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***Original Research Article***

# ASSESSMENT OF BACTERIAL DIVERSITY ASSOCIATED WITH ATHEROSCLEROTIC PLAQUE IN STROKE

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**ABSTRACT**

**Background:**We aimed to investigate the microbial features of cerebral thrombi retrieved via Mechanical thrombectomy in stroke patients. The microbial compositions of all samples were compared using 16S rRNA gene amplicon next-generation sequencing.**Materials and Methods:** Enrollment of 18 Acute ischemic stroke (AIS) patients was done who underwent mechanical thrombectomy in which 12(66.66%) male and 33.3% (n=6) female. The patients' median age was 51±14.6 years old, and they were male and female. During the window of acute stroke with symptomatic carotid stenosis and blockage of the ipsilateral middle cerebral artery, all were submitted to mechanical thrombectomy. After mechanical thrombectomy we extracted the DNA from thrombus sample. **Results:**All of the thrombi retrieved for bacterial DNA in qPCR from the18 patients who had thrombectomy for ischemic stroke were positive. We discovered more than 25 bacteria in plaque that included Pseudomonas putida, Staphylococcus epidermidis, Staphylococcus hominis, and Finegoldia magna and other. Additionally, members of the genera Propionibacterium and the unclassified Burkholderiales were frequently the most important taxa for all atherosclerotic plaque. **Conclusion:**Large bacterial diversity was found in thrombus in ischemic stroke patients in this study, which also indicated that microbes present in thrombus also played role in formation of plaque and Bacteria have direct mechanisms such as acidification and local inflammation of the plaque milieu with lactobacillus, biofilm dispersion leading to inflammation with pseudomonas fluorescence, the gut microbiota, could all lead to thromboembolic and cause stroke.

**Keywords:** Stroke, Mechanical Thrombectomy, Thrombus, Microbiome, Metagenomics

## INTRODUCTION

Stroke is the primary cause of adult long-term impairment in Western countries, and cerebrovascular diseases are substantial causes of death in numerous countries **(1).** Cerebral ischemia, subarachnoid haemorrhage, and intracerebral haemorrhage are the three types of stroke. Every year, 795 000 persons in the United States have a stroke, of which 692 000 are acute ischemic strokes (AISs) **(2).** Coronary artery disease, peripheral arterial disease, and stroke are all primarily caused by atherosclerosis **(1).** It is widely accepted that atherosclerosis develops gradually and forms an atheromatous plaque as a result of low-density lipoprotein (LDL) and plasma cholesterol accumulating beneath the endothelium of artery walls **(3).** The main risk of atherosclerosis is the unexpected rupture of a stable atheroma, which can result in a lesion that can be fatal, called an Atherothrombosis **(4).** The discovery that 76% of all fatal coronary thrombi result from arterial plaque rupture highlights the need of maintaining plaque stability **(4).**

The human microbiome may have an impact on the progression or results of acute ischemic stroke (AIS), according to increasing studies. Brain thrombi are a fresh source of biological data that may shed light on the vascular microenvironment. There are no standards or recommendations for next-generation sequencing (NGS) of the microbiota linked to thrombi. Cerebral thrombi were exposed to amplicon-based bacterial 16S rRNA gene sequencing in this situation to provide proof of concept and technological viability **(5)**

In the present work, we proposed that atherosclerosis might, in at least some instances, be a chronic illness associated with biofilms. To verify this theory, we looked for bacteria in atheromas within diseased human carotid artery explants and conducted microscopic examinations to see if these bacteria had evolved into biofilm deposits**(6).** When norepinephrine was added to cultures of Pseudomonas aeruginosa, a bacterial species that was discovered in this study to be present in atheromas, we tested it to see if it could be made to experience a dispersion response in vitro. This organism was grown as a biofilm in the presence of iron-bound transferrin **(6).**

For many years, researchers have been looking into the viral theory of atherosclerosis. Infections caused by bacteria and/or viruses have the potential to contribute to the pathogenesis of atherosclerosis through both direct and indirect pathways, assuming that both the innate and adaptive immune systems are involved in atherogenesis. We can distinguish between infectious agents that can be found in plaques and have been associated with cardiovascular disease, such as Chlamydia pneumoniae, Helicobacter pylori, some periodontopathogens, such as Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans, and various viruses **(7).** However, despite the fact that numerous researchers were able to identify microbial infections and suggest that they may play a role in the pathogenesis of atherosclerosis, the strength of the evidence for the majority of the pathogens linked to cardiovascular disease is weak. Additionally, many patients with atherosclerosis did not experience better clinical outcomes after receiving antibiotic therapy for specific bacteria **(8)**, raising the possibility that microorganisms play a role in atherogenesis. Rosenfeld and Campbell **(7)** hypothesised, however, that pathogen load and persistent infection in the plaques might possibly be to blame for the failure of the antibiotic trials.

