

ASSOCIATION OF CORONARY ATHEROSCLEROSIS WITH *STUTZERIMONAS STUTZERI* STRAINS (HASA 1, 2, 3, AND 4) OF BIOFILM- FORMING BACTERIA

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ПОВЕЗАНОСТ КОРОНАРНЕ АТЕРОСКЛЕРОЗЕ СА СОЈЕВИМА *STUTZERIMONAS STUTZERI* (HASA 1, 2, 3 И 4) БАКТЕРИЈАМА КОЈЕ ФОРМИРАЈУ БИОФИЛМ

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ABSTRACT

Objective. Coronary atherosclerosis, also known as coronary artery disease, is increasing in developing countries like Iraq. A recent study investigated the presence and potential impact of a bacterial strain called *Stutzerimonas stutzeri* on coronary atherosclerosis. This research aims to provide insights into this medical concern that has seen a significant surge in cases over the past two decades.

Methods. Samples were collected from patients who underwent Percutaneous Coronary Intervention (PCI) at the Mosul Center for Cardiology and Cardiac Surgery between 9/10/2022 and 1/3/2023. The samples were obtained from the fluid (blood) coming out from the guiding catheter of the balloon during angioplasty, and then the balloon samples were placed in transport media (Tryptone Soya Broth). Advanced scientific methods, including biochemical tests, the Vitek-2 system, a Scanning Electron Microscope (SEM), and Molecular methods based on the 16S rRNA gene, were utilized to identify the bacteria. The Nitrogen base sequences were located and compared to those in the NCBI database using BLAST software.

Results. New strains of *Stutzerimonas stutzeri* bacteria were discovered in people with coronary atherosclerosis for the first time. The National Center for Biotechnology Information (NCBI) named these new strains as follows: *Stutzerimonas stutzeri* strain HaSa1, *S. stutzeri* strain HaSa2, *S. stutzeri* strain HaSa3, and *S. stutzeri* strain HaSa4. They have the ability to form biofilms. This was diagnosed with direct smears of balloon samples, smears of isolated bacterial colonies stained with a gram stain, and scanning electron microscope photos of balloon samples.

Conclusion. The newly found *Stutzerimonas stutzeri* strains most likely cause chronic inflammatory responses in people that have atherosclerosis in their coronary arteries by building biofilms.

Key words: *Pseudomonas stutzeri*; percutaneous coronary intervention; atherosclerosis; angioplasty; biofilms.

САЖЕТАК

Увод. Случајеви коронарне атеросклерозе, познате и као коронарна артеријска болест, у порасту су у земљама у развоју, попут Ирака. У недавној студији истраживани су присуство и потенцијални утицај бактеријског соја званог *Stutzerimonas stutzeri* на коронарну атеросклерозу. Ово истраживање има за циљ да пружи увид у медицинску забринутост која је последица значајног пораста броја случајева оболевања у последње две деценије.

Метод. Узорци су прикупљени од пацијената који су подвргнути перкутаној коронарној интервенцији (PCI) у Центру за кардиологију и кардиохирургију у Мосулу између 9. 10. 2022. и 3. 1. 2023. године. Узорци су добијени из течности (крви) која је излазила из балона помоћу водећег катетера током ангиопластике и који су затим стављени у транспортни медијум (Tryptone Soia Broth). За идентификацију бактерија коришћене су напредне научне методе, укључујући биохемијске тестове, систем Vitek-2, скенирајући електронски микроскоп (SEM) и молекуларне методе засноване на гену 16S rRNA. Секвенце азотних база лоциране су и упоређене са онима у NCBI бази података коришћењем BLAST софтвера.

Резултати. Први пут су откривени нови сојеви бактерије *Stutzerimonas stutzeri* код особа са коронарном атеросклерозом. Национални центар за биотехнолошке информације (NCBI) назвао је те нове сојеве на следећи начин: *Stutzerimonas stutzeri* сој HaSa1, *S. stutzeri* сој HaSa2, *S. stutzeri* сој HaSa3 и *S. stutzeri* сој HaSa4. Они имају способност да формирају биофилмове. То је дијагностиковано директним размазивањем узорака балона, размазима изолованих колонија бактерија обојеним по Граму и сликама узорака балона скенираним електронским микроскопом.

