MAJOR ARTICLE

Metagenomic Analysis of Brain Abscesses Identifies Specific Bacterial Associations

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Background. The bacterial flora involved in brain abscess is often complex. In a previous study, using a metagenomic approach based on 16S ribosomal DNA (rDNA) amplification, we demonstrated that the diversity of the microbial flora involved in these infections was underestimated.

Methods. We performed a 16S rDNA-based metagenomic analysis of cerebral abscesses from patients diagnosed from 2006 through 2010. All bacteria present in brain abscess specimens were identified, in view of the clinical and epidemiological characteristics of the patients.

Results. Fifty-one patients were included in our study. By detecting polymicrobial infections in 19 patients, our strategy was significantly more discriminatory and enabled the identification of a greater number of bacterial taxa than did culture and conventional 16S rDNA polymerase chain reaction (PCR) and sequencing, respectively ($P < 10^{-2}$). Data mining discriminated 2 distinct bacterial populations in brain abscess from dental and sinusal origin. In addition, of the 80 detected bacterial species, we identified 44 bacteria that had never been found in brain abscess specimens, including 22 uncultured bacteria. These uncultured agents mostly originated from the buccal or sinusal floras ($P < 10^{-2}$) and were found in polymicrobial specimens ($P < 10^{-2}$).

Conclusions. Cloning and sequencing of PCR-amplified 16S rDNA is a highly valuable method to identify bacterial agents of brain abscesses.

Brain abscesses are focal infections resulting from direct extension from a contiguous suppurative focus (paranasal sinuses, middle ear, or mastoids) in 25%–50% of cases [1–3], from hematogenous dissemination from a distant focus in 15%–30% of cases [1, 2], and from direct inoculation (trauma or neurosurgery) in 8%–19% of cases [4]. In 20%–30% of cases, no source can be identified [2, 3]. Despite advances in diagnostic and neurosurgical procedures, the mortality of this disease remains high (8%–32%) [5–8], and sequelae are observed in 9%–36% of patients [4, 7, 9]. Currently, the detection of bacterial pathogens in brain abscesses is mostly obtained using culture from pus collected by neurosurgical drainage [10, 11]. However, pus culture remains sterile in 9%–63% of cases [5, 9, 12]. Over the past decade, 16S ribosomal DNA (rDNA) amplification and sequencing was successfully used to detect bacteria in brain abscess specimens [13–15]. However, this method was impaired in cases of polymicrobial infection, even when combined with high-resolution polyacrylamide gel electrophoresis [16].

In contrast, metagenomic studies of complex human floras using a combination of 16S rDNA polymerase chain reaction (PCR) and cloning-sequencing of PCR products proved useful to evaluate the bacterial diversity of dental, vaginal, and intestinal floras [17–22]. In a previous study, we applied such an approach to brain abscess specimens [23]. This preliminary study identified 49 distinct brain abscess bacterial agents and enabled the identification of 27 bacteria never detected before in brain abscess, 15 of which were uncultured [23–25].

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Such a high number of bacterial species involved in brain abscess prompted the study of 51 new specimens in an effort to describe further the flora associated with brain abscesses and their etiologies.

PATIENTS AND METHODS

Patients and Clinical Specimens

Brain abscess pus specimens were surgically obtained from 51 patients in Marseille and Nice Hospitals from 2006 through 2010. A brain abscess was defined as a localized intracerebral suppurative lesion for which histological analysis confirmed the presence of pus and excluded the presence of cancerous and lymphomatous cells. Pus specimens were collected using sterile surgical conditions and immediately sent to the bacteriology laboratory. The study was approved by the local ethics committee under reference 07-030.

Culture

Pus specimens were microscopically examined after Gram staining. Specimens were then plated on blood and chocolate agar plates (BioMérieux) and incubated at 37°C under aerobic and anaerobic atmospheres. Plates were examined daily for the presence of colonies for 10 days. Isolated bacteria were identified using the Vitek2 instrument and API identification strips (BioMérieux). When phenotypic identification failed, isolates were identified using 16S rDNA sequencing as described elsewhere [26].

