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CUKUROVA UNIVERSITY
HEALTH SCIENCES INSTITUT
MEDICAL MICROBIOLOGY DEPARTMENT

INVESTIGATION OF THE RELATIONSHIP BETWEEN *CHLAMYDIA PNEUMONIAE* AND ATHEROSCLEROSIS BY 16S rRNA SEQUENCING METHOD

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MEDICAL MICROBIOLOGY DEPARTMENT MASTER PROGRAM
MASTER THESIS

SUPERVISOR
Prof.Dr. Fatih KÖKSAL

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LIST OF SYMBOLS And ABBREVIATION

LPS : Lipopolysaccharide
DNA : DeoxyriboNucleic Acid
RNA : RiboNucleic Acid
LDL : Low-Density Lipoprotein
HDL : High-Density Lipoprotein
TNF-α : Factor-Alpha
IFN-γ : Interferon-Gamma
IL : Interleukin
TH1 : Helper T-Cell
PCR : Polymerase Chain Reaction
Hsp-60 : heat shock proteins
TBE : Tris/Borate/EDTA
CAD : Community-Acquired Pneumonia
IgM : İmmunoglobulin M
IgG : İmmunoglobulin G
RF : Rheumatoid factor
EB : Elementary Body
TW-183 : Taiwan
AR-39 : Acute Respiratory
NAAT : Nucleic Acid Amplification Tests
IFAT : Indirect immunofluorescence assay
MOMP : Major Outer Membrane Protein
MAbs : Monoclonal antibodies
VDIV : Domain IV Region Serovar
C : Cytosine
G : Guanine
µl : Microliter
TAB : Tris-Acetate Buffer
AB : Aberrant bodies
VD : Variable Domain
BAL : Bronchoalveolar lavage

ABSTRACT

Investıgatıon Of The Relatıonshıp Between *Chlamydıa Pneumonıae* And Atherosclerosıs By 16s Rrna Sequencıng Method

Cardiovascular and cerebrovascular diseases are a major public health problem globally, with high morbidity and mortality rates. Atherosclerosis has been blamed for half of the adult deaths in the Western world, especially in the United States. Stenosis, ischemic heart disease due to atherosclerosis of coronary vessels, and cardiac syndromes are among the causes of sudden death due to arterial thrombus formation. The theory that atherosclerosis, which is associated with many reasons such as dietary habits and smoking, which is one of the genetically related vascular wall anomalies, has been increasingly supported since the late 19th century, as a result of chronic inflammation that develops due to chronic infections. After Saikku showed *C. pneumoniae* inclusions in the post-mortem atheroma plaque samples of patients who died of cardiovascular diseases with electron microscopy studies. After Saiku published the results of his study, numerous studies were conducted questioning the relationship between *C. pneumoniae* atherosclerosis. In these studies, different methods such as electron microscopy and direct fluorescent antibody or enzyme immunoassay method were used from endarterectomy samples during post-mortem or cardiovascular surgery. At the end of all these studies, the previously accepted hypothesis of "atherosclerosis develops as a result of infection of the vessel wall" turned into "atherosclerosis develops as a result of infection of the vessel wall and monocytes with *C. pneumoniae*". Later, the results of studies claiming that the frequency and duration of double and triple endpoints were found to be significantly different in the groups in which antibiotics to which *C. pneumoniae* were susceptible were added to the treatment in patients with acute myocardial infarction compared to the control groups began to be published. On the other hand, some researchers claimed that it can cause atherosclerosis in *H. pylori*, CMV, adenovirus, and herpes group viruses using PCR-based methods. But they could not show the microorganism itself directly in the plate, foam cell. As a result, it is highly probable; *C. pneumoniae* may be the cause of the sclerotic lesion and atherosclerotic plaques can be seen in all arteries including cerebrovascular arteries except for the internal mammary artery.

With this study, we aimed to show the possible relationship between atherosclerosis and especially *C. pneumoniae* and *H. pylori* by using PCR-sequencing and IFAT methods. Most of the routine operations could not be performed as a result of the outbreak of the covid-19 pandemic during the sample collection process of the project. For this reason, we could not reach the number of patient samples we planned, but we thought that the results of the 11 samples we evaluated in our study could be a guide for further studies with larger case groups. At the end of the study, 8 (72.7 %) of our patient group with a mean age of 58 years had anti-*C. pneumuniae* antibody response > 1/16 with IFAT, while 7 (63.6 %) of these patients had *C. pneumoniae* in the atheroma plaques. Target sequences of *H.pylori* ureC gene were found in 1 (9.1 %) sample in atheroma plaques. As in previous studies, the incidence of *C. pneumoniae* in atheroma plaques was accepted as a possible association.