Закључак. Новопронађени сојеви *Stutzerimonas stutzeri* највероватније изазивају хроничне инфламаторне одговоре код људи који имају атеросклерозу у коронарним артеријама изградњом биофилма.

Кључне речи: *Pseudomonas stutzeri*, перкутана коронарна интервенција, атеросклероза, ангиопластика, биофилмови

INTRODUCTION

Atherosclerosis is a disease that is difficult to understand. It involves a complex and chronic inflammatory experience as the plaque in patients' arteries builds up. Typically, people blame things such as high cholesterol, inflammation, and their immune system, but new research suggests that bacteria might be the reason, too. The hypothesis behind it states that certain infections can cause or increase the amount of inflammation. Some of the key takeaways and references related to this disease include that the inflammation caused by chronic infection plays a big role in why this disease happens. Furthermore, when your immune system fights off these unwanted guests, it promotes endothelial dysfunction which recruits immune cells and forms plaque (1).

A lot of bacteria have been studied in order to discover how they play a part in causing atherosclerosis. The ones that were found to have this property include *Chlamydia pneumoniae*, *Porphyromonas gingivalis*, *Helicobacter pylori*, and certain species of oral microbiota. These bacteria have all been found in atherosclerotic plaques, and their presence alone has been linked to inflammation and the progression of plaque (2). There are a couple of ways in which microbial agents can contribute to atherosclerosis. Some methods include making pro-inflammatory molecules, activating immune cells, invading vascular cells directly, and inducing an immune response against modified host molecules (3). It is important to note that while the microbial aetiology hypothesis is intriguing, the exact role of specific microbial agents in atherosclerosis and the clinical implications are still being investigated.

Further research is needed to better understand the interplay between infections, inflammation, and atherosclerosis, and for this reason this study aimed to identify the causes leading to coronary artery atherosclerosis. An infection during the life of patients (gastroenteritis and myocarditis) (4, 5) could be a predisposing reason for the infection to reach these sites of the heart leading to arterial stenosis or atherosclerosis (6).

PATIENTS AND METHODS

From 9/10/2022 to 1/3/2023, 100 samples were obtained from patients who were undergoing percutaneous coronary intervention (PCI) after being diagnosed by a cardiologist at Mosul Center for Cardiology and Cardiac Surgery based on chronic coronary syndromes (CCS) guidelines (7). The sample from the angioplasty balloon, used in the PCI, was obtained for further analysis.

The samples were transferred to the laboratory (within 60 minutes), cultivated on Blood agar, McConkey agar, and Nutrient agar, and incubated in aerobic conditions at

37°C for 24-48 hours. After incubation, the bacteria were diagnosed, starting from the shape of the colony, the shape of the bacteria after staining it with a gram stain, and conducting some biochemical tests such as catalase, oxidase, and urease. The confirmation of the diagnosis at the species level was done utilizing two accurate diagnostic techniques - first with Vitek-2 compact (Biomérieux–French) followed by Determination of 16S rRNA primers. The genome of bacteria was isolated using the DNA Extraction kit supplied by Geneaid Company, according to the proven method (8). This was done in the Medical Research Laboratory, College of Medicine, University of Ninevah. The PCR amplification products were forwarded to Korea's Micro laboratory. Using the Basic Local Alignment Search Tool (BLAST) program, the 16s rRNA sequences were determined and compared to the sequences in NCBI.

The pathogenicity of bacteria *Stutzerimonas stutzeri* was determined by its ability to form biofilm by microscopic examination of the direct smear from the balloon samples and from a colony bacterial smear stained with a gram stain, as well as by imaging using the Scanning Electron Microscope technique (9).

