Detection of *Bacillus piliformis* by Specific Amplification of Ribosomal Sequences

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In an effort to explore a sensitive species-specific detection system using the polymerase chain reaction (PCR) for *B. piliformis*, we sequenced 16S ribosomal DNA (rDNA) of the organism (MSK strain) isolated from the mouse and compared it with known rDNA sequences of the RJ strain isolated from the rat. Sequence homology between the MSK strain and the RJ strain was over 97%, but homology between the MSK strain and other bacterial species was less (70~83%). The results indicated that the sequences included *B. piliformis* species-specific regions. On the basis of the sequences, we designed a PCR primer set which amplifies *B. piliformis* rDNA specifically. The PCR with the primer set detected not only these two strains but also an HN strain of hamster origin, although it did not detect other organisms. Therefore, this primer set was considered to be specific for *B. piliformis* species. More than one organism (RJ strain) could be detected by the PCR method. Nine Jcl:Wistar rats were infected perorally with 2×10^4 RJ strain organisms, and three rats each were sacrificed on days 1, 3 and 5 postinoculation (p.i.) to investigate the presence of the organism in the liver, heart, cecum, spleen and mesenteric lymph nodes by PCR and the immunofluorescence test. On days 1 and 3 p.i., *B. piliformis* was not detected in any tissues of the six rats, but *B. piliformis* was detected in two of the three rats sacrificed on day 5 p.i. The presence of the pathogen was seen in both liver and heart (1/3), or in the cecum (1/3) by both methods. The results of this study demonstrate that the 16S rDNA sequence of MSK strain is well conserved at the species level and the PCR with the primers selected is useful for the sensitive and specific species-identification of *B. piliformis*.—KEY WORDS: *Bacillus piliformis*, polymerase chain reaction, 16S rDNA

Tyzzers' disease, which is caused by infection with *Bacillus piliformis*, was first described by Tyzzer in 1917 [17], and is characterized by multifocal necrotizing hepatitis, carditis and/or severe hemorrhagic enteritis [16]. *B. piliformis*, named by Tyzzer, is a gram-negative, spore-forming, obligate intracellular parasite [9], which has not yet been successfully cultivated in artificial media, and whose real classification is still not clear. The diagnosis of *B. piliformis* infection has been based on the microscopic detection of the organism in tissues by histochemical or immunochemical staining and serological assay, such as the complement fixation test, immunofluorescence (IF) test and enzyme-linked immunosorbent assay (ELISA) [2,5,6].

The polymerase chain reaction (PCR) has been shown to be an extremely sensitive tech-

nique for the detection of infectious agents [10]. Wilson *et al.* [18] have recently amplified bacterial 16S rDNA sequences by PCR using synthetic oligonucleotide primers complementary to universally conserved 16S rDNA sequences in eubacteria.

In this study, we initially amplified the 16S rDNA of *B. piliformis* MSK strain isolated from the mouse using the universal primers, sequenced the PCR product and compared the sequence with those of the RJ strain from the rat (Tsuji *et al.*, DDBJ, D14638) to detect conserved sequences at the species level. Using the conserved sequence data and other bacterial sequences, we selected the PCR primers that reacted specifically with the species, and then evaluated the specificity and sensitivity of this technique.

Materials and Methods

Bacillus piliformis: Three strains of *B. piliformis* were used; the RJ strain, which was isolated from the rat [7], MSK strain, which was isolated from the mouse [3], and HN strain, which was isolated from the hamster [12]. The RJ strain was grown in BRL 3A cells as described by Riley *et al.* [14]. The MSK and HN strains were kindly provided by Dr. K. Fujiwara, Department of Veterinary Pathology II, Nihon University Veterinary School, Japan, and each strain was inoculated intravenously into mice with prednisolone-treatment as described previously [8]. The infected BRL 3A cells and liver tissues were harvested 4~7 days postinfection (p. i.) and were frozen at -80°C until use.

Extraction of DNA: Homogenates of infected BRL 3A cells or organ samples were mixed with a solution containing 50mM Tris-HCl (pH 8.0) and 50mM EDTA. After freezing and thawing, 250µg of lysozyme was added to 250µl of the mixture, and kept on ice for 45 min. The mixture was combined with 50µl of STEP buffer (0.5% SDS, 50mM Tris-HCl [pH 7.5], 0.4M EDTA and 1mg/ml proteinase K) and incubated at 50°C for 60min. Then 300µl of TE buffer (10mM Tris, 1mM EDTA, pH 8.0) was added to the mixture. The DNA was extracted by mixing with an equal volume of phenol-chloroform-isoamyl alcohol (50 : 48 : 2). After another extraction with chloroform-isoamyl alcohol, the DNA was ethanol-precipitated and the pellet was dissolved in TE buffer.