Keyword: Cardiovascular, cerebrovascular, *C. pneumoniae,* IFAT, PCR

ÖZET

Chlamydia Pneumoniae Ve Ateroskleroz Arasındaki İlişkinin 16s Rrna Sequncing Yöntemi İle Araştırılması

Kardiyovasküler ve serebrovasküler hastalıklar, yüksek morbidite ve mortalite oranları ile dünya çapında önemli halk sağlığı sorunlarıdır. ateroskleroz batı dünyasında ve özellikle Amerika Birleşik Devletleri'nde yetişkin ölümlerinin yaklaşık yarısından sorumlu tutulmuştur. Stenoz, koroner damarlardaki aterosklerozuna bağlı iskemik kalp hastalığı ve kardiak sendromlar, arteriyel trombüs oluşumuna bağlı ani ölüm nedenleri arasındadır. Beslenme alışkanlıkları ve sigara kullanımı gibi birçok sebeple ilişkilendirilen ateroskleroz teorisi, genetik olarak ilişkili damar duvarı anomalilerinden biri olarak, 19. yüzyılın sonlarından beri "kronik enfeksiyonlara bağlı olarak gelişen kronik inflamasyon'un bir sonucu" olarak kabul edildi. Saikku'nun çalışmasında kardiyovasküler hastalıklardan ölen hastaların postmortem aterom plak örneklerini elektron mikroskobunda inceleyerek foamcell içerisindeki *C. pneumoniae* inklüzyonlarını gösterdi. Saiku, çalışmasının sonuçlarını yayınladıktan sonra, *C. pneumoniae* aterosklerozu arasındaki ilişkiyi sorgulayan çok sayıda çalışma yapıldı. Bu çalışmalarda ölen hastaların ölüm sonrası veya kardiyovasküler cerrahi sırasında endarterektomi örneklerinden elektron mikroskobu ve indirekt floresan antikor veya enzim immunoassay yöntemi gibi farklı yöntemler kullanılmıştır. Tüm bu çalışmaların sonunda daha önce kabul edilmiş olan "damar duvarının enfeksiyonu sonucu ateroskleroz gelişir" hipotezi "damar duvarı ve monositlerin *C. pneumoniae* ile enfeksiyonu sonucu ateroskleroz gelişir" haline dönüşmüştür. Daha sonra akut miyokard infarktüslü hastalarda tedaviye *C. pneumoniae* duyarlı olduğu antibiyotiklerin eklendiği gruplarda ikili ve üçlü sonlanım noktalarının sıklık ve süresinin anlamlı olarak farklı olduğunu iddia eden çalışmaların sonuçları, kontrol grupları yayınlanmaya başlandı. Öte yandan bazı araştırmacılar, PCR tabanlı yöntemler kullanarak *H. pylori,* CMV, adenovirüs ve herpes grubu virüslerinde ateroskleroza neden olabileceğini iddia etti. Ancak mikroorganizmanın kendisini doğrudan plaka köpük hücre içinde gösteremediler. Sonuç olarak, çok olasıdır; *C. pneumoniae,* sklerotik lezyonun nedeni olabilir ve aterosklerotik plaklar, internal meme arteri dışında serebrovasküler arterler dahil tüm arterlerde görülebilir. Bu çalışma ile ateroskleroz ve özellikle *C. pneumoniae* ve *H. pylori* arasındaki olası ilişkiyi PCR-sıralama ve IFAT yöntemleri kullanarak göstermeyi amaçladık. projenin örnek toplama sürecinde covid-19 salgınının bir sonucu olarak rutin işlemlerin çoğu gerçekleştirilemedi. Bu nedenle planladığımız hasta örnek sayısına ulaşamadık, ancak çalışmamızda değerlendirdiğimiz 11 örneğin sonuçlarının daha geniş vaka grupları ile yapılacak ileri çalışmalar için yol gösterici olabileceğini düşündük. Çalışma sonunda yaş ortalaması 58 olan hasta grubumuzun 8'inde (% 72,7) IFAT ile anti-*C. pneumuniae* antikor yanıtı > 1/16 iken, bu hastaların 7'sinde (% 63,6) *C. pneumoniae* aterom plaklarında. *H. pylori* ureC geninin hedef sekansları aterom plaklarındaki 1 (% 9.1) örnekte bulundu. Önceki çalışmalarda olduğu gibi, aterom plaklarında *C. pneumoniae* insidansı olası bir ilişki olarak kabul edildi.

Anahtar Kelimeler: Kardiyovasküler, Serebrovasküler, *C. pneumoniae,* IFAT, PCR

1. INTRODUCTION

C. pneumoniae may be associated with all degenerative disorders of the arteries and heart valves, and are non-rheumatic, and all risk factors Specifically identified so far, the idea that smoking increases the risk of infection 5-6 times by increasing inflammation that enhances cellular immunity by increasing the secretion of IL-4 in the endothelium[1]. These views believe that the history of atheroma plaque formation begins with *C. pneumoniae* infection monocytes in the airways. *C. pneumoniae*, unlike other chlamydia species, *C. pneumoniae* bodies, have the ability to proliferate in infected monocytes and macrophages, rapidly enter the general circulation, and can cause many organ involvement and infections (hepatitis, iritis, etc.), primarily reticuloendothelial system cells, via blood. The results of this study are based on the results of previous clinical studies that proved the presence of infection as evidence. In studies that demonstrated the relationship between *C. pneumoniae* infection and the occurrence of atherosclerosis, objects containing *C. pneumoniae* were shown in plaque samples obtained from the endothelium of the cardiovascular, or in artery biopsy samples after death by electron microscopy and examinations histochemical and immunohistochemical[2]. In these studies, it was shown that the antibody response to *C. pneumoniae* in the sera of patients with atherosclerosis in titrations of 1/64 and above expressed a 237-fold increased risk compared to subjects with normal vessels[3]. In similar studies, the presence of *C. pneumoniae* and nucleic acids in monocytes, coronary arteries, aorta, carotid artery, iliac artery, femoral artery, popliteal artery, and pulmonary arteries, in smooth muscle cells and vascular endothelium has been proven by PCR and EM studies[4]. It has been stated that *C. pneumoniae* is seen not only in necrotic, calcified, fibrous encapsulated mature atheroma plaques but also in monocytes in lesions where small fat deposits (Stary type II-IV) are seen in the initial period of atheromatous changes. Synthesis of matrix metalloproteins such as TNF-α, IL-Iβ, IL-6, muscular growth factor, and matrix metalloproteins increase in infected monocytes induced by *C. pneumoniae* antigens, especially LPS antigens[5]. In addition, lipid metabolism is disrupted in these cells and these cells turn into lipid-rich foam cells. The density of human heat shock proteins (Hsp-60) in foam cells is a diagnostic criterion that has been known for many years and is considered characteristic for atheroma

