Title:

Diagnostics with clinical microbiome-based identification of microorganisms in patients with brain abscesses – a prospective cohort study

Running head: Microbiome analysis of brain abscesses

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Summary


Brain abscesses are often polymicrobial and of unclear primary origin. Here we compare the use of Next Generation Sequencing (NGS) technology with classical microbiological diagnostics for identification of clinically relevant microorganisms and describe the microbiome profiling with respect to the primary source of brain abscess. Thirty-six samples from 36 patients, with primary brain abscesses, were subjected to both culture- and 16S/18S rRNA Sanger sequencing-based diagnostics (“standard methods”) and compared to a 16S/18S amplicon-based NGS, which were also subjected to a microbiome diversity analyses. Forty-seven species were identified with “standard methods” compared to 96 species with NGS, both confirming and adding to the number of species identified (P<0.05). The variation of the brain abscess microbiome diversity was not continuous but could be stratified comparing the presumable origin of infection (“dental”, “sinus”, “disseminated” or “unknown”). Alpha diversity did not differ (P>0.05) between groups while beta diversity differed significantly (P=0.003) comparing disseminated vs. the other presumable origin of infection. Interesting, clustering was also detected between “dental” and “sinusitis”, although not significantly (P=0.07). Microbiome-based diagnostics can increase sensitivity...
The bacterial beta diversity differed between the presumably origin of the brain abscess and might help to clarify the primary source of infection.

**Introduction**

Despite advances in diagnostic and neurosurgical procedures, the mortality and morbidity of brain abscess remains high and occur at any age group (1,2). In a review from 2017 the highest incidence was reported to be between 30-40 years, although a recent study from Denmark reports the median age to be 50 years (3,4). There is a significant male predominance with male to female ratios ranging from 2:1 to 3:1 (3). Abscesses occur most commonly in the frontal lobe but may occur in any location. The location is closely associated with the source of infection (1). Brain abscesses result from direct extension from a contiguous suppurative focus (dental infection, paranasal sinuses, middle ear, or mastoids), from hematogenous dissemination (especially in the case of infective endocarditis) or from direct inoculation (trauma or neurosurgery) (3). The spectrum of pathogens in brain abscesses is diverse and may be caused by bacteria, archae, fungi and parasites (1,5). In most cases, the detection of causative organisms is made by culture from pus collected by neurosurgical drainage. However, culture of pus remains sterile in up to 63% of cases because some species are difficult or impossible to culture or because of prior antimicrobial therapy (6). Culture-independent approaches such as 16S/18S rRNA Sanger sequencing has been a valuable supplement to culture, especially in cases of prior antimicrobial therapy (7). However, the technique is hampered in case of polymicrobial infections, which is very common with brain abscesses. Hence, the microbial spectrum involved in brain abscesses is complex and incompletely characterized. Recently, application of Next Generation Sequencing (NGS) has been introduced as a diagnostic tool and to characterize polymicrobial communities (the microbiome or microbiota), but so far these studies have focused on bacteria with a 16S target (6,8).

In this study, we investigated if a clinical microbiome-based approach with NGS, targeting both the 16S gene (bacteria), and as an add on, the 18S gene (fungi and parasites), could identify additional clinically relevant microorganisms from primary brain abscesses and support the choice of antibiotic therapy.
To expand our knowledge, we also wanted to investigate whether a brain abscess bacterial microflora is correlated to the origin of the brain abscess by carrying out diversity analysis to describe the 16S microbiome profiling.

Preliminary results were presented at the 28th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID), Madrid, Spain, 21-24 April 2018 (Abstract O0449).

**Materials and methods**

**Design**

Single-centre prospective cohort study with microbiological investigation of aspiration specimens from primary brain abscesses. The STARD 2015 guideline was used for reporting of this study, although it is not a diagnostic accuracy study (9).