PCR and gel electrophoresis: The 16S rDNA sequence of *B. piliformis* was amplified by the PCR method [15]. The amplification reaction was performed in a volume of 50µl containing 10µl of the sample DNA, 15.7µl of sterile double-distilled water, 8.3µl of 6x PCR buffer (400mM Tris-HCl [pH8.8], 100 mM ammonium sulfate, 40mM MgCl₂, 60mM 2-mercaptoethanol, 40µM EDTA and 1 mg/ml Bovine serum albumin), 5µl of deoxynucleotides (15mM each), 5µl of 10µM primers and 2.5 units of *Taq* polymerase (GIBCO BRL).

Primers for the first PCR were based on a previously published sequence conserved in bacterial 16s rDNA [11,18] with some modifi-

cations. The universal primers were 16S sense (5'-AACAGGATTAGATACCC-3') and 16S anti-sense (5'-TGACGGGCGGTGTGTACAA-3'). The expected product of amplification of the target DNA produced by PCR with these primers was 625 bp in length. Primers for the *B. piliformis*-specific PCR were developed during the present study. A set of oligonucleotide primers for the second PCR, RJ-1 (5'-GTGCTAGGTGTTGGGAAG-3') and RJ-2C (5'-TACTTTACGTAGCCTGTCAA-3'), was selected on the basis of the subunit 16S rDNA sequences commonly conserved among *B. piliformis* MSK and RJ strains. The expected PCR product was 196 bp in length. These oligonucleotides were synthesized in a DNA synthesizer (391 EP; Applied Biosystems) by the phosphoramidite method. Reaction mixture samples of 50µl were amplified in a DNA thermal cycler (Hybaid), which was programmed for 25 cycles consisting of 2 min at 92°C (denaturation), 2 min at 50°C (primer annealing) and 2 min at 70°C (primer extension-polymerization). The amplified products were subjected to electrophoresis in 4% NuSieve GTG agarose (FMC Bioproducts) gel and stained with ethidium bromide.

DNA sequencing: DNAs from MSK strain amplified by PCR using the universal primer were cloned into the TA cloning™ vector (Invitrogen, U.S.A.). Plasmid preparations from a single colony were sequenced with a Sequenase® kit (USB, U.S.A.).

Experimental *B. piliformis* infection in rats: Nine female Jcl:Wistar rats aged 3 weeks were inoculated perorally with 2×10⁴ bacterial spores. Three rats each were sacrificed on days 1, 3 and 5 p. i., and the liver, heart, spleen, cecum and mesenteric lymph nodes were collected. The organs were divided into two parts. One part of the tissue samples was fixed in 10% buffered formalin, and the sections were processed according to the IF test for detection of *B. piliformis*. The IF test was performed as described previously [6]. The other part of the tissue samples was used for PCR.

Results

Comparison of 16S rDNA sequence of the MSK strain with that of the RJ strain: As shown in Fig. 1, lanes 3 to 5, a DNA fragment

Fig. 1. Agarose gel analysis of PCR products amplified from BRL 3A cells, *E. coli* and *B. piliformis* strains. DNAs of BRL 3A cells, *E. coli* and different *B. piliformis* strains were amplified with a primer set, 16S sense and 16S anti-sense (lanes 2 to 5), and a second primer set, RJ-1 and RJ-2C (lanes 6 to 10). Lanes 2 and 6, BRL 3A cell; lanes 3 and 7, *E. coli*; lanes 4 and 8, RJ strain; lanes 5 and 9, MSK strain; lane 10, HN strain. Lane 1 contains DNA molecular weight markers.

of the expected size (625 bp) was amplified from *Escherichia coli* (*E. coli*), RJ strain and MSK strain using the universal primers, but DNA from non-infected BRL 3A cells was not amplified. The 16S rDNA gene sequences of the 625 bp from two strains of *B. piliformis* and *E. coli* are shown in Fig. 2. The homology of the two strains was over 97%. The rates of homology between the MSK strain and other bacterial species were 79.8% (*E. coli*), 68.9% (*Halobacterium halobium*), 81.8% (*Pseudomonas aeruginosa*) and 82.9% (*Clostridium ramosum*). Additional oligonucleotide primers specific for *B. piliformis* were synthesized on the basis of the sequences as shown in Fig. 2.