The main goals of this study were to contribute to the identification of a major group of atherosclerotic plaque bacteria by pyrosequencing their 16S ribosomal RNA (16S rRNA) gene fragments and to gain a better understanding of the bacterial diversity and abundance in human atherosclerotic plaques derived from distinct patients with atherosclerosis with common carotid arteries.

## MATERIALS AND METHODS

**Subjects:** Patients with acute ischemic stroke (n=18) who also had middle cerebral occlusion and symptomatic carotid stenosis and were hospitalized to the department of neurology at Pacific Medical University & Hospital between April 2019 and March 2022 were taken into consideration as potential study participants. The key qualification for inclusion was an acute ischemic stroke determined by neurologists to have symptomatic carotid stenosis with middle cerebral artery blockage. Brain MRIs and/or computed tomography scans were performed on all patients who presented within a 6-hour timeframe to assess ischemic lesions. They all suffered middle cerebral artery occlusion due to symptomatic carotid stenosis plaque with thromboembolic event. Additionally to the typical magnetic resonance imaging of the brain, a magnetic resonance angiography of the neck vessel and the circle of Willis vessels were carried out. After morphological analysis using carotid Doppler and MRI plaque imaging, it was shown that each of these carotid plaques was weak or unstable. The following conditions were excluded from the study: admission more than six hours after the stroke began; cardio embolic stroke; dissection-associated stroke; patent foramen ovale; vasculitis; history of malignancy; autoimmune disease; chronic kidney disease requiring hemodialysis; parkinson's disease; prior intravenous thrombolysis treatment; cardio embolic stroke; hypercoagulant or genetic underlying cause of stroke; and lack of faecal sample availability. 18 participants were enrolled in the study cohort overall. The mean admission rate for National Institutes of Health Stroke Scale (NIHSS) was 14.75 ±2.58 (range, 11 to 18). This study has received ethical approval from the Pacific Medical University & Hospital Regional Review Board in Rajasthan, India. The use of human subjects in this study has received approval from the regional review board. All participants provided written, informed consent.

### Mechanical Thrombectomy and Thrombus Sample Collection

All procedures were carried out in a cath lab equipped with a biplane angiography system (Allura xper FD 20-15, Phillips). A guiding catheter with a tip balloon up to 9F was inserted into the carotid artery close to the occluded area after an introducer sheath was placed on a femoral artery using the conventional adapted Seldinger procedure. The stent retriever (solitaire; Medtronic) was inserted using the micro catheter with the guide wire (0.021 inches) through the blocked location and across the thrombus. Up to three retrievals in the same vessel and a maximum of two retrievals per unit were accomplished by the patience revascularization devices throughout various retrieval runs. The visible clot was crossed by the micro catheter, which was then pulled back at the desired spot to deploy the stent retrieval device. The Solitaire was resheathed when necessary, but not more than twice. Negative aspiration was used to remove the thrombus under a 20 cc syringe. To evaluate flow restoration (TICI flow), the last angiography was performed. Up to an adequate angiographic outcome, thrombectomy procedures were repeated. The thrombus was split into a 1.5 mL eppendorf micro centrifugal tube for DNA extraction and qPCR analysis.

**DNA Extraction and Quantification**

A commercially available QIAamp DNA Mini Kit (Qiagen, Germany) was used to extract the bacterial DNA from the samples as instructed by the manufacturer. Using a Nanodrop spectrophotometer, the amount of DNA that was quantified.