**Population**

All patients admitted to Rigshospitalet (a tertiary-referral hospital), Copenhagen, Denmark, during a 2-year period, from March 2016 through February 2018 who underwent surgery for primary brain abscesses. Age, sex, prior use of antimicrobial agents, probable primary source of infection as well as any immunocompromising conditions were recorded from the laboratory request form and categorized in the following four groups; “dental”, sinusitis”, “disseminated” or “unknown”. The laboratory request form was completed by the physician before any material was sent for microbiological analysis. Brain abscesses secondary to trauma or neurosurgery were excluded. The study was approved by the Danish Data Protection Agency (2007-41-0229). Informed consent was not required for this study.

**Sample processing**

Samples were investigated by standard culture-based routine diagnostics at the local department of clinical microbiology at Rigshospitalet. Culture-negative samples underwent supplementary 16S/18S rRNA Sanger sequencing. Residual material was stored at 5°C for parallel identification and characterization of pathogens using 16S/18S amplicon based NGS.
Culture

Pus specimens were cultured on 5% horse blood and chocolate agar plates (aerobic conditions and with 5% CO₂), eosin methylene blue plates (aerobic) and on anaerobic agar plates (anaerobic conditions) and incubated at 37°C (all agar plates were from SSI Diagnostica, Hillerød, Denmark). Aerobic and anaerobic incubated plates were cultured for up to 2 and 7 days, respectively. In parallel all samples were incubated in an anaerobic blood culture bottle for up to 7 days. If fungal infection was suspected, pus specimens were also cultured on chromogenic ager plates (bioMérieux, Marcy l’Etoile, France) for up to 2 days and Sabouraud agar plates (SSI Diagnostica, Hillerød, Denmark) for up to 5 days. Isolated bacteria and fungi were identified using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) (Bruker, Bremen, Germany).

Sanger sequencing

Culture-negative samples were subjected to supplementary 16S/18S rRNA Sanger sequencing using the Universal Microbe Detection (UMD) SelectNA kit (Molzym, Bremen, Germany); the routine method used at the local department of clinical microbiology. Samples were processed with the UMD SelectNA assay. Human DNA was removed during the DNA extraction procedure, followed by real-time PCR amplifying the V3-V4 region of the 16S rDNA gene and the V8-V9 region of the 18S rDNA gene and Sanger sequencing as previously described (10). If a mixed chromatogram was found, suggesting DNA from more than one species, we used the Pathogenomix Ripseq software and database to try to separate the species (11).

Microbiome sequencing

Microbiome profiling was done using a recently described 16S + 18S assay, in place at Statens Serum Institut (12-14). Briefly, genomic DNA was extracted from the samples using a DNeasy Blood and Tissue Kit (Qiagen Inc, Düsseldorf, Germany) according to the manufacturer’s instructions. PCRs were carried out using one primer pair targeting the 16S
and three primer pairs targeting the 18S and sequenced on the Illumina MiSeq desktop sequencer (Illumina Inc., San Diego, CA, USA) with the 500-cycle MiSeq reagent kit, V2, in a 2 x 250-nucleotide setup (Illumina Inc., San Diego, CA, USA).

Annotation of sequences reads to taxonomic level was performed using BION (http://box.com/bion), a newly developed analytical semi-commercial open-source package for 16S/18S rRNA, classifying reads predominantly to species level, was used (12). The pipeline accepts raw sequences and includes steps for demultiplexing, primer extraction, sampling, sequence- and quality-based trimming and filtering, dereplication, clustering, chimera checking, identification of similarities to reference data, and taxonomic mapping and formatting. Non-overlapping paired reads were allowed for analysis. The workflow is depicted in Figure 1.

The NGS analysis results in a considerable number of DNA sequences (reads) which requires extensive interpretation for determining a significant clinical pathogen. For the purpose of this study, we used the following interpretation algorithm for a significant clinical pathogen. All reads from species accounting for 0.2% or more of the total reads were included. From the experiences for this study, the number of suspected contaminants, including environmental fungi and bacteria, exceeds the number of significant pathogens, if reads from species below the 0.2% level are included. However, the limit is arbitrary. Following this procedure, all species are evaluated on an individual basis and categorized as significant pathogens or contaminants based on the normal habitat of the species, association with infection and percentage of total reads.

