



## Original Article

# Metagenomic analysis of atheroma plaques for identification of microorganisms indicates presence of *Toxoplasma gondii* as a possible etiological agent



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## ABSTRACT

**Background:** Cardiovascular diseases (CVDs) are the leading cause of death worldwide. Vital organs like the heart are affected by the occlusion of blood vessels due to atherosclerotic plaque formation. However, the role of infectious agents has always been an essential subject of investigation. This study investigated the presence of microorganisms, including nanobacteria, in atherosclerotic plaques removed from human carotid arteries by microbiological and metagenomic examination.

**Methods:** Atheroma plaque samples were obtained from 20 patients with carotid artery stenosis who had atherectomy by surgery or percutaneous intervention. Nanobacteria were grown by culturing homogenates of the atheroma plaques. Whole genome sequencing was done for samples. Because of the high percentage of *Toxoplasma gondii* (*T. gondii*) DNA, PCR investigation was applied to detect *T. gondii* DNA in the samples. **Results:** A molecular analysis of nanobacteria revealed them to be made of human proteins, supporting the theory that they are not living organisms. According to sequencing results, samples showed that more than 50 % of the metagenomic sequences belonged to *Toxoplasma gondii*. PCR investigation indicated that *T. gondii* DNA was positive in 8 (40 %) of 20 plaques.

**Conclusions:** Further evidence regarding the role of *T. gondii* in the etiology of plaque formation may help determine the strategy for prevention and treatment of infections in preventing atheroma plaque formation in the future.

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## 1. Introduction

CVDs including coronary heart, rheumatic heart, and cerebrovascular diseases, are the leading cause of death worldwide [1]. In 2019, 18.6 million people died of cardiovascular diseases [2]. Atherosclerosis has a significant role in the development of all these diseases [3]. Atherosclerosis is defined as a thickening of the arterial wall due to the accumulation of cholesterol, cellular waste products,

macrophages, calcium salts, and other substances. The progression of atherosclerosis leads to plaque formation, which restricts blood flow [4]. Various mechanisms for plaque formation have been proposed. In general, it is considered a chronic inflammatory event [5]. One theory suggests that inflammatory events are triggered by autoimmune diseases [6]. However, microbial agents are also considered one of the leading causes [7]. Although inflammation causing atherosclerosis is regarded as a sterile reaction independent of microbial agents, recent studies have highlighted the possible effects of several microbial agents [8], which have been the subject of a limited number of studies, some showing conflicting results. The potential role of a few organisms such as *Helicobacter pylori*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae*, and the Herpes virus

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family in atherosclerotic disease has been suggested [9], [10], [11], [12]. In one study, *Helicobacter pylori* and *C. pneumoniae* DNA were identified by PCR in specific carotid artery atherosclerotic plaques [13]. Two other studies identified anti-*Toxoplasma gondii* immunoglobulin G (IgG) antibodies with a higher incidence in patients with atherosclerosis [14], [15]. The development of new methodologies, such as epigenetic analysis using next-generation sequencing (NGS), for detecting microbial agents continues to contribute to the identification of unknown pathogens [16]. As emerging pathogens or agents of atherosclerosis, nanobacteria have been proposed as candidate agents capable of forming appetite-coating calcium phosphate precipitates [16], [17], [18]. Nanobacteria may be responsible for pathological calcifications in kidney and dental pulp stones, along with heart valve and arterial calcifications [19]. However, other studies have questioned the microbiological origin of nanobacteria, suggesting that they are formed by the biomineralization of natural molecules.

The main goal of this research was to determine the role of microorganisms, including nanobacteria, in the inflammation process that occurs during the development of atherosclerosis. Many studies have focused on the role of various infectious agents but approaches such as whole genome sequencing, which can cover all possible microorganisms, have remained very limited. This study aimed to investigate the presence of microorganisms and nanobacteria by microbiological culture and genomic examination of atherosclerotic plaques.

## 2. Materials and methods

### 2.1. Sample collection and study design

This study included 20 samples (labelled as A1–A20) of atheroma plaques from patients who underwent atherectomy by surgery or percutaneous intervention. All the plaques included in this study were obtained from the carotid artery of patients. Risk factors for atherosclerosis including gender, smoking status, hypertension, coronary artery disease, diabetes mellitus and dyslipidemia were recorded for each patient in this study (Table 1).

Ethical approval for the study was received from University ethics committee. All patients signed an informed consent form to permit using the biological samples and information related to this study.

The samples with a minimum size of 3 mm obtained from each patient were distributed into four tubes. The first tube contained the DNA/RNA shield solution (Zymo Research, USA), the second contained thioglycollate broth together with 1.0-mm-sized zirconium microbeads, the third contained only thioglycollate broth, and the fourth tube was empty. The tubes were transferred to the laboratory at room temperature.

