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Original Article

Thrombus-associated microbiota in acute ischemic stroke patients

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ABSTRACT

Background: Despite a reduction in stroke incidence and age-standardized death rates, stroke remains a leading cause of death and disability worldwide. Significant interest in recent years has focused on the microbiota-host interaction because accumulating evidence has revealed myriad ways in which bacteria may contribute to risk of stroke and adverse outcomes after stroke. The emergence of endovascular thrombectomy as a treatment provides a unique opportunity to utilize thrombus retrieved from cerebral arteries to fill knowledge gaps about the influence of bacteria on stroke pathophysiology. While bacterial signatures have been confirmed in cerebral thrombi, the exact nature of the pathogenesis has not been established.

Methods: Thrombi were obtained from a cohort of adult ischemic stroke patients during standard of care thrombectomy. After DNA extraction and quantification, thrombi underwent 16S rRNA amplicon-based metagenomic sequencing, followed by bioinformatics processing. Taxonomic identification of bacterial colonies isolated on Agar plates from plated suspension was performed using DNA extraction and full length 16S Sanger sequencing.

Results: A broad diversity of bacterial signatures was identified in specimens, primarily of cariogenic origin.

Conclusion: In this small study, we demonstrate proof of concept and technical feasibility for amplicon-based metagenomic sequencing of arterial thrombi and briefly discuss preliminary findings, challenges, and near-term translational opportunities for thrombus genomics.

Keywords: Metagenomics, Microbiota, Stroke, Thrombectomy

INTRODUCTION

Accumulating evidence suggests that the human microbiota may influence the development or outcomes related to acute ischemic stroke (AIS). Cerebral thrombi represent a new source of biological information and may provide insight into the vascular microenvironment. No standards or guidelines exist for next generation sequencing (NGS) of thrombus-associated microbiota. To demonstrate proof of concept and technical feasibility in this setting, cerebral thrombi were subjected to amplicon-based bacterial 16S rRNA gene sequencing.

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MATERIALS AND METHODS

Materials

Subjects, procedure, and specimens

Thrombi were obtained from cerebral arteries of subjects over age 18 with AIS during standard-of-care endovascular thrombectomy (EVT) at a Comprehensive Stroke Center between January 2020 and October 2020. Analysis was limited to subjects whose thrombi were obtained intact, with full reperfusion (mTICI = 3).[9] To minimize specimen contamination, only thrombi from a single retrieval (first pass[24]) were utilized. Patients with endocarditis or documented active and ongoing bacterial infection were excluded from the study. Specimens from four subjects were enrolled and analyzed. To address technical limitations after lytic therapy, half of the thrombi selected for analysis were obtained from subjects who had received intravenous lytics. Relevant clinical and demographic data were obtained from the medical record including stroke severity on admission using the National Institutes of Health Stroke Scale scores[13] and ischemic stroke subtypes using the Trial of Org 10172 in acute stroke treatment criteria.[1] All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

During the thrombectomy, a combined technique was used. In addition to stent retriever, aspiration was applied through an intermediate catheter as well as a large-bore guide catheter positioned in the cervical carotid to help ensure first pass reperfusion. Because the thrombus is frequently adherent to the devices, it must be washed with sterile saline and gently manipulated for transfer into the sterile collection receptacle. The thrombus was rinsed with sterile water, placed into a pre-prepared sterile collection container containing phosphate buffered saline (PBS), and stored at −80°C until processing.

Methods

DNA extraction and quantification

After homogenization, DNA extraction was performed using the Ultra-Deep Microbiome Prep protocol (Molzym, Portland, OR). DNA was quantified using Qubit® dsDNA high-sensitivity and broad-range fluorometric assays (Thermo Fisher Scientific, Waltham, MA). DNA quality was assessed using Agilent 2100 Bioanalyzer technology (Agilent, Santa Clara, CA).