Conventional 16S rDNA and 18S rDNA PCR Amplification and Sequencing

DNA was extracted from each specimen as described elsewhere [23]. The 16S rDNA and 18S rDNA were amplified using the fD1-rp2 and CUF-CUR primer pairs, respectively (Table 1), as described elsewhere [23]. The efficiency of DNA extraction was verified by amplifying the β-globin gene using the primer pair KM29 and RS42 (Table 1). The 16S rDNA or 18S rDNA PCR products were purified using the NucleoFast 96 PCR kit (Macherey-Nagel) according to the manufacturer's recommendations. Sequencing reactions were performed using the Big-Dye Terminator cycle sequencing kit DNA (version 1.1; PerkinElmer) according to the manufacturer's instructions. The 16S rDNA PCR products were sequenced in both directions using the internal primers 536f, 536r, 800f, 800r, 1050f, and 1050r, whereas the 18S rDNA amplicons were sequenced using the PCR primers (Table 1). Sequences were analyzed on an ABI Prism 3130x genetic analyzer (Applied Biosystems,). The different fragments were assembled using Sequencher version 4.0 software (Applied Biosystems). Obtained sequences were compared with those in the GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) using BLAST software (http://blast.ncbi.nlm.nih.gov).

Table 1. Sequence of Primers Used for Polymerase Chain Reaction and Sequencing

Target, Primer, or Probe Name	Sequence (5′ → 3′)
16S rDNA gene amplification and sequencing	
fD1	AGA GTT TGA TCC TGG CTC AG
rp2	ACG GCT ACC TTG TTA CGA CTT
16S rDNA sequencing	
536r	GTA TTA CCG CCG CTG CTG
536f	CAG CAG CCG CCG TAA TAC
800f	TAG ATA TAC CCG GTT AG
800r	CTA CCA GGG TAT CTA AT
1050r	CAC GAG CTG ACG ACA
1050f	5′-TGT CGT CAG CTC GTG
Insert amplification	
M13d	CAG GAA ACA GCT ATG AC
M13r	GTA AAA CGA CGG CCA G
β-Globin gene amplification	
KM29	GGT TGG CCA ATC TAC TCC CAG G
RS42	GCT CAC TCA GTG TGG CAA AG
18S rDNA gene amplification and sequencing	
CUF	TCCGTAGGTGAACCTGCGG
CUR	GCTGCGTTCTTCATCGATGC

Abbreviation: rDNA, ribosomal DNA

Cloning and Sequencing of 16S rDNA

The 16S rDNA PCR-positive products were cloned using the pGEM-T Easy Vector system with JM109 Competent *Escherichia coli* according to the manufacturer's instructions (Promega). For each sample, 100 white colonies were picked and their DNA was PCR-amplified individually using the M13d and M13r primers (Table 1). Sequencing was performed as described above using the 536r, 536f, 800f, 800r, 1050f, and 1050r primers (Table 1). Pyrosequencing, used elsewhere for the metagenomic study of brain abscesses [23], but time-consuming and costly, was not used in this study.

Data Analysis and Taxa Identification

Sequences obtained from each clone were compared with those in the GenBank database using BLAST software. A sequence similarity level of 98.7% was used as a cutoff for species identification [27]. Chimerical sequences were identified within libraries using the Chimera Check program of the Ribosomal Database Project II [28] and discarded. Complete 16S rDNA gene sequences of novel phylotypes were deposited in GenBank under the accession numbers listed in Table 3.

Analysis of Data

The data from the 51 patients in this study and the 20 patients from our previous study [23] were analyzed with PASW

Statistics software (version 17.0; PASW Statistics) and PASW Modeler software (version 14; PASW Statistics). A univariate analysis was performed to detect statistically significant associations between cloning results, underlying disease, and identified microorganisms. The Mantel–Haenszel or the Fisher exact tests were used according to the expected values of the cells. Differences between diagnostic methods were evaluated using the Fisher exact test. A P value $<.05$ was considered to be significant.

Data Mining

The Auto Cluster node component of the PASW modeler (PASW Statistics) was used to estimate and compare clustering models, which identify groups of records that have similar characteristics without the benefit of prior knowledge about the groups and their characteristics. For the data mining analysis, because of the relatively small number of patients and microorganisms, these were grouped by genus or groups of bacteria (uncultured bacteria, other anaerobes, other streptococci, other staphylococci, enterobacteriaceae, and β-proteobacteria), and those isolated only once were omitted.

RESULTS

Clinical Data

The 51 new studied patients included 37 males (72%) and 14 females (28%). Forty-six were adults, and 5 were children. The age of patients ranged from 9 months to 87 years. The age, sex, and epidemioclinical data of patients are detailed in Table 2. In brief, the abscess developed contiguously following chronic sinusitis in 11 patients (21.5%), otitis media in 6 patients (11.7%), dental infections or treatment in 6 patients (1.1 %), and neurosurgery in 7 patients (13.7%). Abscess developed in 4 patients with cancer (7.8%), in 3 immunocompromised patients (5.9%), and in 11 patients with various conditions (21.5%). No primary source was identified in 3 patients (5.9%). A microbiological diagnosis was obtained in 43 patients (Table 2).