RESULTS

The results showed the number of *S. stutzeri* isolates was 5 from the total of 100 patients with atherosclerosis who underwent angioplasty. It was done by taking a plaque from balloon samples and culturing on media such as blood agar, Nutrient agar, and MacConkey agar, and colonies obtained were rough, dry, wrinkled, rose, and non-hemolytic. The most important characteristic that distinguishes them is the fact that their colonies are sticky and adherent to the culture medium. The bacteria were identified based on their morphological characteristics and biochemical tests, including by staining them with a Gram stain, which showed that they were Gram-negative, non-spore-forming, aerobic, motile, and positive for catalase oxidase test, not fermented for lactose on MacConkey's medium. The diagnosis was confirmed using the VITEK-2 Compact system and it was the result of the isolate named *Pseudomonas stutzeri*. The molecular method for isolation, Amplification of the 16S rRNA gene from the genomic DNA isolated from pure culture, showed that samples that gave positive results using traditional methods all showed positive results using molecular techniques, as expected, but with the new name of bacteria, which is *S. stutzeri*. The results from sequencing the 16S rRNA gene showed that there was a match between diagnosis by conventional methods and molecular methods for all 5 isolates. Accordingly, these samples produced a 1.5 kb band that corresponds to the complete fragment of the 16S rRNA gene (Figure 1).

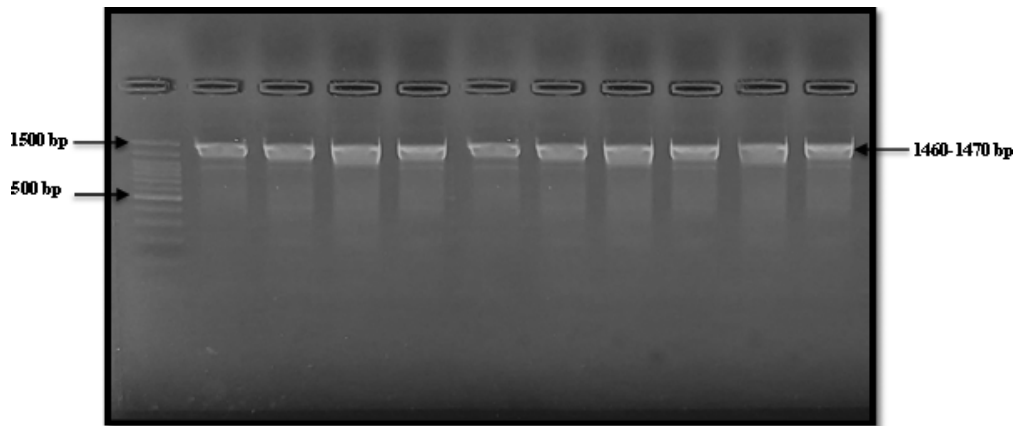


Figure 1. Detection *Stutzerimonas stutzeri* of 16S rRNA gene (1460- 1470bp). Line (L) marker (100 bp). Lines (1- 10) represent positive results. Electrophoresis conditions are 5v/cm².

