PCR-dot blot hybridization based on the neuraminidase-encoding gene is useful for detection of *Bacteroides fragilis*

Tomomi Kuwahara, Haruyuki Nakayama, Tsuyosi Miki, Keiko Kataoka, Hideki Arimochi, and Yoshinari Ohnishi

Department of Bacteriology, The University of Tokushima School of Medicine, Tokushima, Japan

Abstract : *Bacteroides fragilis* is a Gram-negative obligate anaerobe frequently isolated from clinical specimens and sometimes causes severe septicemia in compromised hosts. Increasing interest has been shown in the enterotoxigenicity and drug resistance of *B. fragilis* in the field of medical microbiology. We previously reported rapid detection of this anaerobe by nested PCR targeting a neuraminidase-encoding gene *nanH*. In the present study, we synthesized a digoxigenin-labeled oligonucleotide probe, NH1,which is specific for *nanH* of *B. fragilis*, and we combined the hybridization assay using NH1 with the *nanH*-PCR to detect this anaerobe in a bacteremia model mice. In the specificity test, the oligonucleotide probe, NH1, hybridized only to amplification products from *B. fragilis*. PCR-dot blot hybridization based on *nanH* enabled detection of cells of *B. fragilis* in blood samples even when the number was as low as 2×10^3colony-forming units/ml. These findings suggest that PCR-dot blot hybridization targeting *nanH* is a useful procedure for diagnosis of septicemia caused by *B. fragilis* when viable cells in blood cannot be detected by the traditional culture techniques. J. Med. Invest. 48 : 60-65, 2001

Keywords : *Bacteroides fragilis, neuraminidase, oligonucleotide probe, septicemia, PCR*

INTRODUCTION

The majority of anaerobic isolates from clinical specimens belong to the genus *Bacteroides* (1). The species included in the "*B. fragilis* group" are considered to be clinically important pathogens associated with intra-abdominal infections and abscess formation in soft tissues (2). Among these species, *B. fragilis* is the most virulent because this species accounts for over half of the anaerobes isolated from human infections and often causes severe septicemia with a high mortality rate in compromised hosts (2, 3). Early diagnosis and treatment with appropriate antibiotics are needed for patients infected with *B. fragilis*, but the traditional culture methods for anaerobes are labor-intensive and time-comsuming. In addition, if the clinical samples are not immediately cultured or kept under anaerobic conditions, obligate anaerobes are often not detected in blood cultures. Various techniques, including analysis of electrophoretic patterns of dehydrogenase (4), bacteriophage typing (5), analysis of cellular sugar and lipid compositions (6-8) and serology (9-11), have been used for rapid detection and discrimination of this anaerobe. However, all of these techniques require viable cells and many troublesome steps, and none of them have sufficient specificity and sensitivity to be used for clinical specimens.

Recently, molecular biology-based techniques have been shown to be useful for the rapid identification of many pathogenic microoganisms (12, 13). The polymerase chain reaction (PCR) targeting a specific gene is the most widely used technique in

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Address correspondence and reprint requests to Yoshinari Ohnishi, M.D., PhD., Department of Bacteriology, The University of Tokushima School of Medicine, Kuramoto-cho, Tokushima 770-8503,Japan and Fax : +81-886-33-7069.

diagnostic laboratories because it is quick and it is suitable for the handling of a large number of specimens (14). Furthermore, PCR amplification enables the detection of pathogens even in culture-negative clinical specimens, since this procedure does not necessarily require viable cells (15). However, PCR amplification sometimes produces false-positive results if other bacteria have sequences similar to those of the designed PCR-primers. In a previous study, we synthesized two primer sets, F1-R1 (outer primer set) and F2-R2 (inner primer set), and used them in nested PCR to amplify the neuraminidase-encoding gene *nanH* of *B. fragilis* (16). Although these primer sets specifically amplified a part of the *nanH* gene of *B. fragilis*, one of the *Bacteroides* species, *B. vulgatus*, which possesses high neuraminidase activity, also produced a single band identical to that of *B. fragilis* in size when the primer set F1-R1 was used, and this false-positive band could not be excluded by electrophoresis alone. In such a case, hybridization tests with probes specific for the target are usually required to confirm the specificity of the PCR amplifications.