Culture

Microorganisms were visible microscopically in 23 specimens (45.1%; Table 2). Culture was positive in 30 patients (59%; Table 2). Among these patients, 25 patients were infected with 1 species of bacteria, 4 with 2 species of bacteria, and 1 with 3 species of bacteria (Table 2). Overall, 18 different species were detected by culture.

Conventional PCR and Sequencing

All 51 tested samples were PCR positive by means of β-globin amplification, 39 were 16S rDNA positive, and 4 were 18S rDNA positive. Negative controls remained negative in all PCR experiments. In 7 samples, 16S rDNA sequencing identified polymicrobial infections but failed to discriminate among species. In 32 samples, a single species of bacteria was detected by 16S rDNA sequencing. Overall, 14 different species were identified by conventional 16S rDNA sequencing (Table 2). By means of 18S rDNA PCR, *Toxoplasma gondii* was identified in samples from 3 patients and *Scedosporium apiospermium* was identified in a sample from 1 patient (Table 2).

Metagenomic Analysis Based on 16S rDNA

The 39 PCR-positive samples were cloned and analyzed. Polymicrobial infections were detected in 19 samples. Among these, the number of detected bacterial species ranged from 1 to 14. In all 20 monomicrobial specimens, cloning identified the same bacterium as did conventional 16S rDNA sequencing. In total, 76 different species were identified, 23 of which exhibited greatest sequence similarity to uncultured bacteria (Tables 2 and 3). Of the 76 identified species, 44 had not previously been identified in brain abscesses, including 22 validated species and 22 uncultured bacteria (Table 3).

Comparison Between Diagnostic Methods

All 30 culture-positive specimens were also PCR positive. In 22 of these samples, the identified bacteria were identical using culture and conventional 16S rDNA sequencing. The remaining 8 culture-positive samples included 4 specimens that were polymicrobial using 16S rDNA, 3 in which only 1 of the 2 cultivated bacteria species was detected by PCR and 1 in which the cultivated bacteria species (*Streptococcus constellatus*) differed from that detected by PCR (*Fusobacterium nucleatum*). Twelve specimens were negative using both PCR and culture (Table 2). Nine specimens were PCR positive but culture negative, including 3 polymicrobial specimens detected using conventional sequencing and 6 monomicrobial samples (Table 2).

All bacterial species detected using conventional 16S rDNA sequencing were retrieved using 16S rDNA cloning and sequencing. In contrast, bacteria detected by culture from 6 specimens were not detected by cloning, including both *Enterococcus avium* and *Proteus mirabilis* in 1 specimen; *Staphylococcus epidermidis* in 2 specimens; and *S. constellatus*, *Campylobacter rectus*, and *Haemophilus aphrophilus* in 1 specimen each (Table 2). Overall, 80 bacterial taxa were identified in this study, including 76 detected by 16S rDNA cloning and sequencing (95%), 59 of which were not detected by the other methods (73.7%) (Table 2). Cloning and sequencing identified significantly more polymicrobial specimens than did culture (19 of 51 specimens vs 5 of 51 specimens; $P < 10^{-2}$) and conventional sequencing (19 of 51 specimens vs 7 of 51 specimens; $P < 10^{-2}$). In addition, cloning and sequencing detected significantly more bacterial taxa than did culture (76 of 80 specimens vs 18 of 80 specimens; $P < 10^{-2}$) and conventional sequencing (76 of 80 specimens vs 14 of 80 specimens; $P < 10^{-2}$).

Table 2. Characteristics of the 51 Patients With Brain Abscess Examined in This Study and Bacteria Identified