**Table 1**  
Clinical characteristics of patients with respect to *T. gondii* DNA positivity.

Parameters	Patients that were <i>T. gondii</i> PCR result on atheroma plaques:	
	Positive (n = 8)	Negative (n = 12)
Mean Age	69.25	73.0
Mean Age + Std Dev.	69.25 ± 3.97	73.0 ± 4.95
Sex		
Female	2 (25 %)	2 (12 %)
Male	6 (75 %)	10 (83 %)
Smoking Habit	5 (62,5 %)	4 (33 %)
Hypertension	6 (75 %)	7 (58 %)
Coronary Artery Disease	3 (37,5 %)	8 (66 %)
Diabetes Mellitus	5 (62,5 %)	7 (58 %)
Dyslipidemia	4 (50 %)	7 (58 %)

### 2.2. Sample preparation and culture

#### 2.2.1. Culture of aerobic and anaerobic microorganisms from atheroma plaques

The atheroma plaque collected in the thioglycollate medium was incubated at 37 °C. Samples were incubated for two weeks until turbidity, indicating growth, was observed. If turbidity was not observed, aliquots of the medium were inoculated in two sheep blood agar (SBA) plates, one to eosin methylene blue agar (EMB), Sabouraud dextrose agar (SDA), Löwenstein-Jensen agar, and TK SLC rapid mycobacterial culture medium (TiBo, Türkiye). One SBA plate was incubated under aerobic and the other under anaerobic conditions. All plates were incubated at 37 °C for one week except SBA which was incubated for two weeks. If growth occurred, smears were prepared from the colonies, Gram-stained, and observed by microscopy. The species of the isolated organisms were determined using MALDI-TOF-MS (Bruker, USA).

#### 2.2.2. Culture and analysis of nanobacteria

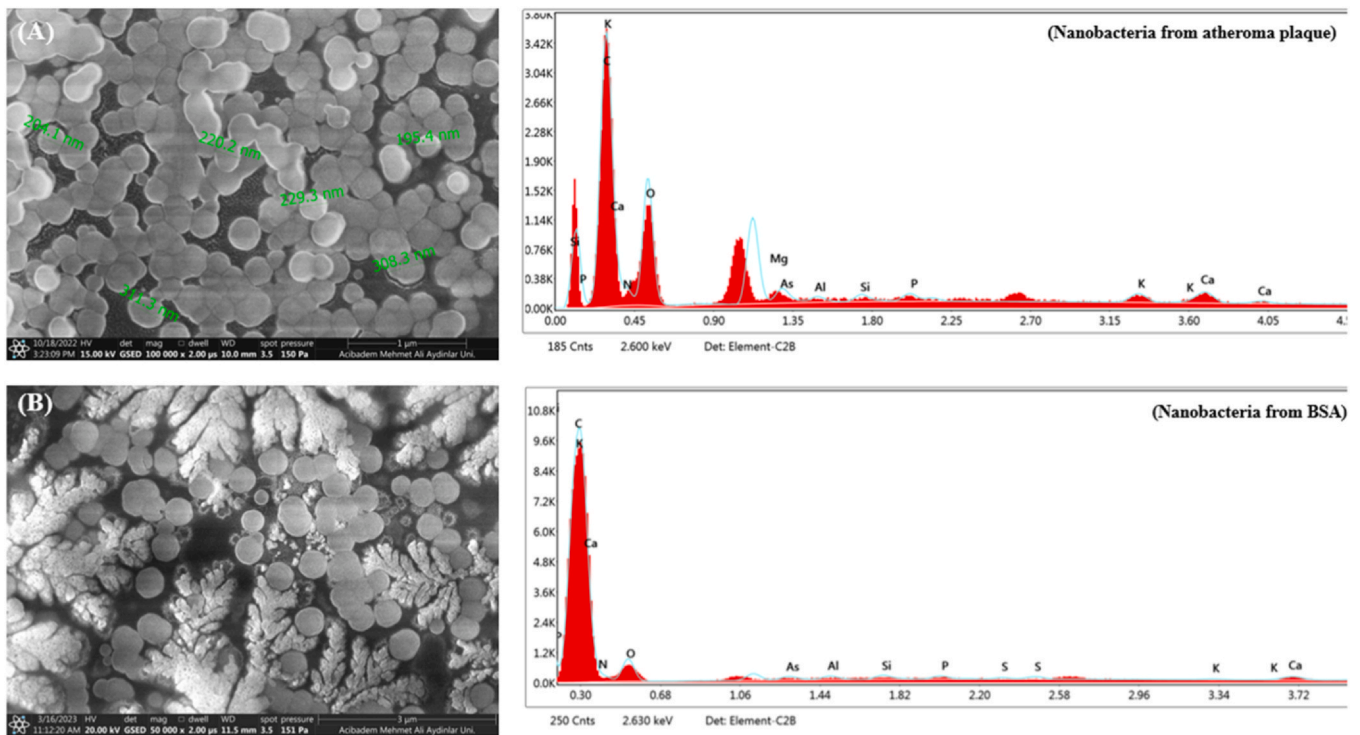
The microcentrifuge tube containing the atheroma plaque sample, microbeads, and thioglycollate medium was placed in a Mini-Beadbeater (Biospec, USA) and homogenized by shaking for 1 min. Then, 400 µl of sterile water was added and pipetted to reduce viscosity and eliminate foam. The contents were transferred to a fresh microcentrifuge tube and centrifuged at 5000xg for 2 min. The supernatant was then filtered through a sterile, 0.22 µm pore-sized filter to eliminate any possible bacteria and inoculated into serum-free Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Thermo Fisher Scientific, USA) in T25 sterile tissue culture flasks (Corning, USA). The flasks were incubated for 4–8 weeks under mammalian cell culture conditions (37 °C; 5 % CO<sub>2</sub>). The control group contained only the culture medium.