Specificity and sensitivity of *B. piliformis*-specific PCR: PCR-products of the same size were amplified from both MSK and RJ strains, when the specific primers were used. The HN strain also showed the same results as the two strains, as can be seen in Fig. 1, lanes 8 to 10. However, the PCR primers used in this study did not amplify 16S rDNA sequences from *E. coli* (Fig. 1, lane 7) and other bacteria (e. g., *Pastrurella multocida*, *Bordetella bronchise-*

Fig. 3. Sensitive detection of *B. piliformis* by PCR. *B. piliformis* (RJ strain) samples were prepared by serial dilutions of infected rat liver, and were subjected to PCR amplification using the specific primers RJ-1 and RJ-2C. The PCR products were analyzed by gel electrophoresis. The number of bacteria contained in each sample was 1×10^2 (lane 2), 10^1 (lane 3), 10^0 (lane 4), 10^{-1} (lane 5), 10^{-2} (lane 6) and 10^{-3} (lane 7). Lane 1 contains DNA molecular weight markers.

ptica, *Streptococcus zooepidemicus*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Corynebacterium kutscheri*) (data not shown) or DNA from non-infected BRL 3A cells (Fig. 1, lane 6). The sensitivity was tested quantitatively by diluting the infected rat liver homogenates. The DNA samples were efficiently amplified up to one organism per PCR solution, as shown in Fig. 3.

Experimental infection in rats: No clinical signs were observed in any rats throughout the experiment. Gross lesions were found in both the liver and heart of one rat on day 5 p. i. As shown in Table 1, *B. piliformis* was detected in the liver and heart (1/3) and the mesenteric lymph nodes (1/3) from two rats sacrificed on day 5 p. i. by both PCR and IF test, but *B. piliformis* was not detected by these methods in any samples from six rats sacrificed on days 1 and 3 p. i. and from one rat sacrificed on day 5 p. i.

(16S S) (RJ-1)

MSK 5' AACAGGATTAGATACCTGGTAGTCCACGCTGTAAACGATG---AGTGCTAGGTGTTGGGAAGAAATTCTCGG
 RJ 5' *****
 E. c 5' *****C*****TCG*C*TGG***TG*CCCTTG*GGCG*G*C 140

MSK 5' TGCCCGCAGCAAACGCAATAAGCACTCCACCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGA
 RJ 5' *****
 E. c 5' *T***G***T***GT***TCGA**G*****G*****A*****T***** 210

MSK 5' CGGGGACCCGCACAAGCGGTGGAGCATGTGGTTAATTCTGAAGCAACGCGAAGAACCTTACCTAAACTTG
 RJ 5' *****GC*****AG*****
 E. c 5' *****T*****GGT*** 280

(RJ-2C)

MSK 5' ACATACCATTGACAGGCTACGTAAAGTAG--CTTTCCTTCGGGGCAATGGATACAGGTGGTGCATGGTTGTC
 RJ 5' *****T*****A*****
 E. c 5' ***-***CG*-**TT*T*AG*G*TG**AATG*G*****AACCGT**G*****C*****C*** 350

MSK 5' GTCAGCTCGTGTCTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTCTTAGTAGCCAGCA
 RJ 5' *****N*****
 E. c 5' *****T***A*****T***C***T***T*****G 420

MSK 5' CTTTGGGTGGGCACTCTAAGGAGACTGCCGTGGATAACACGGAGGAAGGTGGGGATGACGTCAAATCATC
 RJ 5' *C*****G*****
 E. c 5' G*CC**CC***A***A*****AGT*****ACT*****G***** 490

MSK 5' ATGCCCTTATGTTTAGGGCTACACACGTGCTACAATGGCTATAACAAAGTGAAGCGAGACAGTGATGTG
 RJ 5' *****C*****A*****
 E. c 5' ***G*****C*ACC*****GCAT*****A*****CCTC***GAGC 560

MSK 5' GAGCAAAGCACATAAAAGTAGTCCCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGTTGGAATCG
 RJ 5' *****A*****
 E. c 5' A***GG*C*T*****GTGC***GT***C*****G*****C*****C***** 620

(16S A) 620

MSK 5' CTAGTAATCGCGAATCAGAATGTCCGGTGAATACGTTCCCGGTCTTGTACACACCGCCCGTCA
 RJ 5' *****
 E. c 5' *****T*G*****C*A*****C*****

Fig. 2. DNA sequences of *B. piliformis* and *E. coli* 16S rDNA genes and sequences of universal and specific primers. The primers 16S sense (S) and 16S anti-sense (A) were used as universal primers. The primers RJ-1 and RJ-2C were used to amplify specifically 16S rDNA gene sequence from *B. piliformis*. Identical (*) nucleotides are indicated.

