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► **To cite this version:**

Michel Drancourt, Vanessa Demonfort Nkamga, Ndeye Aissatou Lakhe, Jean-Marie Regis, Henry Dufour, et al.. Evidence of Archaeal Methanogens in Brain Abscess. *Clinical Infectious Diseases*, 2017, 65 (1), pp.1-5. 10.1093/cid/cix286 . hal-01573764

HAL Id: hal-01573764

<https://hal.science/hal-01573764>

Submitted on 2 May 2018

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Evidence of Archaeal Methanogens in Brain Abscess

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Background. Methanogens are antibiotic-resistant anaerobic archaea that escape routine detection in clinical microbiology. We hypothesized that methanogens are part of the anaerobic community that cause brain abscess.

Methods. Methanogens were investigated in 1 index sample using specific polymerase chain reaction (PCR) sequencing and culture. The pathogenesis of a methanogen isolate was assessed in a mouse model. Archaea-specific quantitative (q) PCR and metagenomics were used to detect specific archaeal sequences in brain abscess samples and controls.

Results. In 1 index sample, routine culture found *Porphyromonas endodontalis* and *Streptococcus intermedius*, and specific culture found *Methanobrevibacter oralis* susceptible to metronidazole and fusidic acid. Archaea-targeted PCR sequencing and metagenomics confirmed *M. oralis* along with 14 bacteria, including *S. intermedius*. Archaea-specific qPCR yielded archaea in 8/18 brain abscess specimens and 1/27 controls ($P < .003$), and metagenomics yielded archaea, mostly methanogens, in 28/32 brain abscess samples, and no archaea in 71 negative controls ($P < 10^{-6}$). Infection of mice brains yielded no mortality in 14 controls and death in 17/22 *M. oralis*-inoculated mice ($P < 10^{-6}$), 32/95 *S. intermedius*-inoculated mice ($P < 10^{-6}$), and 75/104 mice inoculated with *M. oralis* mixed with *S. intermedius* ($P < 10^{-6}$) 7 days post-inoculation.

Conclusion. Methanogens belong to the anaerobic community responsible for brain abscess, and *M. oralis* may participate in the pathogenicity of this deadly infection. In mice, a synergy of *M. oralis* and *S. intermedius* was observed. Antibiotic treatment of brain abscess should contain anti-archaeal compounds such as imidazole derivatives in most cases.

Keywords. brain abscess; methanogen; *Methanobrevibacter oralis*; microbiology; culture.

Brain abscess remains a serious infection, with a mortality that has fallen from 30%–60% in older series to approximately 15% in more recent series in patients who benefited from computerized tomography scanning, surgical drainage, and combinations of antibiotics [1, 2]. The management of patients diagnosed with brain abscess is guided by the proper identification of the causative organisms [1, 2].

Recent advances in the molecular investigation of brain abscess, including multiple 16S rRNA gene sequencing [3] and metagenomics [4], has steadily expanded the repertoire of bacteria detected in brain abscess by 3-fold and 4.2-fold, respectively, revealing emerging pathogens such as mycoplasma [5]. However, these techniques have overlooked methanogens, which are antibiotic-resistant archaea prokaryotes that escape routine detection in clinical microbiology laboratories. They are highly fastidious organisms cultured in a hydrogen atmosphere

and they resist routine 16S rRNA gene-based detection [6, 7]. Methanogens have been recently detected in the human microbiota associated with the oral, gut, and vaginal mucosae [8, 9]. The methanogen *Methanobrevibacter oralis* has been implicated in periodontal infection [10], but its role as a pathogen remains to be elucidated.

Recently, we developed specific detection and culture techniques in the quest for pathogenic methanogens. We assumed that methanogens, which are strict anaerobes of the oral microbiota, could be detected in diseased tissues also infected by anaerobic oral bacteria, as is the case for brain abscess [4, 11]. Therefore, we thoroughly investigated the presence of methanogens in 1 index brain abscess specimen using methanogen-oriented techniques to confirm the presence of live, pathogenic methanogens in the brain abscess. We then used neutral metagenomics as a second line detection technique in a larger series.

The Ethics Committee of the Institut Fédératif de Recherches 48 approved this study.

METHODS

Patients

One brain abscess index case with a sufficient quantity of abscess material was thoroughly investigated. This brain

Received 7 December 2016; editorial decision 9 March 2017; accepted 28 March 2017; published online April 1, 2017.