When precipitates were observed in the culture flasks by inverted microscopy, they were scraped from the flasks and the collected samples were dropped onto the piece of petri dish. After drying the samples, they were analysed both for morphology and chemical composition using Quattro S SEM (Thermo Scientific, USA) with attached EDAX Energy-dispersive spectroscopy (EDS) analyser (AMETEK, USA) (Fig. 1 A).

20 atheroma plaques that were cultured in T25 flasks at different times for nanobacteria were scraped and pellets with the suspension were collected into each tube. The samples were mixed with 4x protein loading buffer (0,5 M pH 6.8 Tris-HCl, 20 % SDS, 99 % glycerol, 0.005 % bromophenol blue, 355 mM of 2-mercaptoethanol, 1 M NaOH) and heated at 95 °C for 3 min in a heater. Then nanobacterial homogenates were run on 10 % SDS-PAGE gel and macromolecules were detected by silver staining. Their susceptibility to proteinase K, DNase I, and RNase was determined before loading on the SDS-PAGE gel. Due to their susceptibility to proteinase K and resistance to nucleases, these proteins were further analyzed by Q-TOF-LC-MS and identified by accessing human and microorganism protein data banks. Due to the high amount of albumin in the Q-TOF-LC-MS results, BSA was also incubated with DMEM with the same conditions applied to atheroma plaques to observe if this would result in nanobacteria formations.

### 2.3. Whole genome sequencing analysis of atheroma plaques

For isolating genetic material, a 5-mm tissue slice was placed in 2 mL of DNA/RNA shield reagent and was ground with the help of a pestle and mortar. The suspension was collected into a 2 mL centrifuge tube and homogenized using a syringe. DNA was isolated from each sample using a DNA purification kit (GeneMark, China). DNA from samples A1, A2, A3, A6, A8, and A9; and from samples A7, A11, A12, A14, A15, A18, and A19 were pooled into two tubes as Group 1 (G1) and Group 2 (G2). Whole genome sequencing of these



**Fig. 1.** (A) SEM images and EDS analysis of sediment from a culture flask showing nanobacteria-like structures. (B) SEM images and EDS analysis of incubated BSA show the same structures. Bars: A: 1  $\mu$ m; B: 3  $\mu$ m.

two samples was performed (Eurofins Genomics, Germany). Raw sequencing data is processed using fastp (version 0.20.0) software to remove poor quality bases (below Phred Quality 20 were removed) using the sliding window approach. Adapters and reads shorter than 30 bp were also removed by using the same software. MetaPhlan (version 3.0.7) which relies on unique clade-specific marker genes identified from ~17,000 reference genomes (~13,500 bacterial and archaeal, ~3500 viral, and ~110 eukaryotic) were used for the taxonomic profiling. Unclassified reads were subjected to KrakenUniq (version 0.5.8). Kraken classifies reads by breaking each read into overlapping k-mers. Each k-mer is mapped to the lowest common ancestor (LCA) of the genomes containing that k-mer in a precomputed reference database. For each read, a classification tree is found by pruning the taxonomy and only retaining taxa (including ancestors) associated with k-mers in that read. Each node is weighted by the number of k-mers mapped to the node, and the path from root to leaf with the highest sum of weights is used to classify the read. KrakenUniq computes the number of unique k-mers observed for each taxon, which allows it to filter more false positives. Read counts of input samples observed at various taxa levels (Phylum, Genus, and Species) are collected and normalized by using the rarefy function implemented in the vegan bioconductor package (version 2.5.7) to compare species abundances. Rarefied read counts enable better comparisons of operational taxonomic unit (OTU) profiles between samples with different sample sizes. Abundance measured by the percentage of OTU assigned reads from various taxonomic levels is determined. As metagenomic analysis indicated the presence of *T. gondii* DNA, further evidence was obtained by nested PCR specific to this organism.

#### 2.4. Nested PCR for detecting *T. gondii* in atheroma plaque samples

Based on human whole genome sequencing, a high proportion of *T. gondii* DNA sequences were identified in the G1 and G2 sample pools. To obtain direct evidence for the presence of these organisms, two specific primers, 5'-CTGCAGGGAGGAAGACGAAAGTTG-3' as

forward and 5'-CGCTGCAGACACAGTGCATCTGGATT-3', as reverse primers, were used to amplify DNA from the individual samples in the first round of PCR. For nested PCR, 5'-GTGCTGGAGCCACAGAAGG-3' as forward and 5'-CTCCTCTCCCTTCGTCAA-3' as reverse primers were designed and used to amplify a 323-bp product. A ready-to-use PCR Master Mix II Kit (GeneMark, China) was used. The program was: initial denaturation at 95 °C for 1 min; 45 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 45 s; 72 °C for 2 min as final extension. PCR products were analyzed on 1.2 % agarose gel. *T. gondii* DNA grown in mouse peritoneum was used as a positive control, and sterile deionized water was used as a negative control.

#### 2.5. Statistical Analysis

The study data were statistically analyzed using R programming language (version 4.3.3.) was used for all statistical analysis. Fisher's exact test was performed to observe a significance between meta-data variables and *T. gondii*.