plaque formation. Recent studies have shown that these cells express a high rate of specific *C. pneumoniae* Hsp-60. TNF-α increases the production of leukocyte adhesive proteins in the vascular endothelial lumen and initiates angiogenesis. The properties of serum triglycerides increase LDL. IL-Iβ and IL-6 increase the coagulation of platelets and the production of acute-phase proteins such as CRP, fibrinogen, and amyloid-A in the liver[6]. As a result, endothelial procoagulant activity increases. Increased circulating CRP level, for example, 0.3 mg/dl and above, is a predictive finding for ischemia. Increased circulating IL-6 is characteristic of patients with unstable angina[6]. It has been shown that Matrix metalloproteases, whose main task is to dissolve connective tissue during organogenesis and whose nearly 20 species have been identified, are also synthesized by stimulated monocytes and macrophages. These proteins secreted by macrophages tend to encapsulate the fibrous capsule of the atheroma plaque, causing ulceration and fissuring. It has been suggested that fragments detached from here pull the coagulation shunt to activate thrombin and cause the formation of new thrombus foci. Again, reproductive factors produced by infected monocytes, macrophages, endothelial cells, and smooth muscle cells cause proliferation in smooth muscle cells and the migration of smooth muscle cells in the media towards the intima and lumen. In an experimental study on rabbits[7]. The aortic biopsy specimens are taken 6 weeks after injection into the intranasally with *chlamydia* bacteria. Intima thickness was shown to be approximately 0.2 pm thickening in subjects not given azithromycin compared to the group treated with azithromycin immediately after infection, and this thickening was suggested to be due to the production factors provoked by *C. pneumoniae*. On the other hand, DNA sequencing techniques, which began with the Human Genome Project and witnessed amazing developments, have helped discover microorganisms in human and environmental samples that cannot be proven with conventional techniques and which have developed some of our knowledge of the relationship between the microorganism, the host and some of the changes that accompany it. The definition of flora has become replaced with the definition of the microbiome, and the possibility that the body cavities and fluids we define as sterile may also be colonized by microorganisms. The need to make changes in the classification of bacteria with full genome sequencing arose, and the definition of

microbiota gained meaning with metagenomic DNA and 16S rRNA sequencing techniques.

2. GENERAL INFORMATION

Chlamydia is a small group of non-motile Gram-negative bacteria that are obligate parasites within eukaryotic cells[8]. *Chlamydia* is a bacteria that can cause upper respiratory tract and lower respiratory tract infections such as bronchitis and pneumonia in humans[9].

2.1. History And Classification

C. pneumoniae was isolated from the conjunctiva of a child in Taiwan in 1965 and in Iran in 1968, and the isolate in Taiwan was named TW-183[10]. However, although these two roots were isolated from the conjunctiva, it was found that they were not associated with eye infections. In 1943, Joseph E. Smaddle first showed that a human-adapted *Chlamydia* genus origin can be transmitted from person to person via respiration, this claim from Smadel was substantiated 40 years later.

Due to its inclusion morphology and its staining properties in cell culture, *C. pneumonia* was initially considered as *Chlamydophila psittaci* (*C. psittaci*). However, as a result of later studies, it was determined that this organism is different from *C. psittaci* and *Chlamydia trachomatis* (*C. trachomatis*).

C. pneumoniae was isolated from the respiratory tract in 1983 and this isolate is named AR-39. Grayston and Diğ. named this new respiratory pathogen TWAR in 1986 based on TW-183 and AR-39 isolates. This *Chlamydia* strain, called TWAR, was accepted as the third species of the *Chlamydia* genus in 1989 and was named *Chlamydia pneumoniae*. As a result, *C. pneumoniae*, *C. psittaci*, *C. trachomatis*, and *C. pecorum* were included in the new genus *Chlamydophila*[11].