Analysis of thrombus microbiota and quality control

Multiplex 16S rRNA gene (16S) fragment amplification (2 × 300bp paired end) using the Swift Amplicon® panel (SWIFT Biosciences, Ann Arbor, MI) that targets all nine variable regions (V1-V9) of bacterial and archaeal 16S was performed, followed by Swift 2S® Turbo DNA library preparation (SWIFT Biosciences) and deep sequencing through MiSeq® platform (Illumina Inc. La Jolla, CA). Negative controls (water) were included in all sample preparation and sequencing experiments. The R-based DADA2 open-source software package next-generation microbiome bioinformatics algorithm[3] and an in-house computational pipeline were used to quality-filter sequences [Figure 1]. For library preparation, ≥200 ng of DNA was utilized. DNA concentration was confirmed to be ≥20 ng/μl. Purity of specimens was OD260/280 = 1.8–2.0 without degradation or contamination. The amplified region was less than 470 bp. Statistical analysis was done with the QIIME2 next-generation bioinformatics platform.[2,18] Taxonomic identification of bacterial colonies isolated on Agar plates (from plated suspension of each thrombus) was performed using DNA extraction and full length 16S Sanger sequencing.

RESULTS

Subjects

The mean age of subjects was 62, with a standard deviation of 18.8 years. Although all were male, four unique racial/ethnicities were represented.[15] Half the subjects received intravenous lytic therapy before EVT, and half had at least a 20 pack-year history of tobacco smoking. Consistent with most of our AIS population, all subjects had a history of oral or periodontal pathology, resulting in either tooth extraction or hardware implantation within 10 years [Table 1].

Bacterial DNA identified in cerebral thrombi

Deep sequencing of forward and reverse sequence reads representing all nine variable regions of the bacterial 16S amplified from DNA revealed the presence of bacterial signatures. The main bacterial groups associated with specimens in this cohort belonged to the *Acetobacter*, *Streptococcus,* and *Lactobacillus* genera [Figure 2a-d]. Due to poor sequence quality (likely due to DNA degradation), bacterial sequence reads were trimmed to 100 bp to enable accurate sequence alignment and taxonomic classification. The short 16S sequence read length only allowed reliable taxonomic classification at the genus level [Figure 2a], while species level classification remained putative [Figure 2b]. Bacterial cultivation experiments on Agar plates captured growth of *Staphylococcus* species, which was also observed in the DNA extracted from the clots, as revealed by 16S analysis.

DISCUSSION

Validation of prior reports

Analysis of the thrombus-associated microbiota in this cohort supports findings from several previous studies, which

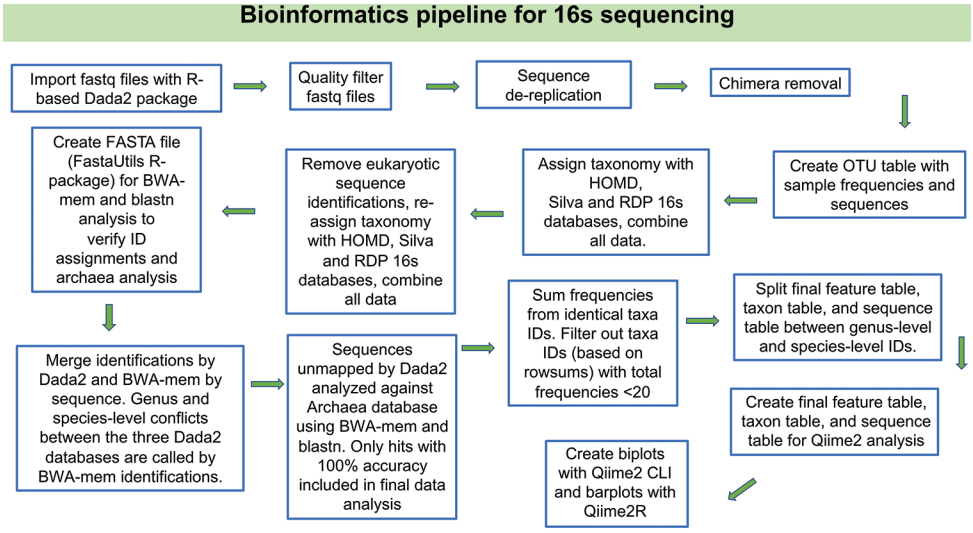

Figure 1: Multiplex 16S rRNA gene (16S) fragment amplification was performed, followed by library preparation and deep sequencing. A next-generation microbiome bioinformatics algorithm and an in-house computational pipeline were used to quality-filter sequences.