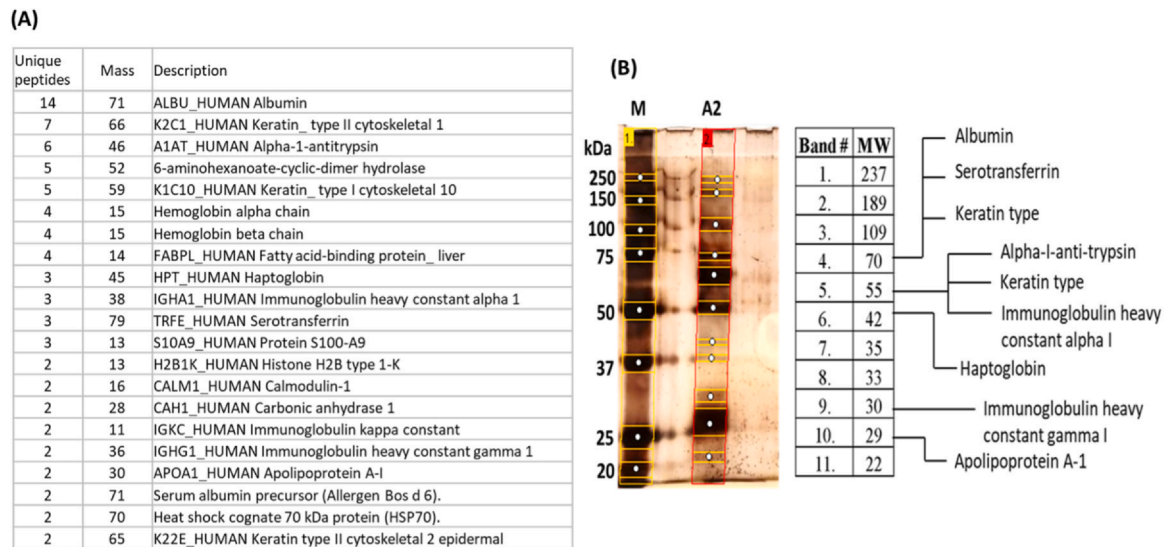
### 3. Results

#### 3.1. Microorganism culture analysis

Among all cultured samples, growth of specimen A14 was observed in only one SBA plate under anaerobic conditions. Gram staining of the isolate showed Gram-negative coccobacilli and was identified as *Cutibacterium acnes* by MALDI-TOF analysis.

#### 3.2. Nanobacterial analysis

When sediment was observed in DMEM media by inverted microscopy, it was analyzed by SEM. SEM revealed spherical structures with sizes ranging from 80 to 900 nm in all samples. These spherical structures with a diameter of 195.4 - 311.3 nm are seen in Fig. 1 A. In addition, elemental analyses (C, N, O, Mg, As, P, S, Ca, Si, Al) of the samples were made. Compared to other elements, a high percentage



**Fig. 2.** (A) The list of proteins identified from nanobacteria by LC-QTOF-MS. (B) SDS-PAGE separated proteins obtained from sample A2. The molecular weights of proteins corresponding to human proteins determined by LC-QTOF-MS are indicated next to the electrophoresis gel. (Lane M: protein marker, A2: nanobacterial proteins cultured from atheroma plaque, MW: molecular weight).

of carbon (C: 52.2%), nitrogen (N: 14%), and oxygen (O: 33%) was detected. DNA extraction was done from cultured atheroma plaque samples for molecular analysis. It revealed the absence of any nucleic acids. Also, SDS-PAGE and silver staining analysis showed the presence of molecules with molecular weights (MWs) ranging from 10 to 250 kDa. All samples revealed the same banding patterns. The molecules were wholly digested by proteinase K but not by DNase I and RNase, indicating they were of protein origin. The nature of these proteins was analyzed by LC-QTOF-MS. The identified patterns were screened using microorganisms and human data banks. All the proteins were identified as human in origin, with albumin being the most abundant (Fig. 2 A). One of the atheroma plaque samples (A2) that was run at SDS-PAGE was compared with LC-QTOF-MS data resulting in the corresponding proteins with molecular sizes of 70, 55, 42, 30 and 29 kDa. Based on the table shown in Fig. 2 A, we predicted that nanobacteria are formed of human proteins, mostly albumin. To test this hypothesis, we incubated bovine serum albumin (BSA) with DMEM medium free of FBS. The same spherical structures as in atheroma plaque samples were observed by SEM. Also, elemental analysis showed similar proportions for carbon, nitrogen, oxygen and the other atoms (Fig. 1B). When we analyzed BSA used in this experiment by SDS PAGE we observed that BSA was not pure but contained several other proteins.

### 3.3. Whole genome sequencing analysis

G1 and G2 were subjected to whole genome sequencing. Sequences belonging to the human genome were eliminated, and those belonging to the metagenome were revealed. In the analysis run, the heatmap and bar graphs representing taxonomic abundance at the species level were formed to compare species richness from all samples (Fig. 3). The results showed that more than 50% of sequences belonged to *T. gondii*.