**Bioinformatics and microbiome profiling (only 16S)**

The BION server not only allows the extraction of FASTA files that can be used to query DNA sequences of interest against the NCBI Database, but also extraction of read counts per taxon per sample which we used to carry out analyses in R (v 4.0.3) and R Studio (R Foundation for Statistical Computing, Vienna, Austria) R Core Team. The R package PhyloSeq was used to describe the sequencing data. A 16S rarefaction threshold was set at 1600 reads. Within-sample (alpha diversity) was illustrated with a ggplot, and individual differences were assessed with Shannon’s Diversity Index and compared between groups.
using the Mann–Whitney rank-sum test. Between-sample (beta diversity) were compared using principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity between samples. Heatmaps were used to detect top-10 most common bacterial genera. A probability (P) value <0.05 was considered to indicate statistical significance.

Results

Clinical data

A total of 36 samples from 36 patients with primary brain abscesses were included from 20 men and 16 women aged 58 years (median; range, three weeks to 84 years) (Table 1). Samples were obtained through stereotactical or ultrasound-guided aspiration. In brief, brain abscess developed in ten patients (28%) following dental infections, in ten patients (28%) following sinusitis, and in three patients (8%) with disseminated infection primary focus was presumed to be neonatal meningitis, urinary tract infection and skin infection, respectively. No primary source could be identified in 13 patients (36%).

Comparison between diagnostic methods

An overview of all 36 primary brain abscesses including a comparison of the species identified by the three methods (culture, Sanger sequencing and microbiome analysis) is presented in Table 1. The combination of culture and Sanger sequencing is referred to as “standard methods”. No samples were positive by standard methods only. A total of 31 samples (86.1%) were positive by both standard methods and microbiome sequencing, while an additional two samples (5.5%) were positive by microbiome sequencing only (Figure 2). Three samples (8.4%) were negative by both standard methods and microbiome sequencing.

Species identification and distribution

The NGS analysis resulted in a number of DNA reads with a median of 104119 reads (from 27826 to 268539 reads). With the microbiome-based diagnostic approach it was possible to identify additional species in 17 samples (47.2%) (Table 1) following the algorithm described
above (excluded contaminants from microbiome sequencing are shown in Supplementary Table S1). A total of 96 significant pathogens were identified with a score at or above the 0.2% level; 78 of these were identified with a score ≥1%. Likewise, 58 contaminants were identified at or above the 0.2% level; 47 were <1%.

The most predominant species identified by both standard methods and microbiome sequencing were those belonging to the *Streptococcus anginosus* group (n=14 and n=14, respectively) and *Fusobacterium nucleatum* (n=6 and n=13, respectively). Species identified by standard methods (n=47) compared to microbiome sequencing (n=96) were divided into the following groups: oral streptococci, anaerobic oral commensals, other oral commensals, skin or intestinal pathogens, and *Aspergillus* species. The distribution is illustrated in Figure 3.

In 16 samples the same single species was found with standard methods and microbiome analysis except for one sample, which showed only partial concordance. Culture identified *Actinomyces oris* (ID 33), which was not identified by microbiome analysis (or by Sanger sequencing) and was considered to be a contaminant from the culture procedure. Most samples showing concordance were monomicrobial (93.8%).

In two samples with several microorganisms, some species detected by standard methods were not identified by microbiome analysis. In one sample (ID 20) from a patient with a brain abscess following a dental infection, *Eikenella corrodens* was cultured but not detected with microbiome analysis, because the percentage of reads for *E. corrodens* were below the 0.2% threshold level (not included in Table 1). In another sample, *Micrococcus luteus* was cultured (ID 21), but was not identified by microbiome analysis, and was most likely a contaminant from the culture procedure.

**Additional results from microbiome sequencing**

Among positive samples, culturing and Sanger sequencing yielded a median of one (range, 1-3) species compared to two (1-10) species identified by microbiome analysis. A total of 18 (50%) polymicrobial samples were detected using a microbiome-based diagnostic approach. The microbiome-based approach identified additional species belonging especially to the
anaerobic oral commensals (13 with standard methods and 52 with microbiome sequencing). In several cases only one species was identified by standard methods, e.g., ID 6, ID 16, and ID 27, but several species were identified with microbiome sequencing. In two cases, ID 18 and ID 30, only microbiome sequencing detected a pathogen. One third of the cases did not present with a probable source of infection, but in all these cases microbiome sequencing either confirmed or added to the number of species identified by standard methods. Also, in seven out of ten culture-negative cases, antibiotic therapy had been administered before sampling; but in six of these cases, sequencing produced clinically relevant results.