Patient	Sex	Age, y	Gram Staining	Antibiotic Treatment Before Specimen Collection	Risk Factor	Culture	Conventional 16S rDNA Sequencing	16S rDNA Cloning and Sequencing (No. of Clones)
1	Male	48	ND	No	I	None	None[a]	ND
2	Male	64	C+	No	M	*Streptococcus constellatus*	Polymicrobial	*Micromonas micros* (42), *Prevotella buccae* (10), *Prevotella baroniae* (18), *Prevotella oris* (12), uncultured BA10 (1), uncultured BA11 (1), uncultured BA12 (2), uncultured BA13 (1%), uncultured BA14 (8), uncultured BA15 (3), uncultured BA16 (1), uncultured BA17 (1), uncultured BA18 (1), uncultured BA19 (2)
3	Male	50	ND	No	K	*Propionibacterium acnes*	*P. acnes*	*P. acnes* (100)
4	Male	50	ND	No	M	*Haemophilus parainfluenzae*	*H. parainfluenzae*	*H. parainfluenzae* (100)
5	Female	51	C+	No	M	*Streptococcus intermedius*	*S. intermedius*	*S. intermedius* (100)
6	Male	56	B+	No	K	*P. acnes*	*P. acnes*	*P. acnes* (100)
7	Male	55	ND	No	N	*Staphylococcus aureus*	*S. aureus*	*S. aureus* (100)
8	Female	87	B+	Yes	L	*P. acnes*	*P. acnes*	*P. acnes* (100)
9	Male	38	ND	Yes	L	None	*Streptococcus pneumoniae*	*S. pneumoniae* (100)
10	Female	56	ND	Yes	O	*Enterobacter cloacae*	*E. cloacae*	*E. cloacae* (57), *Enterobacter hormaechei* (40), *Escherichia coli* (3)
11	Female	25	B+	No	O	*P. acnes*	*P. acnes*	*P. acnes* (100)
12	Male	41	C+	No	L	*S. intermedius, Haemophilus aphrophilus*	*S. intermedius*	*S. intermedius* (70), *H. aphrophilus* (30)
13	Female	81	ND	Yes	K	*Prevotella heparinolytica, Campylobacter rectus*	Polymicrobial	*P. heparinolytica* (96), *Fusobacterium nucleatum* (4)
14	Male	57	ND	No	P	*Nocardia abscessus*	*N. abscessus*	*N. abscessus* (95), *Burkholderia cenocepacia* (3), *Stenotrophomonas maltophilia* (2)
15	Female	1	ND	Yes	L	None	*S. pneumoniae*	*S. pneumoniae* (100)
16	Male	40	B−	No	R	*Enterococcus avium, Proteus mirabilis, Bacteroides fragilis*	Polymicrobial	*Porphyromonas endodontalis* (18), *Porphyromonas asaccharolytica* (2), *Peptostreptococcus stomatis* (8), *M. micros* (12), *Enterococcus raffinosus* (2), *Catonella morbi* (1), *B. fragilis* (30), *Bacteroides thetaiotaomicron* (12), *Bilophila wadsworthia* (8%), uncultured BA20 (1), uncultured BA21 (3), uncultured BA22 (2), uncultured BA23 (1)
17	Male	60	B−	No	O	*Kleibsiella pneumoniae*	*K. pneumoniae*	*K. pneumoniae* (100)
18	Male	70	ND	No	P	None[b]	None	ND
19	Male	70	B−	No	K	*Citrobacter koseri*	*C. koseri*	*C. koseri* (100)
20	Female	61	C+	Yes	Q	*S. intermedius*	*S. intermedius*	*S. intermedius* (100)
21	Male	73	ND	No	S	None	None[a]	ND
22	Male	54	ND	No	M	*H. aphrophilus*	*H. aphrophilus*	*H. aphrophilus* (95), *F. nucleatum* (5)
23	Male	35	B− and C+	No	M	*Staphylococcus epidermidis*	Polymicrobial	*S. intermedius* (2), *F. nucleatum* (65), *Campylobacter gracilis* (6), *Fusobacterium meyeri* (6), *M. micros* (17), uncultured BA24 (4)
24	Female	20	B−	Yes	O	*E. coli*	*E. coli*	*E. coli*
25	Male	48	ND	No	T	None	None	ND

Table 2 continued.