### 3.4. PCR-based detection of *T. gondii* in patients

As metagenome analysis of pooled samples indicated the presence of *T. gondii*, to obtain more concrete direct evidence, the presence of the DNA of this parasite in individual atheroma plaque samples was investigated by nucleic acid amplification using specific primers for *T. gondii* by PCR. A 323-bp product belonging to *T. gondii*

was obtained from eight samples: A3, A4, A12, A14, A15, A17, A18, and A19 among the 20 atheroma plaques by nested PCR (Fig. 4). In conclusion, *T. gondii* DNA was positive in 8 (40%) of 20 patients.

## 4. Discussion

Cardiovascular pathologies are responsible for several clinical conditions that lead to death due to occlusion of the arteries of vital organs [1], [3]. Microorganisms are among the factors that are suspected to play a role in atheroma plaque formations. Although several microorganisms like *Helicobacter pylori*, *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* were claimed to cause atheroma plaque formation, further studies are required to make a strong relation between these organisms and plaque formation.

This study aimed to investigate the possible role of any type of microorganisms in atheroma plaque formation by metagenomic analyses of whole DNA obtained from these plaques. For this purpose, plaque samples from the carotid arteries of 20 patients were inoculated into various culture media and incubated under aerobic and anaerobic conditions to grow any culturable microorganisms. Growth was observed in only one sample identified as *Cutibacterium acnes*, a Gram-positive anaerobic organism. *C. acnes* is an opportunistic pathogen that can infect the damaged endothelium of blood vessels and endocardium. Additionally, *C. acnes* is the second most common pathogen after coagulase-negative Staphylococci isolated from infected internal cerebral ventricular bypasses, which indicates frequent bacteraemia of this organism [20], [21]. There is an ongoing debate regarding whether the isolation of *C. acnes* strains from tissues other than the skin is an actual infection or contamination. In this study, *C. acnes* was identified in only one of the 20 patients, and metagenomic analysis indicated no DNA sequences belonging to this organism in atheroma plaque, making it unlikely to be responsible for atheroma plaque formation. From all of the atheroma plaque samples, it was possible to grow nanobacteria. Nanobacteria, which are formations smaller than bacteria, have been the subject of many discussions regarding whether they are living organisms or not, and have also been proposed as candidate agents for atheroma plaque formation [16], [17], [18]. It is suggested that nanobacteria may be responsible for pathological calcifications in kidney and dental pulp stones, as well as heart valve calcifications and arterial calcifications [22]. However, other studies have questioned the microbiological

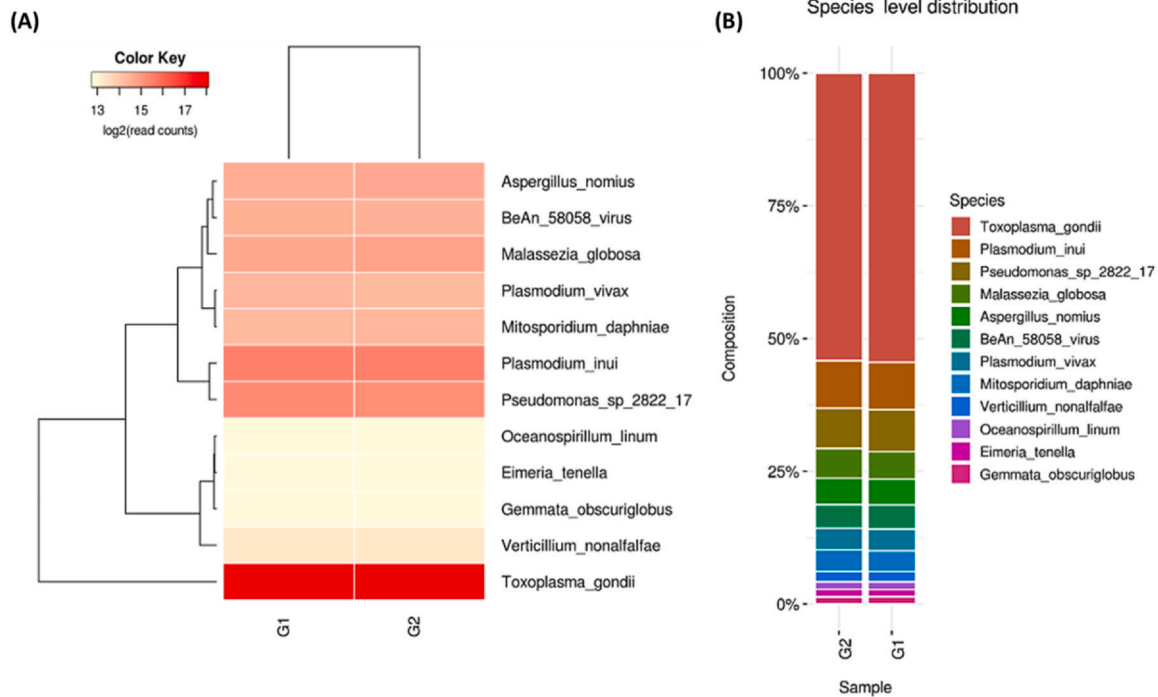


Fig. 3. (A) The various taxonomic levels of group 1 and group 2. (B) Bar plot(s) showing the taxonomic abundance corresponding to G1 and G2.