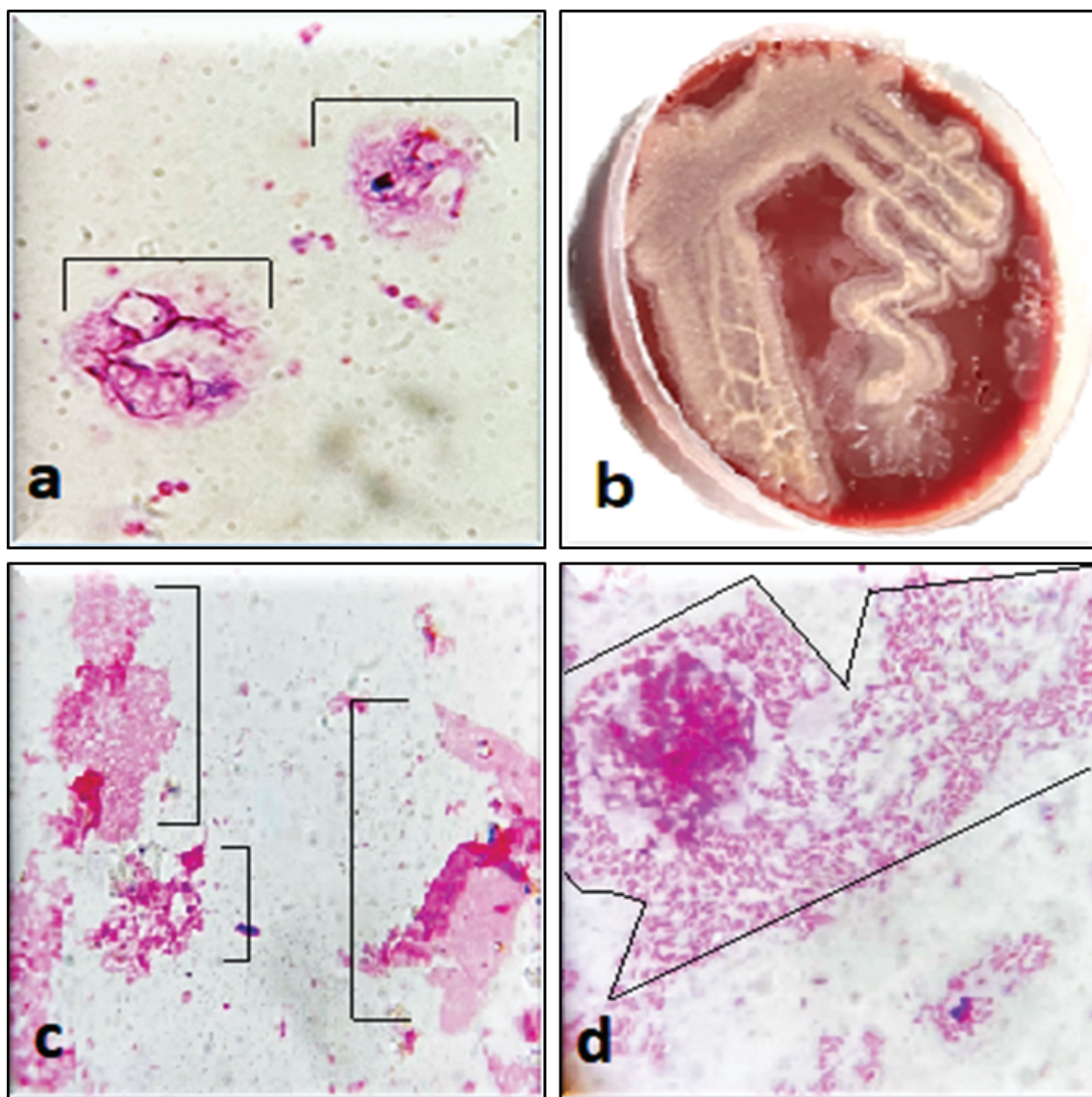


Figure 2. Biofilm of *Stutzerimonas stutzeri*, a: a Light microscope picture, stained with gram stain (1000 X), of a direct smear taken from a balloon sample during angioplasty from patients with atherosclerosis. b: Biofilm formation of *S. stutzeri* on Blood agar with very distinctive mucosal and sticky phenotype and cannot be confused with any other bacterial biofilm. c & d: Light microscope picture, stained with gram stain (1000 X) showing aggregated of *S. stutzeri* to form a biofilm.