C. pneumonia and coronary artery disease were first revealed in 1988 in a study by Saikku with six friends[12].

2.1.1. Lifecycle

The specific developmental cycle of chlamydia is different from all other bacteria. This difference makes an important difference in laboratory diagnosis, clinical course, and antibiotic treatment. During the reproductive cycles, the extracellular infectious form of elementary bodies (EBs) and the intracellular replicative form of

reticular bodies (RBs) play a role. Infectious ECs are 200-400 nm in diameter, adhere to the host cell by electrostatic bonding, and are taken into the cell by endocytosis[13]. Adhesins and adhesin receptors that play a role in cell attachment have not been precisely defined. These EBs remain within the membrane-surrounded phagocytosis of the cell. Bacteria prevents phagocytosis from combining with the lysosome[13].

after 6-8 hours FROM entering the cell, EBs transform into larger (800-1000 nm), metabolically active RBs[14]. Like other bacteria, RBs replicate by dividing in two. During the reproduction process, chlamydia meets high-energy phosphate compounds and some amino acids from the host cell and are therefore also called energy parasites. Approximately 18-24 hours after infection, RB's begins to transform back into smaller EBs. Although 500 to 1000 infectious EB's accumulates in the inclusion, host cell functions are minimally impaired. EBs leave the cell by cytolysis 48-72 hours after entry. This cycle is extremely important in that it enables the microorganism to create a chronic infection. The exit of infectious elemental bodies from the cell enables them to pass to new hosts and infect new cells. The life cycle of *C. pneumoniae* is shown in Figure 2.1

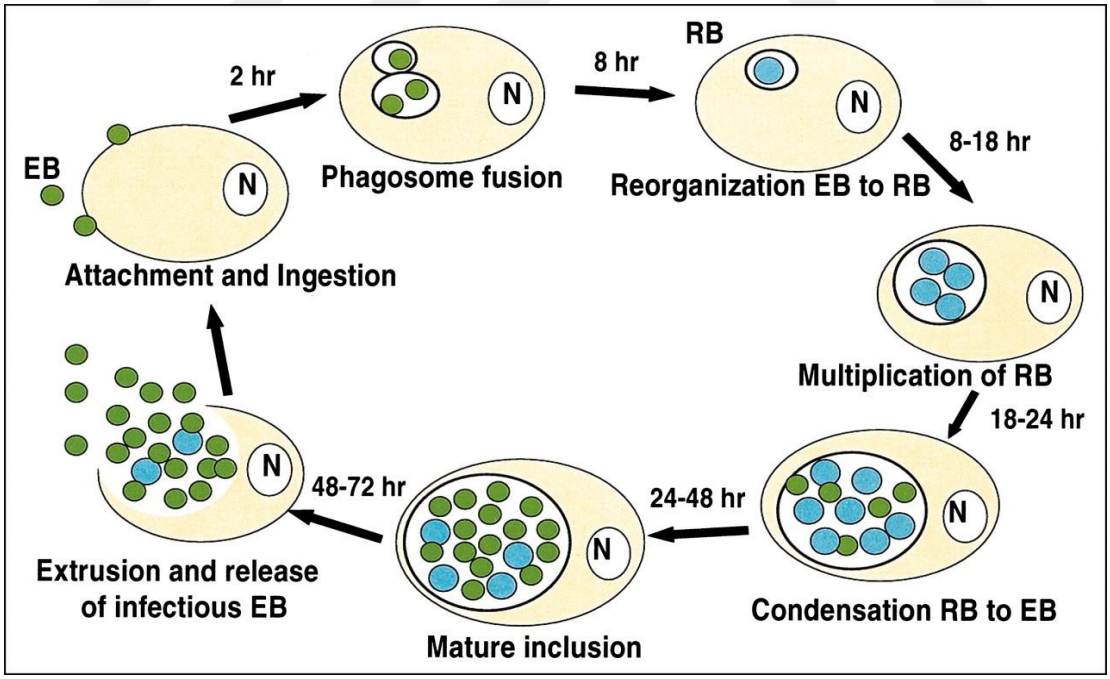

Figure 1. The life cycle of *C. pneumoniae*. EC: Elemental Body, RC: Reticular Body, AC: Aberrant Body[13]

Chlamydias can enter a persistent phase in the presence of certain cytokines such as gamma interferon (IFN-γ), especially some antibiotics such as penicillin, or in the absence of certain nutrients including iron, glucose, and some amino acids. During this phase, metabolic activity decreases and the microorganism becomes more resistant to antibiotic treatment. Persistent chlamydial shapes; Morphologically, they are defined as subtle and expanded RBs that are living but non-infectious, found in small, intracellular inclusions. These RBs are also called aberrant bodies (AB)[13]. It is suggested that persistence is an alternative life cycle that chlamydia develops to escape from the host immune system.

2.1.2. Cell Structure

Chlamydia; They are inert, biphasic life cycle, obligate intracellular pathogenic bacteria. In order for them to survive and develop, they must enter the mucosal epithelium cells. It has inner and outer membranes similar to those of Gram-negative bacteria. All members of the *Chlamydiaceae* family contain a common group of antigens in the lipopolysaccharide (LPS) structure. The lipopolysaccharide layer has lower endotoxic activity than that of other bacteria[15]. The reason for this is thought to be that Lipid A has longer fatty acids than normal. According to the latest genomic analysis, it has been shown that *C. pneumoniae* and *C. trachomatis* synthesize proteins used in peptidoglycan synthesis. These proteins also include penicillin-binding proteins. Therefore, antimicrobials that act on cell wall synthesis inhibit chlamydial cell wall synthesis[3]. In addition, bacteria are sensitive to antibiotics such as tetracyclines, macrolides, and quinolones, which act on DNA and protein synthesis.