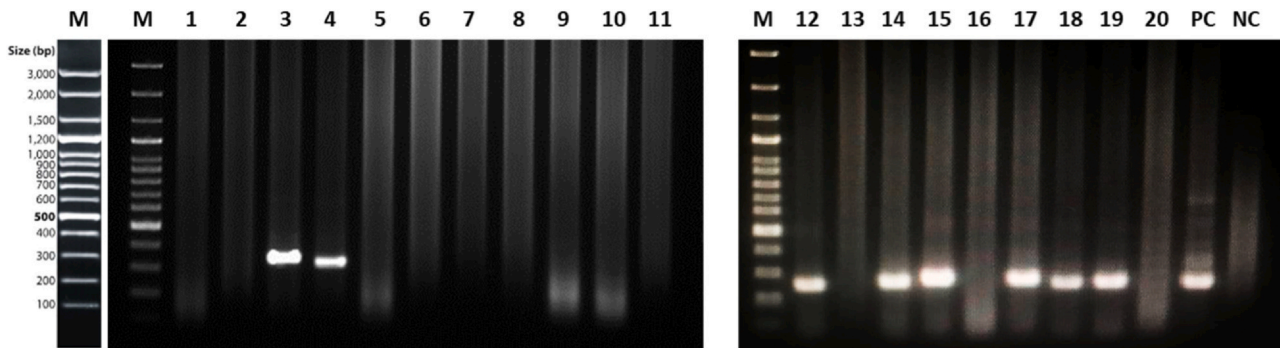


Fig. 4. 1.2% agarose gel showing PCR amplification products of *T. gondii* DNA from the atheroma plaque samples, A1–A20. Lane M: DNA marker, PC: positive control (*T. gondii* DNA), NC: negative control (deionized water).

origin of these particles, suggesting that nanobacteria are formed by the biomineralization of natural molecules. To investigate if nanobacteria may cause the formation of atheroma plaques, the plaques were cultured in DMEM, where nanobacteria can grow. Growth was observed in all samples within a 1–2-month period, as described in the literature [23]. SEM analysis of these formations showed nanobacteria-like structures ranging in size from 80 to 900 nm (Fig. 1), as defined previously [24]. The susceptibility of the molecules from these formations to Proteinase K and resistance to DNase I and RNase indicated their proteinaceous nature, identified as human proteins by LC-QTOF-MS. The samples were scanned in human, bacterial, and viral databases, and only human proteins were identified. A high albumin level was detected based on the same band patterns and MW as albumin detected using SDS-PAGE (Fig. 2). To test the hypothesis that nanobacteria are made of human proteins, mostly albumin, we incubated BSA with DMEM medium and obtained similar nanobacterial structures. When we analyzed the BSA used in this experiment it turned out to contain several other proteins which also may play a role in the formation of nanobacteria. Accumulation of nanobacteria formation may contribute to atheroma plaque formation, however, it may be proposed that

nanobacteria are not the etiological factors that start the plaque formation.

Whole genome sequencing and metagenomic analysis of the pooled plaque samples indicated the presence of *T. gondii* DNA as a large proportion of all DNA. No DNA sequence belonging to *Helicobacter pylori*, *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* was identified. To have more concrete direct evidence for the presence of *T. gondii*, we did further analysis of individual samples by nested PCR and identified *T. gondii* DNA in 8 out of 20 atheroma plaques. Therefore, we concluded *T. gondii* may be an important etiological factor leading to atheroma plaque formation.

As a limitation of the study, it was not possible to include vascular samples without atheroma plaque formations as negative controls. Although atheroma plaque formation mechanism is not expected to be different in other types of arteries, it will be good to include atheroma plaques from other arteries especially from coronary and cerebral regions, in further studies.

In one study, the seroprevalence of anti-*T. gondii* immunoglobulin G (IgG) antibodies were investigated in atherosclerotic patients by ELISA and showed that anti-*T. gondii* IgG was present in; 63.1% of patients with atherosclerosis compared to

46.2% of non-atherosclerotic individuals [14], [15]. We have determined that gender, hypertension, coronary artery disease, diabetes mellitus and dyslipidemia categorical variables had no relationship with *T. gondii* presence (p-value > 0.05). Additionally, the drugs, antiaggregants, statins and beta-blockers that were used by most of the patients did not affect the presence of *T. gondii* in atheroma plaques. The mean age of patients who needed carotid atheroma plaque removal was 71.5 which indicates the chronic nature of the pathology. Although it is hard to conclude because of the low number of patients included in this study, among smokers we identified *T. gondii* in 5 out of 8 patients (62.5%), however in 4 out of 12 patients (33%) in non-smokers. Smoking is known to be a key factor for speeding the development of atherosclerosis which may make vascular endothelium more susceptible to *T. gondii* infection.

*T. gondii* occurs worldwide. It is a very invasive parasite and can infect cells within seconds when it comes into contact. Once it infects people it can stay lifelong in the body. It is the second most frequent foodborne infection in the United States [25]. It causes congenital infections which lead to serious malformations and often death of the fetus. It can cause encephalitis, myocarditis and pneumonitis in immunocompromised patients. It causes asymptomatic chronic infection in healthy people [26], [27]. Its probable role in vascular infections and atheroma plaque formation has not been investigated so far. This is the first study with direct evidence of the presence of *T. gondii* in atheroma plaques.