Microbiome diversity analyses (16S) and source of infection

Crude bacterial richness and alpha diversity comparing the presumably origin of infection (‘‘dental’, sinus’, ‘disseminated’ or ‘unknown’) did not differ (P>0.05; Figure 4). In contrast, beta diversity differed between groups with a very distinct clustering for a presumably disseminated vs. the other groups (P=0.003) as illustrated in Figure 5. The clear distinction between the disseminated and the other groups could also be demonstrated by comparing the top-ten most common bacterial genera identified: Aggregatibacter, Bacteroides, Escherichia, Fusobacterium, Nocardia, Parvimonas, Porphyromonas, Propionibacterium, Staphylococci and Streptococci (Figure 6). No apparent difference in the microbiome profile could be seen between the sinus- and unknown group. Of interest, a clustering between a dental origin of infection vs sinus/unknown foci was apparent (Figure 5), although the difference was not significant (P=0.07) and there were only four genera shared among the ten most common bacterial genera between the “dental” and “sinus”/“unknown” group (Streptococci, Fusobacterium, Porphyromonas and Parvimonas). A higher abundance of sequences belonging to the two genera Actinomyces (P=0.078) and Campylobacter (P=0.05) were especially predictable for a dental origin of infection.

Discussion
In this study, we compared the identification results between standard methods (culture and Sanger sequencing) with microbiome sequencing using NGS. NGS is believed to be more sensitive than with culture, especially in case of previous antibiotic therapy, and Sanger sequencing. In theory, this could be at the cost of specificity; thus, thorough evaluation of the output from NGS is needed. In this study, several reads from environmental contaminants were detected, but it was relatively easy to exclude them from further evaluation. Furthermore, three samples from patients suspected of a brain abscess were negative with NGS, apart from limited output from obvious contaminants. However, it is important to remember that we only included primary brain abscesses. If we had included brain abscesses that were secondary to trauma or neurosurgery, the interpretation would probably be more difficult, because the usual environmental contaminants could be significant pathogens in these cases (15). It is of course very important that the false positive output is easy to exclude and at a low level, if NGS is going to be used in a clinical routine. Several of the cases from this study were monomicrobial according to both culture and NGS, which adds to the reliability of the analysis, i.e., we did not observe a lot of additional output. Also, the sensitivity was apparently superior with microbiome sequencing, as the number of identified species doubled and several fastidious and difficult-to-culture species were detected, e.g., *Campylobacter gracilis* in five samples. The distribution of bacteria from this study, is very similar to a comparable study by Kommedal *et al.*, however, that study only included the 16S target (8). We were able to identify *Aspergillus* species in two cases with microbiome sequencing because the 18S target was also included in this study (also identified with standard methods).

From a clinical point of view, the results did add considerable information to the case history. Identifying the source of a brain abscess, e.g., a dental infection or sinusitis, is a very important aspect of the treatment. In the 12 cases with an unknown source, microbiome sequencing either confirmed or added species information to the standard methods, which would help to identify the primary site of infection. In the cases with none or one species identified by culture, the addition of more species from microbiome sequencing would unequivocally direct the attention towards an oral site of infection or the upper respiratory tract (all the anaerobic species detected with NGS, apart from *Bacteroides fragilis* and
Cutibacterium acnes (formerly Corynebacterium acnes), are commensals of the oral flora (16). The result from microbiome sequencing would also be important for the correct choice of antibiotic therapy. In many cases treatment with penicillin would apparently be sufficient based on culture alone. However, based on microbiome sequencing, it would be reasonable to add metronidazole to the treatment.