All chlamydia encodes a species-specific protein of 40 kDa called the major outer membrane protein (MOMP or OmpA). Major outer membrane protein constitutes approximately 60% of the protein amount found in bacteria. The presence of variable regions of the gene encoding this protein in *C. trachomatis* and *C. psittaci* led to the identification of multiple serovars in these species. On the other hand, *C. pneumoniae* isolates; have a markedly high MOMP affinity. Therefore, different serovars have not been defined for *C. pneumoniae*. In addition to the major membrane protein, Omp2 is a highly conserved outer membrane protein found in all members of the *Chlamydiaceae*

family. This protein, rich in cysteine amino acids, forms the structure of cross-linked disulfide that provide stability in elemental bodies.

Bacteria contain both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and have prokaryotic ribosomes. The genomes of the bacteria in the Chlamydiaceae family vary between 1.0 and 1.24 megabase pairs (Mbp), and their guanine (G) + cytosine (C) content is approximately 40%. *C. pneumoniae* are dependent on host cells for the synthesis of many amino acids and nucleotides[15].

Chlamydias have two different structural forms: the elemental body and the reticular body. EBs is very small (0.2-0.4 μm) and is the infectious particle of *chlamydia.* It has a durable outer membrane that is densely linked by cross-disulfide bonds, so it is resistant to environmental conditions[16]. EBs can be considered as an analogue of the bacterial spore and is metabolically inactive. Unlike other species, the EBs of *C. pneumoniae* are pear-shaped and their periplasmic spaces are very large[7]. The elementary body attaches to the host cell surface and enters the cell and transforms into the vegetative RBs form. The reticulate body is the non-infectious form of *chlamydia*, larger than EBs (0.8-1 μm) and metabolically active, proliferating particle. Unlike EB, its outer membrane is very thin and cross disulfide bonds are less. Both forms contain both DNA and RNA. The ratio of RNA / DNA is 1/1 in EB and 4/1 in RB.

2.1.3. Genome Structure and Properties

The *C. pneumoniae* genome contains 1,230,230 nucleotides (NT) and has an estimated 1052 protein-coding genes[17]. There are no known and defined extrachromosomal elements yet. Its compact genome is suitable for rapid genomic sequence analysis. As a result of the sequence analysis, it was observed that the *C. pneumoniae* genes were significantly similar to the genes encoding the structural, functional, and immunologically important proteins of *C. trachomatis* and *C. psittaci*. Among these, there are ompA, ompB, groEL, and dnaK genes[18].

The gene encoding the major outer membrane protein shows 68% similarity between *C. psittaci* - *C. trachomatis* and *C. pneumonia* - *C. trachomati*s and 71% between *C. psittaci C.pneumoniae*. MOMP, acting as a porin molecule, also contributes to intra and intermolecular disulfide bonds in the structure of the chlamydial envelope.

Four regularly located variable regions (Variable Domain = VD), which determine the serovars of *C. trachomatis*, separate highly conserved regions[16].

Monoclonal antibodies (MAbs) against *C. pneumoniae* MOMP recognize serovar, sub-species, and species-specific epitopes on the protein. The MOMP genes of several strains of *C. trachomatis* and *C. psittaci* were sequenced and found to be largely conserved except for four VDs. The mapping of the epitope showed that three of the variable regions (VDI, VDII, and VDIV) contain serovar, subtypes, and species-specific antigenic determinants. *C. pneumoniae* immunoblot analysis in sera samples taken from patients with *C. pneumoniae* infection or with *anti-C. pneumoniae* rabbit sera revealed that MOMP recognition is genus-reactive. To date, no other antigenic reaction to *C. pneumoniae* MOMP has been identified[19].

Two genes encoding heat shock proteins (HSP; GroEL and DnaK homologs) that are participating in immunopathological and immunoprotective responses in *C. trachomatis* were isolated from *C. pneumoniae*. For *C. trachomatis*, delayed form hypersensitivity response in infections has been associated with HSP60 (GroEL). In contrast, 70-kDa HSP (DnaK) antibodies neutralize infectivity; however, no delayed hypersensitivity activity is elicited from the antigen. In GroEL, the similarity between *C. pneumonia, C. trachomatis, and C.psittaci* has been 95-97% in the amino acid sequence. Whereas, DnaK shared in 87% of *Chlamydia pneumoniae* similarity rate with *C. trachomatis* protein[7].

2.1.4. Antigenic Structure

The presence of species-specific antigens of *C. pneumoniae* was first proved by MAbs in the MIF assay. Protein profiles similar to MOMPs of other *chlamydia* were found in all TWAR isolates. Although most of the proteins within the outer membrane complex (MOMP and 15.5-and 60-kDa proteins) are similar in molecular weight and structure to all other chlamydia species, the 98 kDa cysteine-rich protein is only found in the outer membrane complex of *C. pneumoniae*. The presence of this 98-kDa protein enables the microorganism to have a stronger membrane structure that preserves its pear-like morphology. However, other studies argue that the idea that this protein provides pear-like morphology is not yet certain.