If further evidence is obtained about the role of *T. gondii* in atheroma plaque formation this may change the current classical prevention and treatment strategies of atherosclerosis complications. The presence of *T. gondii* infection in patients with atheroma plaques may be detected by PCR from blood samples if free parasites or their DNA may be found in blood. Although the probability of finding *T. gondii* in blood samples can be expected to be low it may be worth it to do a study to determine the presence of *T. gondii* in patients with atheroma plaques. It is more probable to detect *T. gondii* DNA in atheroma plaques. However, it requires an invasive procedure to obtain a biopsy sample from atheroma plaques which cannot be performed because of the high risk of complications. Therefore, further evidence for the relation between *T. gondii* and atheroma plaque formations can be obtained by similar studies on extracted atheroma plaques or autopsy samples. If strong evidence for the relation between *T. gondii* and atheroma plaque formation is identified, this may change the strategies for prevention and treatment of arteriosclerosis.

Prevention measures against *T. gondii* for seronegative pregnant women and patients with weak immune systems are well-established. Since atherosclerosis is a worldwide common health problem it may be important to use all these prevention measures for all communities. The cysts in the meat can be killed by heating the meat to at least over 67 °C. Hands, all meat-cutting tools, boards and other materials should be washed with soap and water before and after handling meat. Cats are the main reservoir of *T. gondii* in nature [25,26]. It is important to pay attention to hygiene when cleaning cats' faeces sandbox for the people who have cats at home. When gardening, gloves should definitely be worn. Raw eggs and unpasteurized milk should not be consumed, and vegetables and fruits should be washed thoroughly before eating [28]. *T. gondii* infections can be treated with medication using drugs for which their safety is already established. Patients are usually given medications such as pyrimethamine for treatment. In addition, antibiotics such as sulfadiazine, clindamycin, and azithromycin can be used to treat the infection.

In this study among 20 atheroma samples, we detected *T. gondii* DNA in 8 samples. The detection of DNA is strong evidence for infection of this parasite in vascular tissue. Therefore, it may play an important role in the formation of atheroma plaques and vascular

occlusion. It is not possible to predict if *T. gondii* invades vascular tissue before or after atheroma plaque formation. Even if it is not the primary etiological cause of plaque formation it may play an important role in creating inflammation and growth of the plaque. If further evidence can be obtained by further studies including a larger number of patients, prevention or treatment of atheroma plaque formation may become possible. Especially in patients diagnosed with atheroma plaques and vascular occlusion, treatment of *T. gondii* infection may prevent progression of the disease and even ameliorate blood flow by subsiding the inflammation and downsizing of atheroma plaques.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The author is an Editorial Board Member/Editor-in-Chief/Associate Editor/Guest Editor for *Journal of Infection and Public Health* and was not involved in the editorial review or the decision to publish this article.

There is no potential competing interests.

## References

- [1] Tsao CW, et al. Heart Disease and Stroke Statistics-2022 Update: A report from the American Heart Association. *Circulation* Feb. 2022;vol. 145(8):E153-639. <https://doi.org/10.1161/CIR.0000000000001052>
- [2] Roth GA, et al. Global burden of cardiovascular diseases and risk factors, 1990-2019. *J Am Coll Cardiol* 2020;vol. 76(25). <https://doi.org/10.1016/j.jacc.2020.11.010>
- [3] Carrizales-Sepúlveda EF, Ordaz-Farías A, Vera-Pineda R, Flores-Ramírez R. Periodontal disease, systemic inflammation and the risk of cardiovascular disease. *Heart Lung Circ* 2018;vol. 27(11). <https://doi.org/10.1016/j.hlc.2018.05.102>
- [4] Rafeian-Kopaei M, Setorki M, Douidi M, Baradaran A, Nasri H. Atherosclerosis: process, indicators, risk factors and new hopes. *Int J Prev Med* 2014;vol. 5(8).
- [5] Stoll G, Bendzus M. Inflammation and atherosclerosis: novel insights into plaque formation and destabilization. *Stroke* 2006;vol. 37(7). <https://doi.org/10.1161/01.STR.0000226901.34927.10>
- [6] Hansson GK, Hermansson A. The immune system in atherosclerosis. *Nat Immunol* 2011;vol. 12(3). <https://doi.org/10.1038/ni.2001>
- [7] Campbell LA, Rosenfeld ME. Infection and atherosclerosis development. *Arch Med Res* 2015;vol. 46(5). <https://doi.org/10.1016/j.arcmed.2015.05.006>
- [8] Szulc M, Kustrzycki W, Janczak D, Michalowska D, Baczynska D, Radwan-Oczko M. Presence of periodontopathic bacteria DNA in atheromatous plaques from coronary and carotid arteries. *Biomed Res Int* 2015;vol. 2015. <https://doi.org/10.1155/2015/825397>
- [9] B, Farsak, et al. Detection of chlamydia pneumoniae and Helicobacter pylori DNA in human atherosclerotic plaques by PCR. *J Clin Microbiol* 2000;vol. 38(12). <https://doi.org/10.1128/jcm.38.12.4408-4411.2000>
- [10] Ellis RW. Infection and coronary heart disease. *J Med Microbiol* 1997;vol. 46(7):535-9. <https://doi.org/10.1099/00222615-46-7-535>
- [11] Blasi F, et al. Detection of Chlamydia pneumoniae but not Helicobacter pylori in atherosclerotic plaques of aortic aneurysms. *J Clin Microbiol* 1996;vol. 34(11):2766-9. <https://doi.org/10.1128/JCM.34.11.2766-2769.1996>
- [12] Chiu B, Viira E, Tucker W, Fong IW. Chlamydia pneumoniae, cytomegalovirus, and herpes simplex virus in atherosclerosis of the carotid artery. *Circulation* Oct. 1997;vol. 96(7):2144-8. <https://doi.org/10.1161/01.CIR.96.7.2144>
- [13] Kaplan M, et al. Detection of Chlamydia pneumoniae and Helicobacter pylori in atherosclerotic plaques of carotid artery by polymerase chain reaction. *Int J Infect Dis* 2006;vol. 10(2). <https://doi.org/10.1016/j.ijid.2004.10.008>
- [14] Alhussein SM, Saleh NE, El-Zayady WM, Hussein MS, El-Beshbishi SN. Association between Toxoplasma gondii infection and coronary atherosclerosis. *Trans R Soc Trop Med Hyg* 2021;vol. 115(10). <https://doi.org/10.1093/trstmh/trab018>
- [15] Dragomir A, Lupu MA, Lighezan R, Padurarur AA, Olariu TR. Toxoplasma gondii infection in patients with cardiovascular diseases from western Romania: a case-control study. *Life* 2023;vol. 13(7). <https://doi.org/10.3390/life13071575>
- [16] Wainwright M. Nanobacteria and associated 'elementary bodies' in human disease and cancer. *Microbiology* 1999;vol. 145(Pt 10). <https://doi.org/10.1099/00221287-145-10-2623>

