

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