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Clinical Infectious Diseases® 2017;65(1):1–5

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abscess specimen was collected by stereotaxic puncture from a 51-year-old female with a 1-month history of dental avulsion who presented with febrile confusion and a left thalamic lesion that suggested an abscess, documented by magnetic resonance imaging. Her medical history was remarkable for untreated HLA B27-positive rheumatoid arthritis. Cytopathological examination of the brain lesion revealed lymphocytic and monocytic inflammation without polymorphonuclear or malignant cells. Routine 16S rRNA gene-based detection found *Porphyrromonas endodontalis*, with 99.4% similarity to the reference sequence (GenBank, NR_113085), and *Streptococcus intermedius* was documented using specific real-time polymerase chain reaction (RT-PCR) and cultured. The patient responded to 6 weeks of daily treatment, which included ceftriaxone 2 g and metronidazole 1.5 g. At the 3-month medical follow-up, the patient showed no evidence of infection and her cognitive impairment was improving.

PCR Sequencing

Archaea 16S rRNA and *mcrA* genes were PCR amplified in the brain specimen of the index patient, as previously described [12, 13], in the presence of 27 negative controls that consisted of pathologically assessed, anaerobically cultured brain biopsies sampled in patients with brain diseases other than brain abscess. PCR products were purified using the NucleoFast 96 PCR kit (Macherey-Nagel, Hoerd, France), and sequencing reactions were carried out using the Big-Dye Terminator, version 1.1, cycle sequencing kit DNA (Perkin-Elmer, Villebon sur Yvette, France), according to the manufacturer's instructions. All PCR products were sequenced, and the different fragments were assembled using ChromasPro software and compared to the GenBank database using the online BLAST program of the National Center for Biotechnology Information (NCBI).

Isolation and Culture

A 100- μ L volume of the brain abscess specimen from the index patient was cultured in a Hungate tube that contained 5 mL anaerobe methanogenic medium complemented with 100 mg/L imipenem, 100 mg/L vancomycin, and 50 mg/L amphotericin B. This was then incubated using a mixture of 80% H₂ + 20% CO₂ at 2 bar pressure at 37°C, with agitation as previously described [14]. Twenty colonies were picked using a sterile Pasteur pipette in order to perform genome sequencing. The in vitro susceptibility of the methanogen isolate was determined for metronidazole, fusidic acid, chloramphenicol, and ceftriaxone, as previously described [15].

Genome Sequencing

Genomic DNA of the *M. oralis* isolate made from the index patient brain abscess specimen was sequenced using MiSeq Technology [16] (Illumina, Inc., San Diego, California) with paired-end and barcode strategy in order to be mixed with 16

other projects constructed according to the Nextera XT library kit (Illumina). One nanogram of the genomic DNA sample was fragmented by the "tagmentation" step, and fragments were tagged with junction adapter and dual-index barcodes with a limited cycle PCR. Then, limited cycle PCR amplification completed the tag adapters and introduced dual-index barcodes. The libraries were then normalized on specific beads according to the Nextera XT protocol (Illumina), pooled into a single library, then loaded onto the reagent cartridge. Automated cluster generation and paired-end sequencing with dual index reads was performed in a single 39-hour run in a 2 × 251 bp. Total information of 4.7 Gb was obtained from a 486 K/mm² cluster density with 96.1% (9 540 000 clusters) of the clusters passing quality control filters. Within this pooled run, the index representation of *M. oralis* was determined at 6.31%. The 578 627 paired-end reads were filtered according to the read qualities.

Real-Time PCR

DNA was extracted from the pus specimen collected from the index patient, along with pus from brain abscess specimens collected from 17 additional patients with community-acquired brain abscess in the presence of 27 pathologically assessed, anaerobically cultured negative controls. The negative controls consisted of brain biopsies sampled from patients with monomicrobial infection (n = 5) and with diseases other than brain abscess (n = 22; Supplementary Table 1). Extracted DNA was incorporated into blind RT-PCR, targeting *M. oralis*, as previously described [10]. Further RT-PCR detected *Methanobrevibacter smithii* by targeting a repeat region (Supplementary Table 2) using the following primer pair: *M. smithii*-F (5'-ACCATAACyATCAGCAGCATTAT-3') and *M. smithii*-R (5'-AGTATTGGTGAAGGATTTaCTGT-3') (Eurogentec, Seraing, Belgium), and the *M. smithii* probe (6-carboxyfluorescein [FAM]-5'ACCyTTATCAGCTTTACCA TTAATyAAAG-3') (Applied Biosystems, Courtaboeuf, France) under the following PCR conditions: 95°C for 5 minutes, followed by 40 cycles of 95°C for 1 second, 60°C for 35 second, and 45°C for 30 second. The specificity of the *M. smithii* primer and probe were verified in silico using the BLAST program at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST>). The specificity was also experimentally ensured by incorporating DNA extracted from 96 bacteria and DNA extracted from *Methanosphaera stadtmanae* ATCC 43021^T, *Methanomassiliicoccus luminyensis* CSURP135^T, and *M. oralis* DSMZ 7256^T. Eight negative controls that consisted of sterile phosphate buffer were used in this experiment. The RT-PCR assays were all performed using a CFX96 Touch RT-PCR detection system (Bio-Rad, Marnes-la-Coquette, France) and the MasterMix PCR kit (Eurogentec) with 5 pmol of each primer and probe and 5 μ L of approximately 2 μ g of DNA in a 20- μ L final volume. The RT-PCR was regarded as positive for any cycle threshold (Ct) value