In the present study, we developed a digoxigenin-labeled oligonucleotide probe (named NH1), which was specific for *nanH* of *B. fragilis*, and we used it in combination with *nanH*-PCR for a hybridization assay. We could specifically detect *B. fragilis* in blood samples when PCR of a part of the gene *nanH* and dot-blot hybridization using NH1 were applied to model mice with bacteremia induced by challenge of this anaerobe.

MATERIALS AND METHODS

Bacterial Strains

Sixty strains of *B. fragilis*, including two reference strains (ATCC25285 and NCTC9343), were used in this study. The strains of other species used were *B. distasonis* ATCC8503, *B. eggerthii* ATCC27754, *B. ovatus* ATCC8483, *B. thetaiotaomicron* ATCC29148, *B. uniformis* ATCC8492, *B. vulgatus* ATCC8482, *Porphyromonas asaccharolytica* ATCC 25260, *P. endodontalis* ATCC35406, *P. gingivalis* 381 and *Prevotella corporis* JCM8529. All strains were cultured in GAM broth (Nissui Pharmaceutical Co., Tokyo, Japan) at 37°C under anaerobic conditions.

Preparation of Bacterial Cells for PCR

A late log-phase culture (1 ml) of each strain was centrifuged, washed with 1 ml of phosphate-buffered saline, and resuspended in 0.1 ml of distilled water. Each suspension was lysed by heating at 100°C for 10 min, and 10 μl of the lysed preparation was used for PCR amplification of the *nanH* gene. PCR amplification of the *nanH* gene was performed as described previously (16).

Design of an Oligonucleotide Probe

We compared the nucleotide sequences of the *nanH* structural gene of *B. fragilis* strains YCH46 and TAL2480. The oligonucleotide probe NH1 was synthesized on the basis of the common sequence found within F2-and R2-primer annealing sites. NH1 was labeled with digoxigenin using a DIG-labeling Kit (Boehringer GmbH, Mannheim, Germany) according to the manufacturer's instructions. The nucleotide sequence of NH1 was 5'-ATCACTATGAGTGACGGTACTTTGGTATTCCC-3'.

Dot Blot Hybridization

After PCR amplification of the *nanH* gene, each reaction mixture was heated at 95°C for 5 min and chilled on an ice bath. Then, 5 μl of each amplification mixture was spotted on a nylon membrane and UV-fixed. Southern hybridization was performed as described by Sambrook *et al*. (17). The hybridization with digoxigenin-labeled NH1 was perfomed in 10 ml of rapid hybridization buffer (Amersham Co., Ltd.) at 54°C for 1 hour. Post-hybridization washes were performed twice at 54°C with each washing buffer, 2×SSC (1×SSC being 0.15 mM NaCl plus 15 mM sodium citrate)-0.1% SDS and 0.1×SSC-0.1% SDS, respectively. The hybridization signals were detected according to the manufacturer's instructions using an alkaline phosphatase-labeled anti-digoxigenin antibody.

Preparation of Blood Samples

Three C57BL/6J mice were intraperitoneally injected with viable cells of *B. fragilis*. Blood samples (0.2 ml) were collected by cardiac puncture at 1, 3 and 6 hours after injection, and 0.1 ml of each sample was incubated anaerobically on GAM agar plates. The remainder of the samples were centrifuged, washed with 1 ml of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0), and resuspended in 0.1 ml of lysis buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1 mg of proteinase K per ml).

Each suspension was incubated at 65°C for 1 hour, boiled in water for 10 min, and chilled on an ice bath. Ten microliters of each solution was used as a template for PCR amplification of the *nanH* gene.