**16S Sequencing (Metagenomics analysis)**

16S Amplicon-Seq hyper variable regions V1- V9 were amplified using primers, Forward 16S primer 5' - ATCGCCTACCGTGAC - barcode - AGAGTTTGATCMTGGCTCAG–3'and Reverse 16S primer 5' - ATCGCCTACCGTGAC - barcode - CGGTTACCTTGTTACGACTT -3' with gene-specific sequences, MinION, Oxford Nanopore, and molecular barcodes. PCR mixture for the full-length 16S rRNA gene (50 µl total volume) contained 10 ng of DNA template (10ul), 25 µl long Amp Taq 2X master mix (NEB M0287), 1 µl 16S barcode (Barcode 01 to Barcode 04 each for a single sample separately) and 14 µl nuclease-free water as indicated in the 16S barcoding kit (SQK-RAB204). The thermal PCR profile consisted of an initial denaturation of 60s at 95 ° C followed by 25 cycles of 20s at 95 ° C, 30s at 55 ° C, 2 min at 65 ° C and a final stage of 5 min at 65 ° C. The amplicons were washed with the Beckman Coulter (AMPure XP beads) using a ratio of 0.5X. Usage of Nanodrop spectrophotometer was evaluated for each sample quantity. The various bar-coded samples were pooled in equimolar ratio to get a final pool (100–150 ng in 10 µl) to do the sequencing library by adding 1 µl of RAP (Rapid Annealing Primer) to the bar-coded DNA. The prepared sample (11 µl DNA sample) was combined with Library Loading Beads (25.5 µl) and Fuel Mix Running Buffer (35.5 µl) and loaded onto SpotON Flow Cells Mk I (R9.4.1) (FLO-MIN106) for 12 hours using MinKNOWTM 19.06.8.

**Data availability**

Data that support the findings of this study are available upon reasonable request from the Pacific Medical University study investigators.

**RESULTS**

**Patient Characteristics**

All of the study's participants were men and female. The patient's median age was 51±14.6 years. All had mechanical thrombectomy within the window of acute stroke with symptomatic carotid stenosis and ipsilateral middle cerebral artery blockage. Within six hours of the stroke's beginning, all operations were completed. Following the surgery, TICI 3 was reanalyzed in each case. One patient had diabetes mellitus, and two of the patients had a history of hypertension.

Neither patient had a history of septicemia, severe infections, or recent antibiotic use prior to mechanical thrombectomy and thrombus sample collection. Blood, urine and sputum culture were negative in all the patients. Table-1 shows characteristics of the study population.

**Bacterial DNA found in aspirates of Thrombus**

Of the 18 patients undergoing thrombectomy for diagnosis with Ischemic stroke, all thrombi recovered for bacterial DNA in qPCR have been positive. More than 25 bacteria were present in 18 thrombus sample. Majority of bacteria were *Lactobacillus, Staphylococcus,* and *Stenotrophomonas* in all 18 thrombus. Other abundant bacteria were *Pseudomonas putida, Staphylococcus epidermidis, Staphylococcus hominis,* and *Finegoldia magna* as well as many others.

**DISCUSSION**

This is the first study to show that thrombus that was removed via a mechanical thrombectomy included

bacterial DNA. Additionally, we showed that the atherosclerosis plaque contained 80% of bacterial DNA. Our results are consistent with the results of bacterial DNA analysis of thrombus aspirates from the coronary arteries of myocardial infarction patients as well as specimens taken from intracranial aneurysms **(9, 10).**

There is mounting evidence that oral bacteria play a role in the aetiology of atherosclerosis **(11).** The human body's digestive system is home to a huge variety of bacteria, the majority of which are concentrated in the colon. Humans already have more than 700 distinct bacterial species living in their mouths **(12),** which can cause dental biofilms, also known as dental plaque, to form. Both between individuals and different regions in the oral cavity, the precise makeup of the dental biofilm varies. Despite this, a core microbiome that consists of organisms from the following taxa has been proposed: Corynebacterium, Rothia, Actinomyces, Prevotella, Capnocytophaga, Porphyromonas, Veillonella, Granulicatella, Neisseria, Haemophilus, and Fusobacterium **(13).**

Dental caries and periodontal disorders are just two conditions that the dental biofilm can wreak havoc on the teeth and the tissues that support them. Dental plaque must be regularly removed because when the biofilm thickens, bacteria are better protected from saliva's bactericidal effects, which can no longer reach or penetrate the entire tooth (14).Demineralization of the teeth without accompanying inflammation of the surrounding tissues is the hallmark of dental caries. It could, however, progress into inflammatory infections like pulpitis and apical periodontitis if left untreated. Previously thought to be the main causes of dental caries, Actinomyces, Lactobacillus, Dialister, Eubacterium, Olsenella, Bifidobacterium, Atopobium, Propionibacterium, Scardovia, Abiotrophia, Selenomonas, and Veillonella species are now included on the list of caries-associated bacteria along with carbohydrate-fermenting oral streptococci. Many of them are still difficult to cultivate in a lab. Usually, six to 24 months after S. mutans colonises tooth cavities, caries develops **(15)**. S. mutans is cariogenic due to the extracellular polymeric substances (EPSs) it secretes and whose production is in part stimulated by fructose **(16)**.