<38. Results were expressed as the number of *M. oralis* and *M. smithii* gene copies/specimen.

Metagenomic Analyses

Total DNA was extracted from the pus collected from the index patient and from brain abscess specimens collected from an additional retrospective series of 31 patients diagnosed with community-acquired and post-surgical brain abscess. These 31 additional patients were of European origin and aged 8 days to 82 years (mean, 48.3 years). The brain abscess specimens had been referred to our laboratory from 2009 to 2015 and conserved at -80°C . DNA extraction was done using the EZ1 Advanced XL automate and the DNA Tissue kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Each DNA sample was quantified using the Qubit assay (Life Technologies, Carlsbad, California). These samples were sequenced with the MiSeq Technology (Illumina) using the paired-end strategy. Sequencing was performed as described above. Bioinformatic analysis was performed using the MetAMOS V.1.3 pipeline [17]. Reads of poor quality were filtered through FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). The sequence assembly was performed with Velvet software [18]. FragGeneScan allowed searching open reading frames in the assembled sequences [19]. The taxonomic annotation was made by fragment classification package [20]. All other MetAMOS parameters were kept by default.

Experimental Mouse Model of *Methanobrevibacter oralis* Brain Infection

The mouse experimental infection was performed using the *M. oralis* and *S. intermedius* isolates cultured from the index patient's brain abscess. Mice were handled according to the rules of Decree N° 2013–118, 7 February 2013, France, and the experimental protocol (reference APAFIS 2016042113106281) was approved by the Ethics Committee C2EA-14 of Aix-Marseilles University, France, and the French Ministry of National Education, Higher Education and Research. Mice were housed in a specific facility and fed sterile food and water ad libitum. Four groups of newborn mice were used, and the experiment was done in duplicate. The control group was inoculated intracerebrally with a 15- μL volume that contained sterile phosphate buffer, and the experimental group with 104 colony-forming units (CFU) *S. intermedius*, 10^6 CFU *M. oralis*, or 10^1 – 10^4 CFU *S. intermedius* mixed with 10^6 CFU *M. oralis*. Mice were observed twice daily for 7 days for any signs of discomfort or distress, and the mortality was noted. Surviving animals were then euthanized, and the brain was aseptically collected from each mouse. Each sample was conserved at -80°C for *M. oralis* quantitative (q) PCR and isolation. Total DNA extracted as described above served as a template for qPCR using the SYBR Green RT-PCR MasterMix (Qiagen) with 20 pmol of each *mcrA-F* (5'-GGTGGTGTMGGATTCACACARTAYGCWACAGC-3')

and *mcrA-R* (5'-TTCATTGCRTAGTTWGGRTAGTT-3') primers and 5 μL of DNA in a 20- μL final volume. Mouse brain inoculated with 10 μL of *M. oralis* as a positive control and 1 negative extraction control were included in each reaction plate. Results were expressed as the number of *M. oralis* gene copies/specimen. The PCR program was 95°C for 15 minutes, followed by 39 cycles of 95°C for 10 second, 55°C for 30 second, and 72°C for 45 second. A calibration curve was created by measuring the Ct value of a serial dilution of *M. oralis* as positive control.