RESULTS AND DISCUSSION

First, we compared the nucleotide sequences of the *nanH* gene of *B. fragilis* strain YCH46 (18) with strain TAL2480 (19) to design an oligonucleotide probe that was specific for the *nanH* gene of *B. fragilis*. The nucleotide sequences of *nanH* from both strains were highly conserved and showed 91.1% identity (Fig.1). In the present study, we chose the region at 1057-1088 (numbering in the YCH46 *nanH* gene) for a *B. fragilis*-specific probe because (i) this region of more than 30 nucleotides in length is common to both strains, (ii) the guanine plus cytosine content was relatively high (43.8%), and (iii) this region corresponded to the middle of the F2-R2 annealing sites. Since a sufficient amount of the oligonucleotide probe was easily synthesized using a DNA synthesizer and non-radioisotopic labeling is required for use in diagnostic laboratories, we synthesized a digoxigenin-labeled oligonucleotide probe, NH1, based on this region. The nucleotide sequence of NH1 is described in Materials and Methods.

Dot blot hybridization using NH1 was performed against *nanH* PCR products from sixty strains of *B. fragilis* and ten other strains of related species. As shown in Fig. 2, positive spots were obtained from all strains of *B. fragilis* tested except for strain KMS2 (spotted at f-4.). However, strain KMS2, which had initially been identified as *B. fragilis*, was reidentified as *B. vulgatus* when we tested this strain using an API20A system (bioMerieux). In addition, none of the products from other species gave positive spots. Based on these findings, it

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YCH46     701   TTGATGTTGG TTTAAGCCGC AGTACAGATG GTGGAAAAAC TTGGGAAAAA ATGCGTTTGC CTTTGGCTTT
TAL2480     1     GATGTTGG TTTAAGCCGT AGTACAGATG GTGGAAAAAC CTGGGAGAAA ATGCGTTTGC CTTTGGCATT
                                                F-1 →
                *** ** *** ****** *** ********** ****** *** ********** **** ***** **********
YCH46     771   TGGAGAATTC GGTGGTTTGC CTGCCGGTCA GAATGGGGTA GGAGACCCTT CTATTCTTGT TGATACAAAA
TAL2480    69   TGGGGAGTTC GGTGGTCTGC CTGCCGGTCA GAATGGAGTA GGAGACCCTT CTATCCTTGT TGATACAAAA

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YCH46     841   ACAAATAATG TTTGGGTGGT TGCTGCCTGG ACACATGGTA TGGGTAATCA GCGGGCATGG TGGAGTTCAC
TAL2480   139   ACAAATAATG TTTGGGTGGT TGCTGCCTGG ACACATGGTA TGGGTAACCA ACGTGCATGG TGGAGTTCAC

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YCH46     911   ATCCGGGTAT GGATATGAAC CATACAGCAC AACTTGTTTT AGCTAAAAGT ACAGATGATG GTAAAACATG
TAL2480   209   ATCCGGGTAT GGATATGAAT CATACTGCGC AACTTGTTCT TGCCAAAAGT ACGGATGACG GTAAAACGTG
                    F-2 →
                ********** ******** * **** *****  ********  ********** *  *******  ** ******
YCH46     981   GTCTGCACCT ATTAATATTA CAGAGCAGGT GAAAGATCCT TCTTGGTATT TCTTGTTGCA GGGACCGGGT
TAL2480   279   GTCTGCACCT ATTAATATCA CAGAACAGGT AAAAGATCCG TCTTGGTATT TTCTGTTGCA AGGTCCGGGT

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YCH46    1051   AGGGGTATCA CTATGAGTGA CGGTACTTTG GTATTCCCAA CTCAGTTTAT CGATTCGACA CGTGTGCCCA
TAL2480   349   CGGGGCATCA CTATGAGTGA CGGTACTTTG GTATTCCCTA CTCAGTTTAT TGATTCGACA CGTGTACCCA
                                NF1
                **** *****  ***** **  ********** * ** ***** *** ****** ********** * ***** **
YCH46    1121   ATGCCGGTAT TATGTACAGT AAAGATGGTG GCAAGAACTG GAAGATGCAC AATTATGCAC GTACGAACAC
TAL2480   419   ATGCAGGTAT CATGTATAGC AAAGATGGTG GTAAAAACTG GAAAATGCAC AATTATGCAC GCACGAATAC