In contrast to gingivitis, periodontitis causes irreparable tissue damage. Although different Gram-negative rods like Prevotella spp., Porphyromonas gingivalis, and Fusobacterium nucleatum predominate in the subgingival biofilms, deeper layers near the epithelial surface also contain motile bacteria and spirochetes **(17).**

Notably, the biofilm plaque acts as a continuous reservoir for microbes and the inflammatory agents that they produce, both of which can spread repeatedly throughout the body. As a result, dental biofilm bacteria are also a direct or indirect risk factor for a number of other systemic illnesses, including cardiovascular conditions, atherosclerosis, infectious endocarditis, aspiration pneumonia, diabetes mellitus, preterm birth, and low birth weight newborns **(18)**.

A significant human pathogen known as Staphylococcus aureus is responsible for a variety of clinical illnesses **(19)**. A short-term elevated risk of stroke is linked to Staphylococcus aureus bacteremia (SAB), and the risk can last for up to 180 days. Old age, past arterial thromboembolic events, atrial flutter/fibrillation, hypertension, and endocarditis are risk factors for stroke after SAB **(20).**

Although stimulation of the inflammatory response is thought to be the primary cause of stroke, the mechanism by which infection-induced stroke is caused is still not fully understood **(21)**. Pathogens may directly enter the vascular wall while an infection is progressing, along with an increase in the proliferation of smooth muscle cells or the release of inflammatory cytokines. Further, regions distant from the primary infected loci may also be damaged, and this secondary impairment may potentially cause damage to the artery wall. Additionally, the inflammatory response brought on by infection may promote increased platelet aggregation and dysfunctional vasodilation **(22).** High-sensitivity CRP (HS-CRP) blood levels have been shown to be an independent predictor for ischemic stroke, while the precise relationship is still unknown **(23).**

The aetiology of the current patient's stroke was determined to be an embolic stroke with subsequent haemorrhagic transformation caused by migration of the septic emboli caused by systemic Staphylococcus aureus infection to the left internal carotid artery system based on the patient's medical history, physical examination, laboratory, radiology, and pathological examinations. Acute infective endocarditis frequently results from Staphylococcus aureus, and infective endocarditis has been noted as a significant contributor to cardioembolic stroke **(24).**

## CONCLUSION

According to our research, the majority of the bacteria in the thrombi that were removed from stroke patients had thrombus microbiome features. It also showed potential variations in the predominant bacteria, distribution patterns, and bacterial concentrations in thrombi recovered from strokes. Furthermore, higher risks following mechanical thrombectomy for stroke were closely correlated with a higher abundance of opportunistic pathogens in the thrombus. The significance of the microbiota in thrombosis, the occurrence and prognosis of stroke.