Table 1: Subject demographics and metadata.

Subject	Age	Sex	Location	Comorbidities	Smoker	Prior Oral Surgery	tPA	Race/Ethnicity[a]
1	89	M	L M1	Diabetes Hyperlipidemia Hypertension Congestive heart failure L ventricular thrombus Renal failure	Y	Y	N	White
2	53	M	L M1	Intravenous drug use Deep venous thrombosis	Y	Y	Y	Latino
3	38	M	R M1	Optic neuritis	N	Y	N	Asian
4	67	M	L M1	None	N	Y	Y	White

M: Male, L: Left, R: Right, M1: Middle cerebral artery, M1 division, tPA: Tissue plasminogen activator. [a]Self-reported racial categories aligned with the Revisions to the 2015 US Office of Management and Budget Directive 15

report oral bacterial signatures in cerebral,[16] cardiac,[17] and peripheral[22] thrombi. Similar to prior reports,[21] we identified three main bacterial groups, representing *Staphylococcus*,[14] *Lactobacillus*,[4] and *Streptococcus*.[8] Additional bacterial diversity was expected to grow on agar plates, especially from members of streptococci; however, only *Staphylococcus* was isolated.

Technical challenges

Several technical challenges were present, including low biomass specimens, insufficient specimen controls, and sample preservation conditions. Low-biomass samples with high non-microbial (host) nucleic acids may yield small quantities of bacterial DNA that may be insufficient for library construction.[11] Further, bacterial DNA (such as *Ralstonia*[20]) known to be laboratory reagents and extraction kit contaminants can falsely inform the results of microbiota studies, particularly when investigating samples of low microbial biomass such as cerebral thrombi. The use of negative controls (i.e., template-free "blanks" processed with the same DNA extraction and PCR amplification kits as thrombi, sequenced on the same run) help ensures that erroneous conclusions are not drawn from culture-independent investigations such as ours.[20,23] Poor bacterial sequence quality seen across the specimens is likely due to a combination of the factors noted above. Because host DNA can outcompete nonhost DNA in amplification cycles during library preparation steps,[5] the future studies would include techniques like qPCR to separate host and nonhost DNA before analysis. In addition, poor sequence quality has been observed in specimens exposed to multiple freeze-thaw cycles and may have inadvertently occurred during specimen archive and/or transport during unexpected shipping delays. In the absence of clear guidelines for thrombus preservation, PBS was selected as the preservation medium,