Patient	Sex	Age, y	Gram Staining	Antibiotic Treatment Before Specimen Collection	Risk Factor	Culture	Conventional 16S rDNA Sequencing	16S rDNA Cloning and Sequencing (No. of Clones)
26	Female	69	C+	Yes	M	None	*S. intermedius*	*S. intermedius* (100)
27	Male	57	ND	No	P	None	*Nocardia carnea*	*N. carnea* (100)
28	Male	62	ND	Yes	M	None	Polymicrobial	*Tannerella forsythensis* (20), *F. nucleatum* (50), *P. endodontalis* (11), *Dialister pneumosintes* (13), *P. acnes* (2), *Eubacterium brachy* (1), *Filifactor alocis* (1), uncultured BA25 (1), uncultured BA26 (1)
29	Male	44	ND	No	O	None	None	ND
30	Male	14	C+	No	M	*S. intermedius*	*S. intermedius*	*S. intermedius* (93), *H. aphrophilus* (4), *F. nucleatum* (3)
31	Male	12	C+	Yes	L	*S. pneumoniae*	*S. pneumoniae*	*S. pneumoniae* (100)
32	Male	69	ND	No	J	None	None	ND
33	Female	75	ND	Yes	U	None	None	ND
34	Male	26	ND	Yes	O	*E. cloacae*	*Enterobacter* species	*E. hormaechei* (98), *E. cloacae* (1), *Tepidimonas arfidensis* (1)
35	Male	70	ND	Yes	V	None	None	ND
36	Female	44	ND	No	O	None	None	ND
37	Female	69	ND	No	V	None	None	ND
38	Male	55	ND	Yes	L	*S. pneumoniae*	*S. pneumoniae*	*S. pneumoniae* (100)
39	Male	23	ND	No	I	None	None[a]	ND
40	Male	38	C+	No	W	*S. intermedius*	*S. intermedius*	*S. intermedius* (61), *M. micros* (31), *E. brachy* (5), *F. nucleatum* (3)
41	Male	42	C+	No	J	None	Polymicrobial	*C. rectus* (1), *Treponema maltophilum* (1), *Bacteroides heparinolyticus* (15), *Porphyromonas gingivalis* (33), *Fusobacterium alocis* (27), *F. nucleatum* (9), *Eubacterium nodatum* (4), *Campylobacter showae* (2), uncultured BA27 (8)
42	Male	54	ND	Yes	O	None	Polymicrobial	*Clostridium cllostridiforme* (27), *Massilia timonae* (15), uncultured BA28 (9), *Streptococcus parasanguinis* (8), *Pseudomonas trivialis* (10), *Micrococcus luteus* (3), *Enhydrobacter aerosaccus* (1), *Petrobacter succinimandens* (1), uncultured BA29 (12), uncultured BA30 (1), uncultured BA31 (13)
43	Male	58	C+	No	M	*S. constellatus*	*F. nucleatum*	*F. nucleatum* (95), *H. aphrophilus* (5)
44	Female	84	ND	No	V	None	None	ND
45	Male	54	ND	Yes	J	None	*S. intermedius*	*S. intermedius* (100)
46	Male	65	ND	No	J	None	*S. intermedius*	*S. intermedius* (67), *M. micros* (34), uncultured BA32 (7)
47	Female	9 mo	C+	No	X	*S. aureus*	*S. aureus*	*S. aureus* (100)
48	Male	15	C+	No	M	*S. intermedius*, *S. epidermidis*	*S. intermedius*	*S. intermedius* (99), *Pseudomonas lutae* (1)
49	Male	36	C+	No	Y	*S. aureus*	*S. aureus*	*S. aureus* (100)
50	Male	47	C+	No	M	*S. intermedius*, *H. aphrophilus*	*S. intermedius*	*S. intermedius* (97), *M. micros* (3)
51	Male	62	C+	No	J	*S. intermedius*	*S. intermedius*	*S. intermedius* (95), *M. faucium* (5)

Abbreviations: B+, Gram-positive bacilli; B−, Gram-negative bacilli; C+, Gram-positive cocci; I, human immunodeficiency virus positive; J, dental infection; K, infection of brain metastasis from cancer; L, otitis media; M, chronic sinusitis; N, skull base fracture and cerebrospinal fluid rhinorrhea; ND, not done; O, brain surgery within the past month; P, renal graft recipient; Q, right lung pneumonia; R, colic diverticulitis; rDNA, ribosomal DNA; S, rheumatoid arthritis treated with rituximab; T, herpes simplex encephalitis; U, acute pyelonephritis; V, no risk factor identified; W, dental treatment prior to cardiac surgery; X, post-cardiac surgery; Y, venous catheter-associated bacteremia.

[a] Toxoplasmosis (positive by 18S rDNA polymerase chain reaction)

[b] *Scedosporium apiospermium* infection (positive by 18S rDNA polymerase chain reaction)

Table 3. Bacterial Species Detected in 51 Patients Using 16S Ribosomal DNA Cloning and Sequencing[a]