Studies have shown that MOMP in the outer membrane complex provides the strength of the cell wall as bound with disulfide bonds as in other chlamydia species. Although *C. pneumoniae* MOMP has the same structural function, the antigenic analysis revealed that this protein has different properties than other *chlamydial* MOMPs. In contrast. to *Chlamydia trachomatis* and *Chlamydia psittaciensis*, where MOMP is the immune antigen identified during infection, immunohistochemical analysis of proteins identified by human sera from *C. pneumoniae.*

Many proteins specific to *C. pneumoniae* have been identified by the immunoblot method. 43-kDa protein with activities specific to *C. pneumoniae* and proteins with molecular weights ranging from 50-kDa to 60-kDa have been shown in human and animal sera (13). In one study, it was found that the specific activity of *chlamydia pneumoniae* of 54 kDa protein was predominant in immunoblot in positive sera[20]. In another study, it was revealed that 43, 46, and 53-kDa proteins were immunodominant antigens specific to *C. pneumoniae* during human infection[21].

GroEL from other proteins expressed during infection, from folding and stabilization; From PgK and GlgP glycolysis; Amn amino acid biosynthesis; From SctN type III secretion; From RpoA and PnP transcription and RNA degradation; From GyrA DNA replication; Rrf is responsible for translation and the structure of the cell wall such as Amib and MOMP[4].

2.1.5. Pathogenesis

C. pneumoniae can infect monocytes, smooth muscle cells, macrophages and mucosal epithelial cells as well as endothelial cells. This feature suggests the possibility that the bacteria could spread systemically. Animal studies with *C. pneumoniae* also support this situation. *C. pneumonia*e attaches to surfaces using glycosaminoglycan, prevents phagosome-lysosome fusion and prevents apoptosis in the host cell.

In animal models of acute lung infection caused by *C. pneumoniae*, in rabbits inoculated intratracheally and intranasally with the bacteria, bronchiolitis and pneumonia developed within one week. Histopathological examination of the lungs of these animals revealed infiltrates consisting of macrophage, lymphocyte, and plasma cells in the interstitium, alveolar space, and bronchial lumen. In addition, *C. pneumoniae*

antigen was found in the spleen, liver, and aortic tissue. In animals, the infection usually heals between 20-28 days[22].

The role of *C. pneumoniae* in the etiopathogenesis of atherosclerosis has been clearly proven, but it is not known exactly by what mechanism it causes damage after it is systemically spread by monocytes and macrophages and settled in the vascular endothelium *C. pneumoniae* can reach the veins through the lower respiratory tract. Infected macrophages move along the mucosal barrier and allow the pathogen to reach the lymphatic system, systemic circulation and atheromas. On the other hand, *C. pneumoniae* can infect many cells commonly found in atheroma, including macrophages, coronary artery endothelial cells ,and aortic smooth muscle cells. It has also been shown that *C. pneumoniae* can affect atheroma biology by controlling the macrophage-lipoprotein relationship. In these conditions, *Chlamydial* lipopolysaccharide (LPS), a strong endotoxin component in the outer surface of Gram-negative bacteria, induces the release of cytokines that promote leukocyte adhesion, leukocyte migration, and internal inflammation. LPS was shown to have a major role in lipid metabolism. LPS mediates the capture of low-density lipoprotein (LDL) cholesterol by *C. pneumoniae*-infected macrophages[23]. Thus, macrophages transform into cholesterol-filled foam cells, which are key cells of newly formed atherosclerotic lesions[2]. Moreover, in vitro studies have shown that upregulated molecules by *C. pneumoniae*, 1-integrins and including -2, adhesion molecules (ICAM-1, VCAM-1), tissue factor (TF), early growth response factor (EGR-1), appear to contribute to these events. Some of these molecules together with matrix metalloproteinases contribute to destabilizing atheroma plaques and thrombus formation. Thus, they cause arterial thromboembolic complications. The pathogenesis of *C. pneumoniae* is summarized in figure 2.2.

2.1.6. Immunity

Much less is known about *C. pneumoniae* immunity than *C. trachomatis* immunity. However, according to the limited number of data, *C. pneumoniae* shares a similar immunobiological paradigm with *C. trachomatis*. *C. pneumoniae* grows inside epithelial cells, like *C. trachomatis*, in a specialized vacuole. However, in tissue culture,

C. pneumoniae has more absolute nutritional requirements and, again different from *C. trachomatis*, it has the ability to spread from cell to cell[24].

C. pneumoniae stimulate both cellular and humoral immune response. Cellular immunity and helper T cell (Th1) response play a major role in infections caused by this bacterium since they are intracellular bacteria[25]. At the same time, cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), interleukin 1 (IL-1), and lymphotoxin have been shown to prevent chlamydial infections. This inhibition is due in part to the activation of the enzyme nitric oxide synthase, which, besides being a cell-signaling molecule, releases nitric oxide (NO), an important antimicrobial and tumoricidal agent.