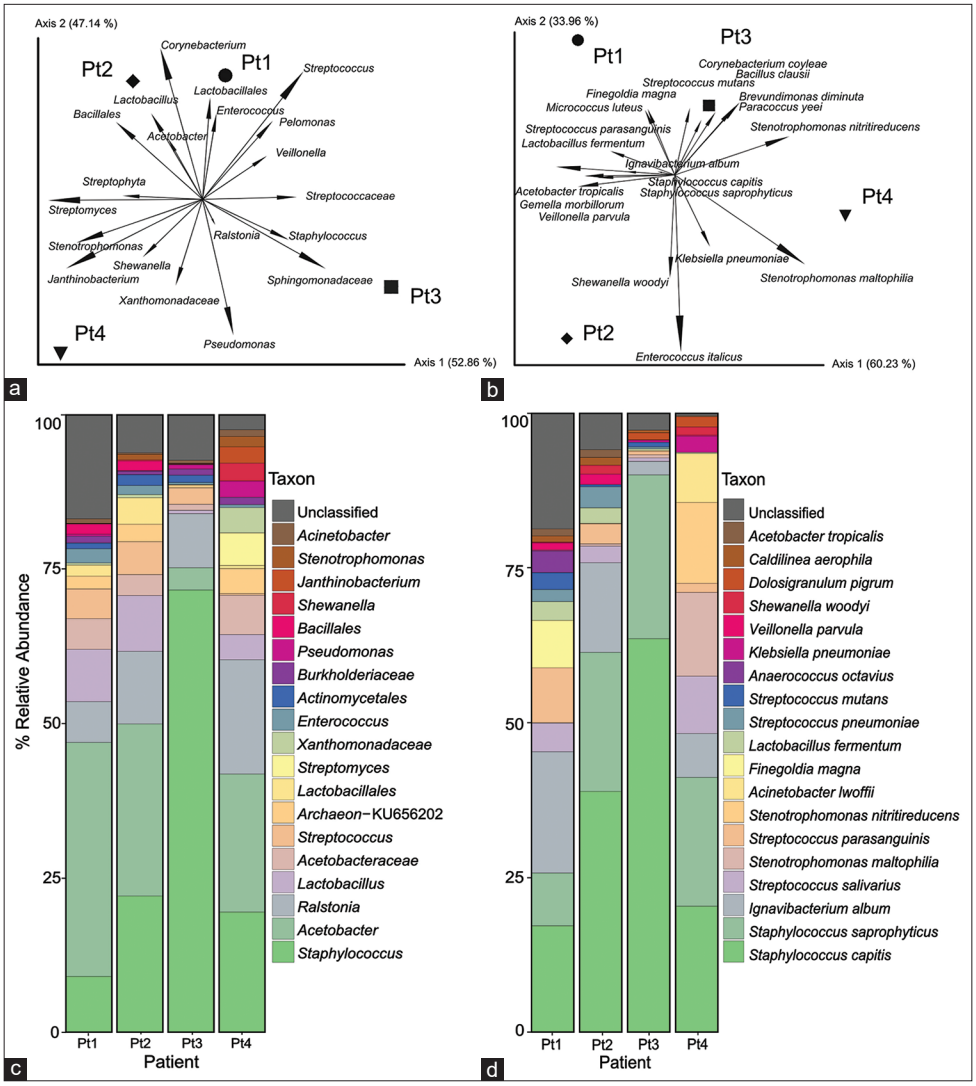

Figure 2: Biplot analysis of bacterial taxa based on 16S fragment sequencing (a and b) reveals the presence of a diverse bacterial community within cerebral thrombi. Signatures of oral pathogens, specifically cariogenic *Staphylococcus*, *Streptococcus,* and *Lactobacillus* were revealed in thrombi (c and d).

but other media designed for features like room temperature stability may be more effective. Traditional formalin-based preservation may, in fact, be destructive to the DNA and RNA within specimens.[10,25] Therefore, in specimens of low biomass requiring DNA/RNA stability, the development of optimal storage and amplification methods specific to cerebral thrombi is necessary to further validate these findings.[12]

CONCLUSION

Analysis of the thrombus microbiota from cerebral thrombi is technically feasible and demonstrated the presence of multiple taxonomic groups. The confirmation of bacterial signatures representing the human microbiota, specifically the oral microbiota,[6,21] is significant and merits further investigation. While the presence of bacteria alone is unlikely to drive specific thrombotic events, strain-specific virulence such as secretion of toxic metabolites or clot inducing factors may promote thrombosis in susceptible individuals.[7,19] While this study supports basic feasibility for NGS, well-controlled future benchmarking studies of the thrombus-associated microbiota would be greatly enhanced by optimizing protocols that better preserve DNA/RNA in low biomass and high host-contaminant specimens. Alternative approaches for analyzing complex microbial communities, such as shotgun metagenomic or metatranscriptomics, may provide more robust information on the host-microbiota interaction. Although the specific findings in this preliminary report should be interpreted with caution, cerebral thrombi offer valuable new information to further investigate the role of human microbiota in AIS.

Declaration of patient consent

Institutional Review Board (IRB) permission obtained for the study.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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