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Detection of a Mixed Infection in a Culture-Negative Brain Abscess by Broad-Spectrum Bacterial 16S rRNA Gene PCR$^{\triangledown}$†

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We describe the identification of two bacterial pathogens from a culture-negative brain abscess by the use of broad-spectrum 16S rRNA gene PCR. Simultaneous detection of *Fusobacterium nucleatum* and *Porphyromonas endodontalis* was possible due to a 24-bp length difference of their partially amplified 16S rRNA genes, which allowed separation by high-resolution polyacrylamide gel electrophoresis.

Broad-spectrum 16S rRNA gene PCR is a universal and highly versatile technology for the detection and identification of bacterial pathogens (11). Microbiological examination of life-threatening brain infections relies on microscopic examination and culture of abscess material. A recent study by Al Masalma et al. (1) explored an expanded technology applying multiple 16S rRNA fragment cloning and subsequent sequencing to analyze mixed floras in brain abscesses and found that the number of species associated with brain abscesses is much larger than previously noted. Based on a recent case and an *in silico* feasibility study, we present a modified technique for the molecular analysis of polymicrobial infections. After broad-spectrum 16S rRNA gene amplification, amplicons are separated electrophoretically and excised from a polyacrylamide gel for subsequent sequence identification. In comparison to the previously described labor-intensive cloning and sequencing strategies used to resolve polymicrobial infections by PCR (1, 4), the technique presented herein is less time-consuming and less expensive, further allowing its introduction in the routine diagnostic laboratory.

Case report. A 29-year-old male was hospitalized after a first seizure, which developed in the context of frontal sinusitis. Three days before hospitalization, the patient was diagnosed with acute frontal sinusitis, which initially was treated symptomatically. Because of severe persistent frontal headache, amoxicillin-clavulanic acid (875 mg/125 mg) every 12 h was prescribed the day before hospital admission. A computed tomography (CT) scan of the skull performed at the hospital showed pansinusitis with a bone defect of the dorsal wall of the frontal sinus and an adjacent brain abscess. The antibiotics ceftriaxone (2-g dose, twice daily) and metronidazole (500-mg dose, three times daily) were given intravenously. On the fourth day of hospitalization, because of the progressive nature of the infection, surgical excision of the brain abscess was performed. Intraoperatively, three samples (designated I, II, and III) of abscess material were obtained and investigated by conventional and molecular bacteriological diagnostics (Table 1). Microscopic analysis showed the presence of leukocytes in samples I and III but no microorganisms in any of the samples. Aerobic media (Columbia blood agar, MacConkey agar, CNA blood agar, and Crowe agar) as well as anaerobic media (*Brucella* agar, kanamycin-vancomycin agar, phenylethyl alcohol agar, and thioglycolate medium) inoculated with the samples showed no bacterial growth after 3 days of incubation under aerobic (ambient atmosphere and 5% CO_2 at 37°C) and anaerobic (atmosphere of 85% N_2, 10% CO_2, and 5% H_2 at 37°C) conditions. A single PCR for broad-spectrum bacterial 16S rRNA genes using primers 5′-AGT TTG ATC MTG GCT CAG-3′ (BAK11w; *Escherichia coli rrsA* nucleotide [nt] positions 10 to 27) and 5′-GGA CTA CHA GGG TAT CTA AT-3′ (BAK2; *E. coli rrsA* nt positions 787 to 806) was performed as previously described (2). PCR resulted in two distinguishable fragments each for samples I and III, respectively, as visualized by CleanGel (GE Healthcare, Zurich, Switzerland) polyacrylamide gel electrophoresis (Fig. 1). The two PCR products of the two samples were purified after gel excision and reamplified in a seminested PCR using primers BAK11w and BAK553r (5′-TTA CCG CGG CTG CTG GCA C-3′, *E. coli rrsA* nt positions 515 to 533) and sequenced using the amplification primers. DNA sequence homology analyses were done using the SmartGene IDNS database and software (SmartGene, Zug, Switzerland). The sequences of the two PCR products reamplified using primers BAK11w and BAK553r showed highest homologies to the 16S rRNA gene of *Fusobacterium nucleatum* (1 mismatch in 499 bp, 1 ambiguous base indicating the presence of multiple 16S rRNA gene copies in the genome; GenBank accession number FJ638888.2) and *Porphyromonas endodontalis* (1 mismatch in 507 bp; GenBank accession number FJ638887.2), respectively.

In silico calculation of amplification fragment lengths using the BLASTN algorithm for *F. nucleatum* and *P. endodontalis* resulted in lengths of 774 and 798 bp, respectively. In polyacrylamide gel electrophoresis, the calculated lengths correspond to the amplicons of a direct PCR from a pure culture of either species (Fig. 1C, lanes 1 and 2). In previous studies, 16S rRNA gene amplifications resulting in two different fragments could

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TABLE 1. Analysis of patient samples taken from the cerebral abscess

Sample	Microscopy results		Culture result[c]	Identification by bacterial 16S rRNA gene PCR analysis	No. of mismatches/ total no.	% Homology
	Leukocytes[a]	Microorganisms[b]				
I	>25 leukocytes	Negative	Negative	*Fusobacterium nucleatum*	1/499	99.8
				Porphyromonas endodontalis	1/507	99.8
II	Negative	Negative	Negative	ND[d]		
III	Negative	Negative	Negative	*Fusobacterium nucleatum*	1/499	99.8
				Porphyromonas endodontalis	1/5071	99.8