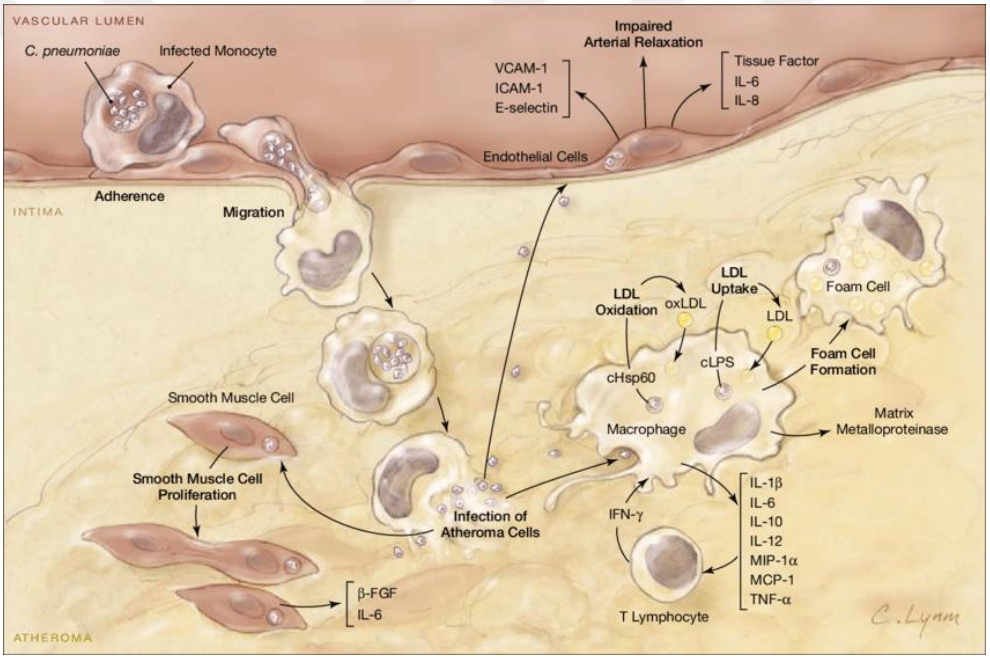

Figure 2. ***C. pneumoniae*** **atherosclerosis pathogenesis**

C. Pneumoniae has also been shown to produce cytokines of this type IFN-, TNF-, IL-1, IL-6, IL-8, IL-12, and intercellular Molecule adhesion-1 in various systems such as alveolar macrophages, human peripheral blood mononuclear cells. *C. pneumoniae* infection suppresses the expression of major tissue matching complex class I (MHC-1) molecules and stimulates the production of anti-inflammatory cytokines such as IL-10[26]. Epithelial cells and peripheral mononuclear cells infected with *C.*

pneumoniae become resistant to apoptosis induced by chemicals or death receptors with IL-10 induction[5].

Bronchial epithelial cells constitute the first step of the defense system during respiratory tract infection caused by *C. pneumoniae*. Epithelial cells on the mucosal surface are known to have the capacity to secrete pro-inflammatory cytokines and chemoattractant, which are important mediators of lung defense against bacterial infection. These properties reveal that epithelial cells can act as an early warning system for regional and inflammatory cells. In addition, these epithelial cells have properties to strengthen the phagocytosis process[27]. However, macrophages can join the circulatory system and spread the infection. The same may be true for T cells. This is an indication that *C. pneumoniae* can use the defense system to its advantage[4].

C. pneumoniae infection produces strong serum antibody response. In some cases, the antibody response that occurs during *C. pneumoniae* infection may be immunopathological. Immune complexes that contain *C. pneumoniae* antigen and antibody and circulate in the blood can be detected in the serum of people with chronic or acute coronary heart disease[28].

It takes approximately 2-3 weeks for IgM antibodies and 6-8 weeks for IgG antibodies to reach a diagnostic titer after the onset of the disease[27]. *C. pneumoniae* infection does not produce an overprotective immune response and re-infection may occur. In case of re-infection, IgM antibodies may not be seen and the IgG antibody level rises rapidly within 1-2 weeks and reaches the level of 1: 512.

Similar to *C. trachomatis* infection, people with *C. pneumoniae* infection usually also experience a lymphoproliferative response[29]. Lymphoproliferative studies of *C. pneumoniae* are largely species-specific. The T cell lines used in these studies have the ability to recognize multiple antigens of the microorganism, including 98, 53, and 32-kDa antigens.

2.1.7. Epidemiology

C. pneumoniae is a common respiratory pathogen in humans and has also been isolated from other animal species such as horses, koalas, and frogs. However, since no zoonotic infection has been reported to date, it is thought that *C. pneumoniae* caused an infection by transmitting from person to person. Transmission by respiratory secretions,

aerosols, and contaminated food is suggested among humans[30]. *C. pneumoniae* can survive for 15-30 hours in non-living environments and for 15 minutes in the skin. Epidemics caused by this factor can occur in military units, nursing homes, and other closed communities.

It is known that *C. pneumoniae* causes different upper and lower respiratory tract infections ranging from asymptomatic infections to sinusitis, pharyngitis, pneumonia, and bronchiolitis. According to many seroprevalence studies, *chlamydia* increases from school age to adolescence and reaches 30-45%. As the age progresses, the rate of antibody detection increases, and this rate increases to 40-95% in the adult population[30].