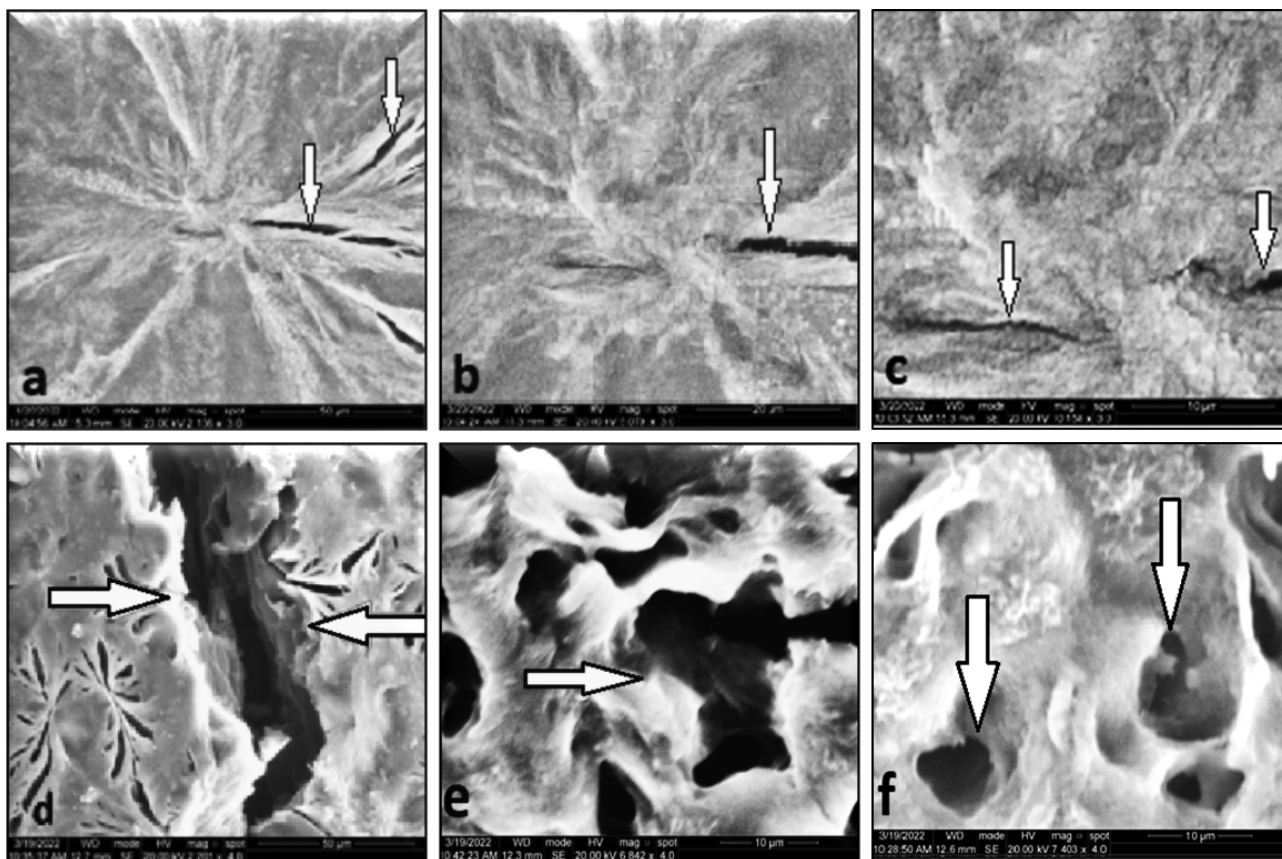


Figure 3. Biofilm of *Stutzerimonas stutzeri*, a: a Scanning Electron Microscope, with magnification power (a: 2 136X; b: 5079X; c: 10 158X; d: 220 1X; e: 6842X; f: 7403X), taken from a balloon sample during angioplasty from patients with atherosclerosis. (The marked areas refer to the areas of cracking of the biofilm).

The PCR products containing the 16S rRNA gene detected from balloon angioplasty samples used in this study were subjected to gene sequencing, and their sequences were compared to sequences submitted to the NCBI database to identify the type of bacteria. The results also showed that bacteria *S. stutzeri* can form biofilms, as this was noticed by making a direct smear of the balloon sample taken from the patient with atherosclerosis during the angioplasty process, as the biofilm appeared clearly and as in the figure 2:a. This was also noticed through the shape of the bacterial growth colonies on the blood agar medium, as the colonies appear sticky and mucous as in figure 2:b and this was confirmed by making a smear from the growing colonies on the agar media in which bacterial clusters associated with each other with membranes also appeared (Figure 2).

This was shown very clearly with an examination by Scanning Electron Microscope photographs of the balloon samples taken during angioplasty, in which there are pieces of plaque or thrombus formed in the atheroma plaques and this indicates the involvement of bacteria in the proceeding of atherosclerosis (Figure 3).