RESULTS

Laboratory Investigations of the Index Patient With Brain Abscess

In the thoroughly investigated brain abscess pus collected in the index patient, RT-PCR yielded *M. oralis* (gene copies/specimen, $3.29\text{E}+03$) and no *M. smithii*, while the negative controls remained negative. In this specimen, PCR sequencing yielded a 16S rRNA gene sequence exhibiting 99% sequence similarity with the reference *M. oralis* (GenBank accession number HE654003), and the PCR sequencing of the *mcrA* gene exhibiting 99% sequence similarity with the reference *M. oralis* (GenBank accession number DQ251045), while the 27 negative controls remained negative. The sequences of the different genes reported here have been deposited in GenBank (accession numbers LN876655 and LN876656). The culture in aerobic atmosphere remained sterile, but culture in anaerobic conditions using a blood agar plate enriched with antioxidant molecules (ascorbic acid, 1 g/L; uric acid, 0.5 g/L; and glutathione, 0.5 g/L) [21, 22] yielded *S. intermedius*, identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) with scores of 1.907 and 2.043, respectively. In the presence of 2 negative controls that remained sterile, culture of the pus in SAB-medium, as previously described [14], generated methane in 7 weeks, and subculture yielded colonies in 12 weeks. Complete genome sequencing of the isolate confirmed the identification of *M. oralis*, with 99% genomic sequence similarity with the reference genome [23]. The isolate, grown in the presence of 100 mg/L imipenem, 100 mg/L vancomycin, and 50 mg/L amphotericin B in the SAB medium, was susceptible in vitro to metronidazole (minimum inhibitory concentration [MIC] ≤ 1 mg/L) and fusidic acid (MIC ≤ 0.5 mg/L) and resistant to chloramphenicol (MIC ≥ 50 mg/L) and ceftriaxone (MIC, 100 mg/L).

Real-Time PCR

In a collection of 18 brain abscess specimens, RT-PCR detected *M. oralis* in 7/18 (38.9%) specimens, with an average gene copies/specimen of $1.01\text{E}+03 \pm 1.14\text{E}+03$, and *M. smithii* in 1/18 (5.6%), with a gene copies/specimen of $1.88\text{E}+02$. In the control group, the same experiments detected *M. oralis* in 1/27 (9.4%) specimens, with an average gene copies/specimen of $1.59\text{E}+03 \pm 1.97\text{E}+03$ and no *M. smithii*. The difference in the

prevalence of *M. oralis* between the control group and the brain abscess group was significant ($P < .003$; χ^2 , 8.8). In particular, 5 patients with presumptive monomicrobial brain abscess were negative for archaea by RT-PCR.

Metagenomic Analyses of a Series of Brain Abscesses

After MiSeq sequencing, the average number of reads was 611c215 reads/patient. The size of the reads varied from 105 bp to 300 bp. Reads belonging to archaea were found in all patients, whereas negative controls yielded *Halomonas* spp. (52.06%), *Shewanella algae* (30.26%), miscellaneous bacterial species, and no archaeal reads. Excluding *Homo sapiens*, the proportion of reads belonging to bacteria was $72\% \pm 6.4\%$ (65%–93%), and the proportion of reads belonging to viruses was $0.3\% \pm 0.4\%$ (0%–2%). Archaea reads were found in 28/32 samples, including the index patient specimen; the proportion of reads belonging to archaea was $26\% \pm 1.4\%$ (range, 25%–27%) in brain abscesses of traumatic origin, $24.5\% \pm 2.38\%$ (range, 21%–26%) of dental origin, $22.5\% \pm 2.1\%$ (range, 21%–24%) of cancer origin, $21.5\% \pm 8.4\%$ (range, 9%–27%) linked with a bacteremia, and $21.3\% \pm 7.2\%$ (range, 9%–27%) in post-surgical brain abscess. Reads belonging to archaea represented 25% in 1 patient with otitis, 25% in 1 patient with Rendu-Osler disease, and 23% in 1 patient with sinusitis. By contrast, no archaeal read was found in 45 plasma and serum samples, 12 lymph node biopsies, 8 pericardial fluid, and 6 ocular fluid specimens (using a 70% cutoff sequence similarity) ($P < 10^{-6}$; χ^2 , 98). The phylum *Euryarchaeota* represented the majority of archaeal reads (64.2%). The genera most represented were *Methanothermobacter*, *Methanobrevibacter*, and *Methanobacterium*, and *Methanothermobacter thermoautotrophicus* and *Methanothermobacter marburgensis* were the most common species (Supplementary Table 3).