The results from this study emphasize the role of anaerobic bacteria in brain abscesses. Thus, metronidazole should be administered in the absence of NGS testing when culture results indicate an oral or intestinal source of infection, even if anaerobic bacteria have not been cultured. Metronidazole resistance is still rare, and the drug is still considered to be an efficient antibiotic for anaerobic bacteria (17). Almost all the anaerobic species detected by NGS in this study have been reported to be highly susceptible to either penicillin or metronidazole (18). Accordingly, empirical treatment with a combination of metronidazole and a beta-lactam antibiotic is usually recommended (1,3,17). From this study, however, the choice of benzylpenicillin as the beta-lactam antibiotic would not provide sufficient coverage in several of the cases, e.g., Haemophilus influenzae, and a third-generation cephalosporin, ceftriaxone or cefotaxime, should be part of empirical treatment (1,3).

We did also investigate whether a brain abscess bacterial microflora is correlated to the origin of the brain abscess (dental vs sinus vs disseminated vs unknown) by carrying out diversity analysis to describe the 16S microbiome profiling. The top-ten most common bacterial genera were Aggregatibacter, Bacteroides, Escherichia, Fusobacterium, Nocardia, Parvimonas, Porphyromonas, Propionibacterium, Staphylococci and Streptococci. We did not detect any significant difference regarding alpha diversity (Shannon’s diversity index) between origin groups. However, beta diversity (PCoA based on the Bray-Curtis distance) revealed that the variation of the brain abscess microbiome diversity was not continuous but could be stratified comparing the presumable origin of infection in three clusters with a very distinct and significant cluster in the disseminated group compared to the other origin of infection. Of note, we did also notice clustering between the presumably “sinus” origin vs “dental” origin, although not significantly (P=0.07) and there were only four genera shared among the ten most common genera between the “dental” and “sinus” group (Streptococci, Fusobacterium, Porphyromonas and Parvimonas). A higher abundance of sequences...
belonging to the two genera *Actinomyces* and *Campylobacter* were especially predictable for a dental origin of infection. Interestingly, beta diversity was very similar between the “dental” and “unknown” group suggesting the primary source of brain abscess in these patients originated from a dental infection.

Limitations of the study include the fact that the sample size was relatively small and that the access to precise clinical information was limited to the information received on the laboratory request form. Another obvious limitation with the used NGS technique is the lack of susceptibility testing. This means that treatment directed at species detected with NGS will be based on historical susceptibility data. Hopefully in a near future, prediction of susceptibility based on shotgun metagenomic data will be possible (19). In one case, ID 19, a methicillin-resistant *Staphylococcus aureus* (MRSA) was detected by culture. Denmark is a low-prevalence country for MRSA, and empirical treatment for brain abscesses would usually not cover this pathogen. Another limitation is an apparent inability of using the 16S target to differentiate between closely related species. It is noticeable that only *S. intermedius* is identified by NGS and not *S. anginosus* or *Streptococcus constellatus*, which have all been implicated in brain abscesses. This is a known weakness of using the 16S target for species identification of streptococci. Finally, microbiome-based diagnostics with NGS is still a relatively complex and expensive method and results are not available within a few hours (20).

In conclusion, microbiome-based diagnostics may play a role in clinical microbiology diagnostics for brain abscesses in the future. The technique appears to be more sensitive than standard methods without losing specificity, as many more species are being identified, e.g., in case of prior antibiotic therapy and species which are difficult to culture. This may add information to the source of infection and supports the choice of antibiotic therapy. Microbiome-based diagnostics can increase sensitivity without losing specificity, supporting the choice of antibiotic therapy. The bacterial beta diversity differed between the presumably origin of the brain abscess and might help to clarify the primary source of infection.
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Declarations

Author contributions statement:
Katrine Hartung Hansen: Data curation, Investigation, Project administration, Visualization, Writing - original draft
Ulrik Stenz Justesen: Conceptualization, Methodology, Project administration, Supervision, Validation, Visualization, Writing - review & editing
Jesper Kelsen: Investigation, Resources, Supervision, Writing - review & editing
Kirsten Møller: Investigation, Resources, Supervision, Writing - review & editing
Jannik Helweg-Larsen: Investigation, Resources, Supervision, Writing - review & editing
Kurt Fuursted: Conceptualization, Formal analysis, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing - review & editing

All authors reviewed and approved the final manuscript.