[a] Average of 10 visual fields at a 100× magnification.
[b] Average of 10 visual fields at a 1,000× magnification.
[c] Samples were streaked on seven different agar media (Columbia blood agar, MacConkey agar, CNA blood agar, Crowe agar, brucella agar, kanamycin-vancomycin agar, and phenylethyl alcohol agar) and incubated under aerobic and anaerobic conditions as described in Materials and Methods.
[d] ND, not done due to insufficient sample volume.

not be resolved due to poor fragment separation (6). The progress in separation of the primary amplicons was achieved by using polyacrylamide instead of agarose gel electrophoresis for the improved resolution of amplicons. Our study shows that polymicrobial infections can be identified by broad-range bacterial 16S PCR when variable amplicon lengths allow electrophoretical separation. *In silico* calculation of the partial 16S rRNA gene amplicon of 156 human pathogenic bacterial species revealed that the fragments amplified by the universal primers BAK11w and BAK2 can range between 738 and 910 bp (22 species are listed in Table 2; see the supplemental material for a list of all 156 species), providing the opportunity for efficient separation in a significant number of polymicrobial infections.

Anaerobes play an important role in brain abscess formation and infections spreading from chronic sinusitis (3). In most mucous membranes, anaerobic Gram-negative bacilli (AGNB) outnumber aerobic and facultative bacteria in ratios ranging from 10:1 to 10,000:1 (5). *F. nucleatum* and *P. endodontalis* belong to the group of strictly anaerobic bacteria (8) presumably associated with periodontal disease (12). In our patient, brain abscess arose from acute sinusitis and is a well-known complication of this infection. Progression of the abscess under empirical antibiotic treatment is due to either inadequate antibiotic diffusion into the abscess or inappropriate antimicrobial spectrum of the prescribed antibiotics. Ceftriaxone and metronidazole show penetration in the brain, are active against the commonly described pathogens responsible for brain abscess, and are generally recommended as empirical therapy (9). In progressive disease despite correct antibiotic prescription, surgical drainage or excision, which also permits sampling for microbiological analyses, is mandatory.

Bacterial 16S rRNA gene PCR often outperforms anaerobic culture in the detection of fastidious and anaerobic pathogens in brain abscess material (7, 10, 11). This may be explained due to the difficult and time-critical sample treatment in plating for anaerobic cultures. Microorganisms were not found by microscopy. This is mainly due to the low sensitivity of the microscopic technique (cutoff, $>10^4$ bacteria per ml) and might also be attributable to bacterial lysis in the presence of granulocytic proteinases. Microscopy showed differing amounts of leuko-

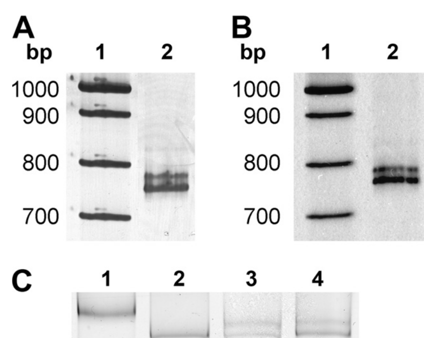

FIG. 1. Polyacrylamide gel electrophoresis of 16S rRNA gene PCR products. (A) Lane 1, molecular mass ladder XIV (Roche Diagnostics); lane 2, 16S rRNA gene amplicons obtained with patient sample I. (B) The setup for sample III is identical to that described for panel A. (C) Lane 1, amplicon of *Porphyromonas endodontalis* ATCC 35406^T culture; lane 2, amplicon of *Fusobacterium nucleatum* subsp. *nucleatum* culture; lane 3, amplicons obtained with patient sample I; lane 4, amplicons obtained with patient sample III.

TABLE 2. 16S rRNA gene amplicon length variability of 22 human pathogens

Taxonomic name	Amplicon length (bp)[a]	NCBI accession no.
Agrobacterium radiobacter K84	738	NC_011985.1
Bacillus anthracis strain A0248	806	NC_012659.1
Bartonella henselae strain Houston-1	741	BX897699.1
Bordetella pertussis strain Tohama I	791	BX640418.1
Borrelia burgdorferi ZS7	792	CP001205.1
Brucella melitensis ATCC 23457	738	NC_012441.1
Campylobacter jejuni RM1221	771	CP000025.1
Clostridium cellulolyticum H10	910	NC_011898.1
Clostridium difficile 630	758	NC_009089.1
Corynebacterium diphtheriae NCTC 13129	772	NC_002935.2
Enterococcus faecalis strain HN-S7	815	FJ378704.1
Escherichia coli BW2952	797	NC_012759.1
Francisella tularensis subsp. *tularensis* WY96-3418	785	NC_009257.1
Mycobacterium leprae Br4923	797	NC_011896.1
Mycoplasma hominis	786	M96660.1
Mycoplasma pneumoniae M129	793	U00089.2
Nocardia asteroides strain ATCC 19247	749	DQ659898.1
Nocardia farcinica IFM 10152	769	AP006618.1
Pseudomonas aeruginosa LESB58	791	FM209186.1
Salmonella enterica subsp. *enterica* serovar Paratyphi C	797	NC_012125.1
Staphylococcus aureus strain ATCC 14458	804	DQ997837.1
Streptococcus pneumoniae 70585	801	NC_012468.1

[a] Amplicon lengths were determined using universal primers BAK11w and BAK3 as start and end sequences, respectively.

cytes, indicating different levels of inflammation in the material sampled (Table 1). Even though great care and rapid manipulations were employed during plating, cultures remained sterile in our case. The failure to grow the corresponding pathogens is more likely due to the previous antibiotic treatment of the patient (1). The causative agents in this case of a life-threatening brain abscess could be identified only by broad-spectrum PCR and a successful separation of the PCR amplicons. The patient was treated for 6 weeks with ceftriaxone and metronidazole and recovered fully with the exception of residual headache.

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