Mouse Model

Seven days after inoculation, the experimental infection of mice brains yielded no mortality in the 14 control mice inoculated with buffer, whereas 17/22 (77.3%) *M. oralis*-inoculated mice died ($P < 10^{-6}$), 32/95 (33.7%) *S. intermedius*-inoculated mice died ($P < 10^{-6}$), and 75/104 (72.1%) mice inoculated with *M. oralis* mixed with *S. intermedius* died ($P < 10^{-6}$). The mortality in mice coinoculated with mixed *M. oralis* and *S. intermedius* was significantly higher than the mortality in mice inoculated with *S. intermedius* alone ($P < 10^{-6}$). Culture of the liver and spleen indicated no methane production, but 2/22 tubes seeded with mice brains inoculated with *M. oralis* produced methane gas. The methanogen was identified as *M. oralis* on the basis of a 99% 16S rRNA and *mcrA* gene sequence similarity with the reference *M. oralis*. The same 2 tubes cultured *S. intermedius* after a 2-day incubation on Colombia agar + 5% sheep blood at 37°C under anaerobic conditions; the isolate was identified by MALDI-TOF-MS. In all PCR-based experiments, negative controls remained negative, whereas the quantification plasmid

introduced as an internal control to monitor the absence of PCR inhibition yielded positive amplifications in 100% of specimens. In *M. oralis*-infected mice, PCR quantifications of the *mcrA* gene specific for methanogens showed an average cell number of $4.39E+02 \pm 4.58E+01$ (Ct, 33.7 ± 2.47), while no archaea were detected in mice inoculated with sterile phosphate buffer or *S. intermedius* only.

DISCUSSION

We show that archaea are part of the microbiota responsible for some cases of brain abscess. This fact was confirmed by several lines of evidence, including RT-PCR detection of *M. oralis* with a prevalence 8 times higher in 18 patients than in 27 controls, including 5 cases of monomicrobial brain abscess; the metagenomics detection of archaea in brain abscess specimens from all 32 studied patients; and the direct observation of *M. oralis* in 1 thoroughly investigated index brain specimen by RT-PCR, PCR sequencing, and isolation in coculture with *S. intermedius*. Accordingly, metagenomics data indicate that archaea form 5%–27% of the brain abscess microbiota. These archaea mostly belong to the Euryarchaeota (64.2%) and Crenarchaeota (29.7%), the most represented organisms belonging to the methanogenic species *M. thermoautotrophicus* and *M. marburgensis*. The fact that we did not detect reads of archaea in a recently investigated series of 71 tissue and biopsy specimens in our laboratory further reinforces the specificity of the metagenomics study of brain abscesses. Being retrospective, these data had no direct impact on the medical management of these patients. Furthermore, all patients investigated in this series were of European origin, consequently, the prevalence and nature of brain abscess methanogens detected here may not reflect those observed in other geographical settings, and further series should be investigated in other reference centers.

Archaea have not previously been detected in brain specimens because they are fastidious organisms that do not grow using routine protocols in the microbiology laboratory and are not amplified by bacteria-dedicated 16S rRNA PCR assays. Accordingly, previous multiple sequencing investigations that used bacterial 16S rRNA gene PCR, cloning, and sequencing overlooked archaea [4, 24, 25]. Metagenomic data agree with the isolation and culture of *M. oralis*, a methanogen in the oral cavity and also isolated from the digestive tract [23]. This isolate was clearly identified after we sequenced its complete genome. The mouse model indicated that *M. oralis* alone was responsible for an acute mortality significantly higher than controls. This observation suggests that *M. oralis* alone is pathogenic for neural tissue. Indeed, *M. oralis* produces methane, a potent neurotoxic gas [26], suggesting that methanogens may participate in the pathogenicity of brain lesions in part by neuronal toxicity.

The *M. oralis* brain isolate studied here was resistant to ceftriaxone, imipenem, and vancomycin, which are routinely used to treat brain abscess [1]. This pattern of resistance agrees with previous in vitro susceptibility data that indicate that methanogens are broadly resistant to antibiotics, including many agents often prescribed as empirical treatment of brain abscess [27]. However, this isolate was susceptible to metronidazole, which is in agreement with previously published data [6, 27]. Metronidazole revolutionized the prognosis of brain abscess in the 1970s, and this has been attributed to its activity against anaerobes [1]. In fact, part of the clinical effectiveness of metronidazole may also be due to its potent activity against methanogens. In the series reported here, the sole recorded death was of a patient infected by *M. smithii* who did not receive metronidazole.

CONCLUSIONS

These data suggest that, given the probable involvement of methanogens in brain abscess, the medical treatment of brain abscess must incorporate agents active against these microorganisms, such as the imidazole derivatives [27].

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Financial support. V. D. N. received a PhD grant from the IHU Méditerranée Infection, Marseille, France. This work was funded by Unité de recherche sur les Maladies Infectieuses et Tropicales Emergentes, Marseille, France

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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