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Data availability statement: All relevant data are within the manuscript and supplemental material files.

Ethics approval

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The study was approved by the Danish Data Protection Agency (2007-41-0229). Informed consent was not required for this study.

References


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Table 1. Characteristics of the 36 patients with brain abscess form this study and the microorganisms identified

<table>
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<tr>
<th>ID</th>
<th>Sex/Age (years)</th>
<th>Probable source of infection</th>
<th>Culture</th>
<th>Sanger sequencing</th>
<th>Significant pathogens identified by microbiome analysis (% reads)</th>
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<td>F/74</td>
<td>Disseminated</td>
<td>Streptococcus dysgalactiae</td>
<td>Ciprofloxacin, clarithromycin, dicloxacillin, Meropenem, penicillin</td>
<td>P. micra (3.9), Prevotella oris (2), S. intermedius (17.8)</td>
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<tr>
<td>18</td>
<td>F/76</td>
<td>Sinus</td>
<td>0</td>
<td>Ampicillin, ceftriaxone</td>
<td>Campylobacter showae (1.3), F. nucleatum (22.2), P. endodontalis (34.6), Prevotella baroniae (2.3)</td>
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<td>19</td>
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<td>Sinus</td>
<td>Staphylococcus aureus</td>
<td>Cefuroxime, dicloxacillin, metronidazole, penicillin, rifampin, vancomycin</td>
<td>Staphylococcus aureus (43.6)</td>
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<td>20</td>
<td>F/54</td>
<td>Dental</td>
<td>A. aphrophilus, E. corrodens, S. anginosus group</td>
<td>Meropenem, metronidazole</td>
<td>A. meyeri (1.2), A. aphrophilus (5.4), C. gracilis (1.3), Candida albicans (0.9), F. nucleatum (36.3), P. micra (11.7), P. conceptionensis (0.2), P. endodontalis (1.8), S. intermedius (13.6)</td>
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<td>Unknown</td>
<td>Aspergillus fumigatus, Micrococcus luteus</td>
<td>Meropenem, metronidazole, fluconazole</td>
<td>Aspergillus fumigatus (2.8), S. aureus (0.8)</td>
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<td>22</td>
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<td>Dental</td>
<td>S. anginosus group</td>
<td>Piperacillin-tazobactam</td>
<td>A. aphrophilus (0.3), S. intermedius (46.2)</td>
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<th>Organism(s)</th>
<th>Antibiotics/Therapy</th>
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<td>F/76</td>
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<td>0, 0</td>
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<td><em>F. nucleatum</em>, <em>Prevotella</em></td>
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<td>Gender</td>
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<td>Condition</td>
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<td>M/54</td>
<td>54</td>
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<td>F. nucleatum, P. micra</td>
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</tbody>
</table>

477 a ID, study identification number
478 b F, female; M, male
479 c Immunocompromised
480 d Neonatal meningitis
481 e Skin infection
482 f Urinary tract infection
483 g ND, not done for Sanger sequencing

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h NS, no sequences=negative
**Figures**

**Figure 1.** Overview of microbiome sequencing and bioinformatic analysis.

**Figure 2.** Overview of results of diagnostic method of 36 samples from primary brain abscesses. A, samples positive by standard methods only (culture and/or Sanger sequencing); B, samples positive by both standard methods and microbiome sequencing; C, samples positive by microbiome sequencing only; D, samples negative by both standard methods and microbiome sequencing.
Figure 3. Significant pathogens identified by standard methods (culture and/or Sanger sequencing) (n=47) compared to microbiome sequencing (n=96) divided into the following groups: oral streptococci, anaerobic oral commensals, other oral commensals, skin pathogens, intestinal pathogens, and *Aspergillus* species.
Figure 4. Alpha (within samples) diversity between patients with either a presumably dental, sinus, disseminated or unknown origin of infection measured by Shannon’s Diversity Index.
Figure 5. Beta (between samples) diversity between patients with either a presumably dental, sinus, disseminated or unknown origin of infection as measured by Bray–Curtis dissimilarity analysis.
Figure 6. The top-ten most common genera identified in samples from patient stratified according to the presumably origin of infection, as detected by heat map analysis.