- [17] Kajander EO, Çiftçioğlu N. Nanobacteria: an alternative mechanism for pathogenic intra- and extracellular calcification and stone formation. *Proc Natl Acad Sci USA* Jul. 1998;vol. 95(14):8274–9. <https://doi.org/10.1073/PNAS.95.14.8274>
- [18] Puskás LG, Tiszlavicz L, Rázga Z, Torday LL, Krenács T, Papp JGY. Detection of nanobacteria-like particles in human atherosclerotic plaques. *Acta Biol Hung* 2005;vol. 56(3–4):233–45. <https://doi.org/10.1556/ABIOL.56.2005.3-4.7>
- [19] S.M. Grundy et al., “Cardiovascular and risk factor evaluation of healthy American adults. A statement for physicians by an Ad Hoc Committee appointed by the Steering Committee, American Heart Association,,” *Circulation*, vol. 75, no. 6, pp. 1340A–1362A, Jun. 1987, Accessed: Apr. 22, 2024. [Online]. Available: <https://www.scholars.northwestern.edu/en/publications/cardiovascular-and-risk-factor-evaluation-of-healthy-american-adu>.
- [20] L.N. Walti, A. Conen, J. Coward, G.F. Jost, and A. Trampuz, “Characteristics of infections associated with external ventricular drains of cerebrospinal fluid,” *J. Infect.*, vol. 66, no. 5, 2013, doi: 10.1016/j.jinf.2012.12.010.
- [21] Coenye T, Spittaels KJ, Achermann Y. The role of biofilm formation in the pathogenesis and antimicrobial susceptibility of *Cutibacterium acnes*. *Biofilm Dec* 2021;vol. 4. <https://doi.org/10.1016/J.BIOFLM.2021.100063>
- [22] Çiftçioğlu N, McKay DS. Pathological calcification and replicating calcifying-nanoparticles: General approach and correlation. *Pediatr Res* 2010;vol. 67(5). <https://doi.org/10.1203/PDR.0b013e3181d476ce>
- [23] Yaghobee S, Bayani M, Samiei N, Jahedmanesh N. What are the nanobacteria? *Biotechnol Biotechnol Equip* 2015;vol. 29(5). <https://doi.org/10.1080/13102818.2015.1052761>
- [24] Jelic TM, et al. Calcifying nanoparticles associated encrusted urinary bladder cystitis. *Int J Nanomed* 2008;vol. 3(3):385–90. <https://doi.org/10.2147/IJN.S3559>
- [25] Almeria S, Dubey JP. Foodborne transmission of *Toxoplasma gondii* infection in the last decade. An overview. *Res Vet Sci* 2021;vol. 135. <https://doi.org/10.1016/j.rvsc.2020.10.019>
- [26] Layton J, et al. Clinical spectrum, radiological findings, and outcomes of severe toxoplasmosis in immunocompetent hosts: a systematic review. *Pathogens* 2023;vol. 12(4). <https://doi.org/10.3390/pathogens12040543>
- [27] Smith NC, Goulart C, Hayward JA, Kupz A, Miller CM, van Dooren GG. Control of human toxoplasmosis. *Int J Parasitol* 2021;vol. 51(2–3). <https://doi.org/10.1016/j.ijpara.2020.11.001>
- [28] Khairullah AR, et al. A comprehensive review of toxoplasmosis: serious threat to human health. *Open Public Health J* 2024;vol. 17(1). <https://doi.org/10.2174/0118749445281387240202094637>