Species Name	Phylum	No. of Patients/ No. of Clones
Previously reported taxa (GenBank accession no.)		
Streptococcus intermedius	Firmicutes	11/890
Fusobacterium nucleatum	Fusobacteria	8/234
Micromonas micros	Firmicutes	6/139
Propionibacterium acnes	Actinobacteria	5/402
Streptococcus pneumoniae	Firmicutes	4/400
Haemophilus aphrophilus	Proteobacteria	4/134
Staphylococcus aureus	Firmicutes	3/300
Escherichia coli	Proteobacteria	2/103
Enterobacter cloacae	Proteobacteria	2/58
Porphyromonas endodontalis	Bacteroidetes	2/29
Eubacterium brachy	Firmicutes	2/6
Citrobacter koseri	Proteobacteria	1/100
Haemophilus parainfluenzae	Proteobacteria	1/100
Kleibsiella pneumoniae	Proteobacteria	1/100
Nocardia abscessus	Actinobacteria	1/95
Porphyromonas gingivalis	Bacteroidetes	1/33
Streptococcus constellatus	Firmicutes	1/30
Bacteroides fragilis	Bacteroidetes	1/30
Prevotella baroniae	Bacteroidetes	1/18
Dialister pneumosintes	Firmicutes	1/13
Prevotella oris	Bacteroidetes	1/12
Prevotella buccae	Bacteroidetes	1/10
Peptostreptococcus stomatis	Firmicutes	1/8
Bilophila wadsworthia	Proteobacteria	1/8
Campylobacter gracilis	Proteobacteria	1/6
Actinomyces meyeri	Actinobacteria	1/6
Micrococcus luteus	Actinobacteria	1/3
Stenotrophomonas maltophilia	Proteobacteria	1/2
Campylobacter rectus	Proteobacteria	1/1
Mycoplasma faucium	Tenericutes	1/5
Treponema maltophilum	Spirochaetes	1/1
Uncultured BA21 (GU592691)	Firmicutes	1/3
Taxa not reported previously (GenBank accession no.)		
Enterobacter hormaechei	Proteobacteria	3/138
Nocardia carnea	Actinobacteria	1/100
Prevotella heparinolytica	Bacteroidetes	1/96
Clostridium clostridiforme	Firmicutes	1/27
Fusobacterium alocis	Firmicutes	1/27
Tannerella forsythensis	Bacteroidetes	1/20
Massilia timonae	Proteobacteria	1/15
Bacteroides heparinolyticus	Bacteroidetes	1/15
Bacteroides thetaiotaomicron	Bacteroidetes	1/12
Pseudomonas trivialis	Proteobacteria	1/10
Streptococcus parasanguinis	Firmicutes	1/8
Eubacterium nodatum	Firmicutes	1/4
Burkholderia cenocepacia	Proteobacteria	1/3
Porphyromonas asaccharolytica	Bacteroidetes	1/2
Enterococcus raffinosus	Firmicutes	1/2
Campylobacter showae	Proteobacteria	1/2
Filifactor alocis	Firmicutes	1/1
Catonella morbi	Firmicutes	1/1
Pseudomonas lutea	Proteobacteria	1/1
Enhydrobacter aerosaccus	Proteobacteria	1/1
Petrobacter succinimandens	Proteobacteria	1/1
Tepidimonas arfidensis	Proteobacteria	1/1
Uncultured BA10 (GU592680)	Bacteroidetes	
Uncultured BA11 (GU592681)	Bacteroidetes	
Uncultured BA12 (GU592682)	Bacteroidetes	
Uncultured BA13 (GU592683)	Firmicutes	1/1
Uncultured BA14 (GU592684)	Synergistetes	1/1
Uncultured BA15 (GU592685)	Firmicutes	1/2
Uncultured BA16 (GU592686)	Bacteroidetes	1/1
Uncultured BA17 (GU592687)	Bacteroidetes	1/8
Uncultured BA18 (GU592688)	Bacteroidetes	1/8
Uncultured BA19 (GU592689)	Bacteroidetes	1/3
Uncultured BA20 (GU592690)	Firmicutes	1/1
Uncultured BA22 (GU592692)	Firmicutes	1/1
Uncultured BA23 (GU592693)	Firmicutes	1/1
Uncultured BA24 (GU592694)	Proteobacteria	1/2
Uncultured BA25 (GU592694)	Bacteroidetes	1/1
Uncultured BA26 (GU592696)	Bacteroidetes	1/2
Uncultured BA27 (GU592697)	Firmicutes	1/1
Uncultured BA28 (GU592698)	Firmicutes	1/4
Uncultured BA29 (GU592699)	Firmicutes	1/1
Uncultured BA30 (GU592700)	Bacteroidetes	1/1
Uncultured BA31 (GU592701)	Proteobacteria	1/8
Uncultured BA32 (GU592702)	Firmicutes	1/9

Table 3 continued.

[a] Bacterial taxa were classified according to their previous identification (if any) in brain abscesses in the scientific literature.

Identification of Disease-Specific Floras

The combination of data from 71 patients (51 from the present study and 20 from our previous study [23]) enabled the identification of several associations. Univariate analysis demonstrated a significant association between monomicrobial infections and otitis media (6 of 29 specimens vs 2 of 42 specimens; $P = .04$); between polymicrobial infections and sinusitis or dental defects (18 of 29 specimens vs 6 of 42 specimens; $P < 10^{-2}$); and between polymicrobial infections and *Streptococcus* species, *Prevotella* species, *Peptostreptococcus* species, *Staphylococcus* species, *Campylobacter* species, *Fusobacterium* species, *Porphyromonas* species, other anaerobes, *Mycoplasma faucium,* and uncultured bacteria ($P \leq .02$ for all microorganisms).