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YCH46    1191   CACAGAAGCC CAGGTAGCTG AGGTCGAACC CGGAGTGTTG ATGTTGAATA TGCGTGATAA TCGTGGAGGA
TAL2480   489   TACAGAGGCT CAGGTTGCCG AGGCCGAGCC CGGAGTATTG ATGTTGAATA TGCGTGATAA TCGTGGTGGA

                ** ** **** ********** ****** *** ********** **** ***** ********** ** *******
YCH46    1261   AGCCGCGCTG TGGCTATTAC AAAAGATTTG GGTAAAACAT GGACGGAACA TGAATCTTCT CGTAAGGCAT
TAL2480   559   AGTCGTGCTG TGGCTATTAC AAAAGACTTG GGTAAAACAT GGACAGAACA TGAATCTTCT CGCAAGGCAT

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YCH46    1331   TGCCGGAATC TGTTTGTATG GCTAGTTTGA TCAGTGTGAA AGCAAAAGAT AATGTGTTGG GCAAGGATTT
TAL2480   629   TGCCGGAATC GGTTTGCATG GCTAGTTTGA TAAGCGTGAA AGCCAAAGAT AATGTTTTGG GCAAGGATTT

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YCH46    1401   ATTGATTTTC TCTAATCCTA ATACGACAAA AGGACGCTAT AATACTACTA TTAAAATAAG TTTGGATGGC
TAL2480   699   ATTGATATTC TCTAATCCTA ATACGACGAA AGGACGTTAT AATACTACTA TTAAAATCAG TCTGGATGGT
                        ← R-2
                ********** ********** *** ** **
YCH46    1471   GGTGTGACTT GGTCACCCGA ACATCAGCTG
TAL2480   769   GGTGTGACTT GGTCACCCGA ACACCAACTT
                     ← R-1
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Fig. 1. Comparison of the nucleotide sequences of *nanH* from *B. fragilis* YCH 46 and TAL2480 using Genetyx-Mac software Ver8.0.5 (Software Development Co., Ltd., Tokyo, Japan). In the case of strain TAL2480, only a partial nucleotide sequence was available in the DDBJ/EMBL/Genbank database. The common residues are indicated by asterisks above the nucleotide sequences. The positions of the PCR-primers (underlined) and oligonucleotide probe, NH1 (double underlined), are shown below the sequence.