DISCUSSION

The recently proposed *Pseudomonadaceae* genus *Stutzerimonas* includes lineages from *Pseudomonas stutzeri*'s more ancient evolutionary group. There are 22 genomes and at least 16 species of *S. stutzeri* in this genus (10). It is important to note that *S. stutzeri* is not considered a primary pathogen in healthy individuals and is more commonly found in the environment, but the clinical significance of *S. stutzeri* infections is still being studied, and more research is needed to fully understand the spectrum of diseases associated with this bacterium. At this point, the *S. stutzeri* is considered to be opportunistic bacteria that cause many nosocomial infections including bacteremia, osteomyelitis, arthritis, endocarditis, meningitis, pneumonia, and infection of immunosuppressed patients (10, 11).

Biofilms constitute an architecture with spatial dimensions (12) that are of great importance in classifying the types of biofilms formed and determining the extent of their danger to human health, as they constitute a community of microorganisms responsible for many diseases. These diseases are linked to chronic infection in humans, as research has shown that these gatherings of

biofilms release many virulence factors including toxins and proteases (13, 14) thus damaging host tissues. Biofilm infection also poses additional risks to the host due to its low exposure to antibiotics and its ability to compete with the host for oxygen and nutrients (15, 16). One of the most important activities of biofilms is the response Dispersion which releases bacteria from within the biofilm by decomposition of the biofilm matrix (17). The role of bacteria in atherosclerosis is the bacteria's ability to access the vascular system through multiple mechanisms, including the type of eating, oral hygiene, bowel movements, and other routine events. Immunoglobulins and antibiotics can stick to damaged endothelial cell receptors using intercellular adhesion molecules (ICAM) and vascular adhesion molecules (VCAM) (18). After adhesion, the biofilms can produce a highly inflammatory environment to be an engine that leads to the occurrence of atherosclerosis, and this occurs by stimulating the immune response and the production of inflammatory cytokines: interleukin-1 β , interferon- γ , and tumour necrosis factor- α , among others, interleukin-8 and interleukin-6, coupled with excessive neutrophils and macrophages. Since the formation of atherosclerosis involves the deposition and modification of apoB-containing lipoproteins, primarily low-density lipoprotein (LDL) particles, within the arterial walls. Here's a general overview of how the bacteria and apoB-containing lipoproteins contribute to the development of atherosclerosis (19).

Bacterial infection can contribute to the development of atherosclerosis, a condition characterized by the build-up of plaque in the arteries. Atherosclerosis is primarily considered a disease of the blood vessels, and emerging evidence suggests that certain bacterial infections can play a role in its initiation and progression. The exact mechanisms by which bacteria contribute to atherosclerosis are still being studied, but here are a few possible ways, including chronic inflammation (20), dysregulation immune response (21-23), endothelial dysfunction (24, 25). Certain bacteria can modify lipid metabolism or the cellular surrounding the pro-inflammatory status of cytokines (19, 20).

The macrophages accumulate excessive amounts of cholesterol, transforming into foam cells. Foam cells contribute to the formation of fatty streaks, which are the earliest visible signs of atherosclerosis. The formation of atherosclerotic plaques: over time, foam cells pile up and alongside other cells like smooth muscle they form a fibrous cap over fatty streaks. This is what this leads to the development of atherosclerotic plaques. These can grow and cause arteries to narrow, which ultimately slows down blood flow (2).

In conclusion, the results show that the presence of these bacterial strains could be linked with coronary

atherosclerosis. If this link is proven, then it would have important implications for the diagnosis and treatment of this condition. These bacteria are able to form biofilms, which increase resistance to antibiotics and host immune responses. Suggesting they may play a vital role in chronic inflammation and plaque formation that is seen in atherosclerosis. Even though more research is needed to fully understand how these bacteria do this, it highlights the importance of exploring microbial communities in cardiovascular disease pathogenesis. Ultimately, this knowledge could lead to developing new diagnostic tools and treatments to help improve patient outcomes and reduce burden on public health.

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