Otitis media was associated with *Streptococcus pneumoniae* (4 of 6 specimens vs 0 of 65 specimens; $P < 10^{-2}$). Sinusitis or dental defect was associated with *Streptococcus* species, *Prevotella* species, *Peptostreptococcus* species, *Streptococcus intermedius,*

Table 4. Classification of the 71 Patients According to Data Mining Analysis[a]

Variable	Group 1 (N = 49 [69])	Group 2 (N = 18 [25.4])	Group 3 (N = 3 [4.2])	Group 4 (N = 1 [1.4])
Microorganisms				
β-Proteobacteria	1 (2)	0 (0)	0 (0)	1 (100)
Bacteroides species	0 (0)	0 (0)	3 (100)	0 (0)
Enterobacteriacae species	6 (12)	1 (6)	2 (67)	0 (0)
Fusobacterium species	1 (2)	12 (67)	1 (33)	0 (0)
Micrococcus species	0 (0)	0 (0)	0 (0)	1 (100)
Mycoplasma faucium	0 (0)	2 (11)	2 (67)	0 (0)
Other anaerobes	0 (0)	5 (28)	1 (33)	1 (100)
Peptostreptococcus species	0 (0)	11 (61)	1 (33)	0 (0)
Porphyromonas species	0 (0)	3 (17)	1 (33)	0 (0)
Pseudomonaceae species	0 (0)	0 (0)	0 (0)	1 (100)
Pseudomonas species	0 (0)	1 (6)	0 (0)	1 (100)
Streptococcus intermedius	8 (16)	10 (56)	1 (33)	0 (0)
Uncultured bacteria	0 (0)	7 (39)	3 (100)	1 (100)
Polymicrobial infection	7 (14.3)	18 (100)	3 (100)	1 (100)
Demographic and clinical data				
Mean age, years	49.6	50.4	48.7	54
Male sex	33 (67)	16 (89)	3 (100)	1 (100)
Brain surgery	10 (20)	0 (0)	0 (0)	1 (100)
Sinusitis or dental treatment	6 (12)	16 (89)	2 (67)	0 (0)

[a] Data are no. (%) of patients, unless otherwise indicated. The 8 patients for whom no etiology was identified were all included in group 1.

Campylobacter species, *Fusobacterium* species, other anaerobes, *M. faucium*, and uncultured bacteria ($P \leq .04$ for all microorganisms). Brain surgery was associated with enterobacteriaceae and β-proteobacteria (5 of 12 specimens vs 7 of 59 specimens; $P = .02$), and immunodeficiency was associated with toxoplasmosis and *Nocardia* species (6 of 10 specimens vs 0 of 61 specimens; $P < 10^{-2}$)

The PASW modeler autocluster node found that the K-means node clusters was the method that provided the best results for the identification of clusters in the database. The K-means method classified the patients into 4 groups according to the demographic and clinical data as well as according to the microorganisms isolated alone or in combination (Table 4). The first group included 49 (69%) patients, the second included 18 (25.4%) patients, the third included 3 (4.2%) patients, and the fourth included only 1 patient. The first group included patients for whom only cultured bacteria were detected and most infections were monomicrobial, and patients without any etiology identified. In group 2, most patients had suffered sinusitis or dental treatments and had polymicrobial infections including uncultured bacteria in only 39% of cases. The most common identified microorganisms were *Peptostreptococcus* species, *Fusobacterium* species, and *S. intermedius*. In group 3, most patients had also suffered sinusitis and polymicrobial brain abscesses, but they differed from patients in group 2 by the systematic presence of *Bacteroides* species and uncultured bacteria, and that of *M. faucium* and *Enterobacteriaceae* in 2 of 3 patients. Group 4 was constituted by a single patient who developed, following brain surgery, a polymicrobial infection made of bacteria belonging to at least 5 different genera and/or species. No statistical link could be made between these microbial groups and clinical or epidemiological characteristics.

DISCUSSION

In this study, by identifying 80 distinct bacterial taxa, including 44 (55%) not previously described in brain abscess, we demonstrated that the microbial flora of brain abscesses is far from being fully known and is differentially distributed depending on the abscess etiology.