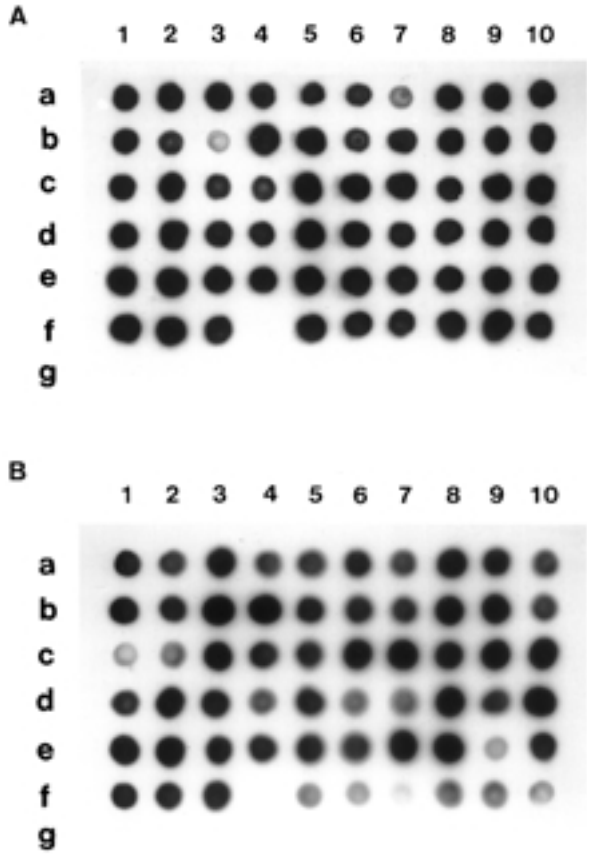

Fig. 2. Dot blot hybridization using digoxigenin-labeled oligonucleotide probe NH1 against the *nanH* amplification products generated by primer F1-R1(A) and F2-R2(B). The spots from a-1 to e-10 correspond to *B. fragilis* strains YCH1 to YCH50. The other spots are as follows : f-1, *B. fragilis* B1 ; f-2, *B. fragilis* B2 ; f-3, *B. fragilis* KMS1 ; f-4, *B. fragilis* KMS2 ; f-5, *B. fragilis* KMS3 ; f-6, *B. fragilis* KMS4 ; f-7, *B. fragilis* KMS 5 ; f-8, *B. fragilis* TDP-101 ; f-9, *B. fragilis* ATCC25285 ; f-10, *B. fragilis* NCTC9343 ; g-1, *B. distasonis* ATCC8503 ; g-2, *B. eggerthii* ATCC27754 ; g-3, *B. ovatus* ATCC8483 ; g-4, *B. thetaiotaomicron* ATCC29148 ; g-5, *B. uniformis* ATCC8492 ; g-6, *B. vulgatus* ATCC 8482 ; g-7, *Porphyromonas asaccharolytica* ATCC25260 ; g-8, *Porphyromonas endodontalis* ATCC35406 ; g-9, *Porphyromonas gingivalis* 381 ; g-10, *Prevotella corporis* JCM8529.

was suggested that (i) the digoxigenin-labeled oligonucleotide probe, NH1, was specific for *nanH* of *B. fragilis* and (ii) PCR-dot blot hybridization targeting *nanH* was useful not only for the identification of *B. fragilis* but also for that of *B. vulgatus* (PCR with F1-R1 was positive, but dot blot hybridization with NH1 was negative.).

To assess the usefulness of *nanH*-PCR and the dot blot hybridization assay in clinical specimens, *B. fragilis* bacteremia model mice were constructed. Three 8-week-old male C57BL/6J mice were intraperitoneally injected with *B. fragilis* strain YCH 46, and 0.2 ml blood samples were collected by cardiac puncture at 1, 3 and 6 hours after injection. Table 1 shows the results of the blood culture. Rapid translocation of *B. fragilis* cells from the peritoneal cavity to the blood stream was observed. This finding might represent the pathogenic potential of this species, but almost all *B. fragilis* cells appeared to be cleared from the blood stream within 24 hours in healthy mice.

Nested PCR of the *nanH* gene was performed using each blood sample. Fig. 3 shows the findings of the PCR-dot blot hybridization assay using blood samples from mouse A in Table 1. The 518 bp bands of expected size were detected in the samples at 1, 3 and 6 hours (Fig. 3A), while no band was found in the 0 time control. We confirmed that these were *B. fragilis*-specific amplification products by dot blot hybridization with digoxigenin-labeled NH1 (Fig. 3B). These findings suggested that PCR-dot blot hybridization based on *nanH* enables detection of *B. fragilis* cells in clinical specimens with a cell number as low as 2×10^3cfu/ml, even if the sample tested contains non-viable cells. As shown in Fig. 3, the intensity of the PCR bands was not in proportion to the strength of the hybridization signals. The reason for this might be that the excess amount of template produced a large amount of intermediate amplification products in the early cycles of amplification and reduced the amount of specific bands, or that NH1 directly reacted with genomic DNA contained in blood samples. The latter case would mean that NH1 would enable direct detection of *B. fragilis* cells in blood samples

Table 1. Results of blood cultures from a bacteremia model mice with *B. fragilis*.