Table 1. Detection by PCR of *B. piliformis* in organs from experimentally infected rats

	Days p. i.		
	1	3	5
Liver	0/3	0/3	1/3
Heart	0/3	0/3	1/3
Cecum	0/3	0/3	1/3
Spleen	0/3	0/3	0/3
Mesenteric lymph nodes	0/3	0/3	0/3

Discussion

In this study, one third of the 16S rDNA sequence of *B. piliformis* (MSK strain) was determined, and we utilized the sequence data for *B. piliformis*-specific PCR. The homology between the MSK strain and RJ strain was over 97%, but when the sequences were compared with those of other bacteria using the GenBank data base, the homology was less (70~83%). It was suggested that the regions that were well conserved at the *B. piliformis* species level were included in the sequence determined here.

The *B. piliformis*-specific origonucleotide primer set used in this study was designed using sequences to amplify conserved regions shared by two strains of *B. piliformis* isolated from a mouse and a rat. Using the primer set, not only the two strains but an antigenically different HN strain (hamster origin) [12] could be detected, but other organisms were not detected. The fact that at least three strains of *B. piliformis* could be detected by this PCR indicated the possibility that the primer set reacts widely with *B. piliformis* strains.

The most remarkable advantage of the PCR system is its sensitivity. We were able to detect one organism per PCR mixture using PCR which was readily detectable by ethidium bromide staining. The sensitivity of the *B. piliformis*-PCR was similar to that of other bacterial PCR [1,13]. We assume that this high sensitivity is due to multiple copies of the 16S rDNA gene in the bacteria. *B. piliformis* could be detected by both PCR and IF test in 2 of 3 experimentally infected rats sacrificed on day 5 p. i.

Diagnosis of *B. piliformis* infection in rats is generally based on clinical and histopath-

ological observations, and serological tests. However, Tyzzer's disease is frequently subclinical [4], and histopathological examinations are very time consuming. Serological tests for *B. piliformis* that are useful in confirming the infection are not applicable to diagnosis in the early stage of this infection, and serological tests such as ELISA and the IF test may not be sufficiently sensitive to detect antibodies to heterologous antigens, unless the isolates are closely related antigenically [14], since many reports have suggested antigenic diversity among the isolates [3,14]. On the other hand, PCR may be more useful in detecting the presence of *B. piliformis* species in the very early stages of infection. The combination of both PCR and serological tests may provide additional help in the diagnosis of this infection.

In conclusion, we have demonstrated that the 16S rDNA-based PCR established here is useful for sensitive and specific identification of *B. piliformis* in infected animals.

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リボゾーム DNA 増幅によるティザー菌の検出

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ティザー菌を PCR 法を用いて、高感度かつ特異的に検出する目的で、マウス由来ティザー菌 (MSK 株) の 16S リボゾーム DNA (rDNA) の塩基配列を決定し、その配列を RJ 株 (ラット由来) の配列と比較した。決定された配列は MSK 株および RJ 株で相同性が高かったが (97%以上)、他の菌種との相同性は低かった (70~83%) ことからティザー菌種に特異的配列を含んでいると考えられた。そこで、PCR 法で Tyzzer 菌種を特異的に検出するプライマーをこれらの塩基配列をもとに選択した。選択されたプライマーを用いた PCR 法では、ティザー菌 2 株 (MSK 株および RJ 株) の他、ハムスター由来の HN 株を検出できたが、ティザー菌以外の細菌は検出されず、ティザー菌種に対して高い特異性を示した。また、1 個以上のティザー菌

(RJ 株) が検出可能であった。さらに、9 匹の Jcl: Wistar ラットに 2×10^4 の菌を経口的に実験感染させ、接種後 1, 3 および 5 日目にそれぞれ 3 匹ずつ肝臓、心臓、盲腸、脾臓および腸間膜リンパ節を採材し PCR 法および蛍光抗体法を用いて菌検索を行った。接種後 1 および 3 日目の材料からはいずれの方法においても菌は検出されなかった。しかし、接種後 5 日目のラットのうち 1 匹は肝臓および心臓から、他 1 匹は盲腸からそれぞれ両方法によって菌が検出された。これらの結果から、今回明らかにされた MSK 株 16S rDNA の塩基配列はティザー菌種間でよく保存されていること、さらに選択されたプライマーを用いた PCR 法が高感度かつ特異的なティザー菌診断法として有用であることが示唆された。