Combining cloning and sequencing enabled us to identify a significantly greater number of polymicrobial specimens and bacterial taxa (76) than with culture (18) and conventional sequencing (13; $P < 10^{-2}$ for both). The observed differences in output between cloning and culture might be because culture may be impaired by early antibiotic use and/or delay between sampling and inoculation, and it may not retrieve fastidious microorganisms, even if they are the most abundant in the studied specimen [29]. In addition, the discriminatory power among taxa of conventional 16S rDNA sequencing is limited in complex floras, often yielding uninterpretable sequences, as described elsewhere for brain abscess specimens [13, 15]. However, in 5 specimens, 6 bacterial species were cultivated but not detected by cloning. These bacteria were *C. rectus*, *E. avium*, *P. mirabilis*, *S. epidermidis*, *H. aphrophilus*, and *S. constellatus*. Such a discrepancy may result from biases in 16S rDNA gene cloning and sequencing. In particular, it is unlikely that the cell lysis [30] and DNA extraction and purification methods used, choice of primers [31], PCR [32, 33], and cloning conditions [34], notably the broad range of primers used, will be equally suitable to all bacteria present in a polymicrobial specimen. In addition, because DNA from all bacteria in a sample compete for the same reagents, those present at the lowest concentrations might be underamplified in the PCR [35, 36]. As a consequence, the number of sequenced clones may not enable characterization of all bacterial taxa present in polymicrobial samples.

Our strategy enabled us to detect 44 bacteria species (56.4%) that had not been identified previously in cerebral abscess. Similar results have been obtained in previous studies using comparable tools and investigating brain abscess, endodontic infections, dental caries and periodontitis, and pulmonary

infections [21–23, 37, 38], thus demonstrating the power of metagenomic studies to characterize complex floras. In addition, data mining and statistical analyses demonstrated a strong association of immunodeficiency with toxoplasmosis and nocardiosis ($P < 10^{-2}$); of brain surgery with Enterobacteriaceae and β-proteobacteria ($P = .02$); of monomicrobial infections, mainly by *S. pneumoniae,* with otitis ($P = .04$); and of polymicrobial infections with sinusitis or dental defects ($P < 10^{-2}$). These data suggest that otogenic brain abscesses may be empirically treated with antipneumococcal antibiotics, whereas those associated with dental diseases or sinusitis should be treated with the usual empirical treatment of brain abscesses, including a β-lactam and metronidazole. In addition, among patients with sinusitis or dental treatment, 2 microbial groups were identified (Table 4), including one made of *Peptostreptococcus* species, *Fusobacterium* species, and/or *S. intermedius* but no uncultured bacteria, and a second group made of *Bacteroides* species, uncultured bacteria, *M. faucium*, and Enterobacteriaceae. Although we acknowledge the fact that the numbers of patients in both groups are small, we did not expect to identify distinct microbial associations in brain abscess following sinusitis or dental treatments. We did not find any epidemiological or clinical specificity associated with these microbial populations, but such a finding may not have any impact on the management of patients, because the association of a β-lactam and metronidazole may cover most of the microorganisms identified in these 2 patient groups.

Of the 23 sequences matching those of uncultured bacteria, described as belonging to the periodontal or intestinal microflora, only 1 (uncultured BA21) had been detected previously in a brain abscess [23]. The uncultured bacteria, including 10 Bacteroidetes species (43.5%) and 9 Firmicutes species (39.1%), were detected from 7 specimens and significantly associated with polymicrobial floras ($P < 10^{-2}$), in particular in patients with sinusal or dental risk factors ($P < 10^{-2}$), thus highlighting the underestimated diversity of potential pathogens in this flora. Our data are consistent with previous metagenomic studies of the oral flora, in which it was estimated that approximately 50% of the bacteria are uncultivable [17, 20–22, 39] and belong to the Bacteroidetes and Firmicutes phyla.

We identified *M. faucium* in 1 specimen. In our previous study, *M. faucium* was detected in 3 of 20 specimens (15%) and was thus thought to be a quite common brain abscess agent [23], prompting the development of a specific PCR assay. However, in the present series, only 1 specimen was positive for *M. faucium* (1.9%). This difference, which is nearly significant ($P = .06$), likely results from sampling differences. After combining data from both studies, we observed that *M. faucium* was significantly found in polymicrobial specimens ($P = .02$) and in patients with sinusal or dental defects ($P = .01$). Although this bacterium is not susceptible to the usual empirical treatment of brain abscesses [40], all 4 patients infected with *M. faucium* in our series recovered without any specific treatment.

When combined with our previous study [23], metagenomic analysis of 16S rDNA sequences amplified from brain abscesses from 71 patients proved to be an extremely powerful tool, identifying 71 bacterial taxa that had not been previously detected in these infections, including 37 that are as yet uncultured, and highlighting the sinusal and dental floras as the main source of new bacterial taxa in brain abscess.

Notes

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