Experimental mouse	Number of *B. fragilis* cells used in *i. p.* injection (cfu)	Number of *B. fragilis* cells detected by blood culture (cfu/ml of blood)			
		0 h[a]	1 h[a]	3 h[a]	6 h[a]
A	8.0×10^6	0	1.0×10^5	4.3×10^3	2.1×10^3
B[b]	5.3×10^6	0	5.1×10^5	5.2×10^5	3.8×10^3
C[b]	5.3×10^6	0	2.2×10^5	6.6×10^3	2.5×10^3

[a] The time when the blood samples were collected after *i.p.* injection, and 0 h means before injection.
[b] In these experiments, the same culture of *B. fragilis* was used for injection into two independent C 57/BL/6 J mice.

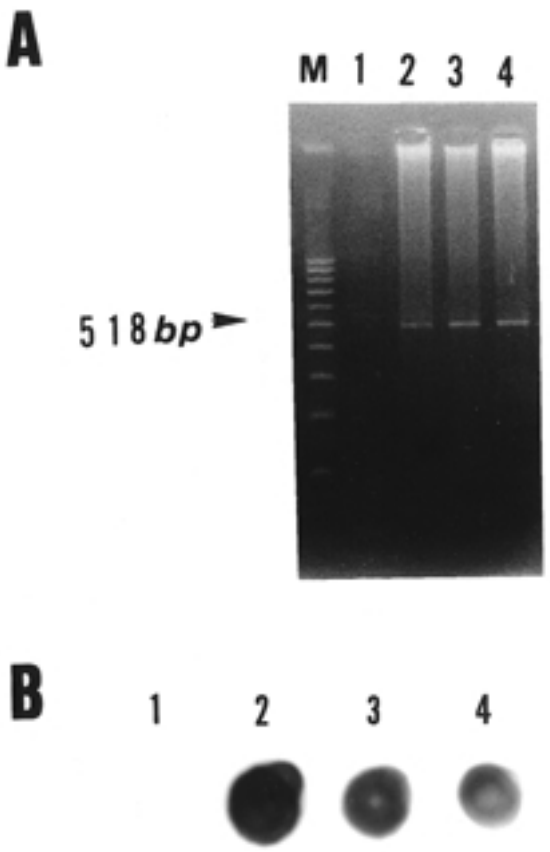

Fig. 3. Detection of amplification products by nested PCR of the *nanH* gene of *B. fragilis* cells in blood samples (A) and results of a specificity test by dot blot hybridization with a digoxigenin-labeled oligonucleotide probe, NH1(B). Lane1, before bacterial injection ; lane 2, 1 hour ; lane 3, 3 hours ; lane 4, 6 hours after injection, and M, molecular size marker.

if there was a sufficient number of cells in the sample. It was suggested that the PCR-dot blot hybridization assay using *nanH* was useful for the detection and quantification of *B. fragilis* present in clinical specimens. Of course, NH1 can be applied for the rapid identification of cultured *B. fragilis*, but there are many cases in which the culture of blood from a patient is negative even when septicemia is suspected from clinical signs due to the administration of antibiotics or, particularly in cases of anaerobic infection, due to storage of anaerobes under inappropriate conditions. Furthermore, in almost all cases, clinical samples do not contain a sufficient number of cells to enable detection using direct hybridization assay. Therefore, an amplification step is needed before performing the hybridization assay to obtain accurate results.

In the present study, we demonstrated the usefulness of PCR-dot blot hybridization based on the neuraminidase-encoding gene for specific detection of *B. fragilis* cells using bateremia model mice. It was suggested that a combination of the method used in the present study with a traditional culture method would be useful for a clinical survey of the accurate incidence of *B. fragilis* infection and for differential diagnosis in patients with fever of unknown origin. However, it is necessary to determine whether this procedure is useful in the clinical setting by performing tests using various clinical samples such as pus, sputum and drainage fluid in a future study.

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