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## REFRENCES


1. Roger VL, Go AS, Lloyd-Jones DM, Adams RJ, Berry JD, Brown TM, et al. Heart disease and stroke statistics—2011 update: a report from the American Heart Association. Circulation. 2011 Feb 1;123(4):e18-e209. doi: 10.1161/CIR.0b013e3182009701, PMID 21160056.
2. Lusis AJ. Atherosclerosis. Nature. 2000;407(6801):233-41. doi: 10.1038/35025203, PMID 11001066.
3. Falk E, Shah PK, Fuster V. Coronary plaque disruption. Circulation. 1995 Aug 1;92(3):657-71. doi: 10.1161/01.cir.92.3.657, PMID 7634481.
4. Falk E. Pathogenesis of atherosclerosis. J Am Coll Cardiol. 2006 Apr 18;47(8):C7-C12. doi: 10.1016/j.jacc.2005.09.068.
5. Walker M, Uranga C, Levy SH, Kelly C, Edlund A. Thrombus-associated microbiota in acute ischemic stroke patients. Surg Neurol Int. 2022;13:247. doi: 10.25259/SNI_104_2022, PMID 35855159.
6. Lanter BB, Sauer K, Davies DG. Bacteria present in carotid arterial plaques are found as biofilm deposits which may contribute to enhanced risk of plaque rupture. mBio. 2014 Jul 1;5(3):e01206-14. doi: 10.1128/mBio.01206-14, PMID 24917599.
7. Rosenfeld ME, Campbell LA. Pathogens and atherosclerosis: update on the potential contribution of multiple infectious organisms to the pathogenesis of atherosclerosis. Thromb Haemost. 2011;106(5):858-67. doi: 10.1160/TH11-06-0392, PMID 22012133.
8. Andraws R, Berger JS, Brown DL. Effects of antibiotic therapy on outcomes of patients with coronary artery disease: a meta-analysis of randomized controlled trials. JAMA. 2005 Jun 1;293(21):2641-7. doi: 10.1001/jama.293.21.2641, PMID 15928286.
9. Pessi T, Karhunen V, Karjalainen PP, Ylitalo A, Airaksinen JK, Niemi M, et al. Bacterial signatures in thrombus aspirates of patients with myocardial infarction. Circulation. 2013 Mar 19;127(11):1219-28. doi: 10.1161/CIRCULATIONAHA.112.001254, PMID 23418311.
10. Pyysalo MJ, Pyysalo LM, Pessi T, Karhunen PJ, Lehtimäki T, Oksala N, et al. Bacterial DNA findings in ruptured and unruptured intracranial aneurysms. Acta Odontol Scand. 2016 May 18;74(4):315-20. doi: 10.3109/00016357.2015.1130854, PMID 26777430.
11. Leishman SJ, Do HL, Ford PJ. Cardiovascular disease and the role of oral bacteria. J Oral Microbiol. 2010 Dec 21;2. doi: 10.3402/jom.v2i0.5781, PMID 21523220, PMCID PMC3084572.
12. Dekaboruah E, Suryavanshi MV, Chettri D, Verma AK. Human microbiome: an academic update on human body site specific surveillance and its possible role. Arch Microbiol. 2020 Oct;202(8):2147-67. doi: 10.1007/s00203-020-01931-x, PMID 32524177.
13. Saini R, Saini S, Sharma S. Biofilm: A dental microbial infection. J Nat Sci Biol Med. 2011 Jan;2(1):71-5. doi: 10.4103/0976-9668.82317, PMID 22470238.
14. Seneviratne CJ, Zhang CF, Samaranayake LP. Dental plaque biofilm in oral health and disease. Chin J Dent Res. 2011 Jan 1;14(2):87-94. PMID 22319749.
15. Balakrishnan M, Simmonds RS, Tagg JR. Dental caries is a preventable infectious disease. Aust Dent J. 2000 Dec;45(4):235-45. doi: 10.1111/j.1834-7819.2000.tb00257.x, PMID 11225524.
16. Forssten SD, Björklund M, Ouwehand AC. Streptococcus mutans, caries and simulation models. Nutrients. 2010 Mar 2;2(3):290-8. doi: 10.3390/nu2030290, PMID 22254021.
17. Larsen T, Fiehn NE. Dental biofilm infections–an update. APMIS. 2017 Apr;125(4):376-84. doi: 10.1111/apm.12688, PMID 28407420.
18. Kumar S, Chandra N, Singh L, Hashmi MZ, Varma A, editors. Biofilms in human diseases: treatment and control. Cham: Springer; 2019 Nov 19.

19. Tong SY, Davis JS, Eichenberger E, Holland TL, Fowler Jr VG. Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. Clin Microbiol Rev. 2015 Jul;28(3):603-61. doi: 10.1128/CMR.00134-14, PMID 26016486.
20. Mejer N, Gotland N, Uhre ML, Westh H, Schønheyder HC, Petersen A, et al. Increased risk of arterial thromboembolic events after Staphylococcus aureus bacteremia: a matched cohort study. J Infect. 2015 Aug 1;71(2):167-78. doi: 10.1016/j.jinf.2015.03.010, PMID 25936743.
21. Elkind MS, Ramakrishnan P, Moon YP, Boden-Albala B, Liu KM, Spitalnik SL, et al. Infectious burden and risk of stroke: the northern Manhattan study. Arch Neurol. 2010 Jan 1;67(1):33-8. doi: 10.1001/archneurol.2009.271, PMID 19901154.
22. Epstein SE, Zhou YF, Zhu J. Infection and atherosclerosis: emerging mechanistic paradigms. Circulation. 1999 Jul 27;100(4):e20-8. doi: 10.1161/01.cir.100.4.e20, PMID 10421626.
23. Emerging Risk Factors Collaboration, Kaptoge S, Di Angelantonio E, Lowe G, Pepys MB, Thompson SG et al. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. Lancet. 2010 Jan 9;375(9709):132-40. doi: 10.1016/S0140-6736(09)61717-7, PMID 20031199.
24. Dickerman SA, Abrutyn E, Barsic B, Bouza E, Cecchi E, Moreno A, et al. The relationship between the initiation of antimicrobial therapy and the incidence of stroke in infective endocarditis: an analysis from the ICE Prospective Cohort Study (ICE-PCS). Am Heart J. 2007 Dec 1;154(6):1086-94. doi: 10.1016/j.ahj.2007.07.023, PMID 18035080.

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