Community-acquired pneumonia rates associated with *C. pneumoniae* infection in children and adults vary from 0% to 44% according to the geographical region, age group, and the diagnostic method used. While the primary infection is more common in school-age children, recurrent infections are seen in adults. *C. pneumoniae* infection in the elderly has a more severe course and constitutes 32% of pneumonia. It has a fatal course in patients with underlying diseases such as *C. pneumoniae* infection, congestive heart failure, and chronic obstructive pulmonary disease.

The term atypical pneumonia is used to distinguish pneumonia caused by *C. pneumoniae*, *Mycoplasma pneumoniae*, Legionella, and viruses from pneumonia caused by classical type bacteria. Atypical pathogens are responsible for 2-30% of community-acquired pneumonia occurring in adults and children.

2.1.8. Clinic

2.1.8.1. Respiratory Infections

C. pneumoniae is a pathogenic microorganism that primarily settles in the respiratory tract and causes respiratory tract infections. It has been proven that *chlamydia pneumoniae* causes infection asymptomatic, in the studies that were conducted when taking a swab from the nasopharynx of people who did not show any symptoms indicating inflammation[31]. *C. pneumoniae* causes lower and upper respiratory tract infections such as pharyngitis, bronchitis, sinusitis, and pneumonia[24].

There are no specific symptoms and signs of respiratory tract infections caused by *C. pneumoniae*, but many features of the clinical picture can be used to distinguish these infections from others. For *C. pneumoniae* infections, the time from the onset of the disease to admission to the hospital is longer than for other acute respiratory infections.

2.1.8.2. Community-Acquired Pneumonia (CAP)

Numerous serological studies confirm that *C. pneumoniae* causes community-acquired pneumonia in every region of the world. Most cases of *C. pneumoniae pneumonia* are mild and do not require hospitalization. However, even in mild cases, complete recovery is slow despite appropriate antibiotic therapy, cough and weakness may persist for weeks after acute illness. There is evidence that *C. pneumoniae* may cause more serious infections in the presence of concurrent infections and underlying diseases with other bacteria such as pneumococci[32]. Elderly patients, on average, show a more severe clinical course than younger patients. According to studies conducted with hospitalized patients with *C. pneumoniae* pneumonia, most patients have either one or more underlying diseases[24]. In addition, most of the deaths associated with *C. pneumoniae* infection occur in patients with underlying diseases and complications such as *pneumococcal* bacteremia. The disease progresses as epidemics in closed communities.

Early studies emphasized that *C. pneumoniae*-associated pneumonia is clinically similar to weak atypical pneumonia associated with *Mycoplasma pneumoniae (M.pneumoniae)*. However, although *M. pneumoniae* infection is always an acute disease, *C.pneumoniae* exhibits a more chronic. On either hand, *Chlamydia pneumoniae* is more analogous to *Streptococcus pneumoniae* in terms of the patient's clinical symptoms and underlying health conditions.

Chlamydia pneumoniae infection can begin with a sore throat such as *M. pneumoniae* infection; however, unlike this, most of the cases also have hoarseness. Pharyngitis may end before the more typical bronchitis or pneumonia syndrome develops. Cough is very common and lasts long. fever usually disappears. Sinusitis is common with *C. pneumoniae* infection. Pericarditis, myocarditis, and endocarditis may develop.

C. pneumoniae also has chronic respiratory tract carriage after acute respiratory tract infections, and according to culture studies, asymptomatic infection lasts for at least one year[33]. In studies conducted, *C. pneumoniae* could be isolated from the nasopharynx of 4.7% of healthy individuals[34].

2.1.8.3. Acute Bronchitis

Other than pneumoniae, it has been proposed that *C. pneumoniae* causes inflammation in the lower and upper respiratory tracts. In a study conducted, it is reported that *C. pneumoniae* is the trigger factor in approximately 20% of acute bronchitis[35]. The percentage of infection varies depending on the diagnostic procedure used, as in community-acquired pneumonia trials. Most studies are based on serology and the MIF test is used. Polymerase chain reaction and culture methods have been used to detect *chlamydia pneumoniae* in samples taken from patients with bronchitis[34]. As with *C. pneumoniae* pneumonia, there is no characteristic clinical picture. Cough, sore throat, and weakness are seen in patients. In the study conducted by Falck et al., It was stated that the presence of weakness, not cough and, sore throat, was significantly more common in patients with *C. pneumoniae* infection.

The relationship between *C. pneumoniae* and other upper respiratory tract infections such as sinusitis, pharyngitis, otitis media is more ambiguous than the relationship between them and acute bronchitis.

2.1.8.4. Coronary Artery Disease and Atherosclerosis

Coronary artery disease is an indicator of atherosclerosis and referred to as a multi-cause disease. Genetic and environmental factors that cause this disease are specified. Smoking, hypertension, and high cholesterol are among the risk factors. The opinion that *C. pneumoniae* infections are associated with chronic coronary heart and acute myocardial infarction disease was first put forward by Saikku et al in 1988. Later, the same researchers obtained results supporting their first study with the “Helsinki Heart Study[36]”, a large-scale study, and also demonstrated that this relationship was independent of other risk factors that predispose to coronary heart disease. Following these two studies, studies using other tests based on serology, culture, antigen search, and nucleic acid search were conducted to support the obtained data, and it was shown

that *C. pneumoniae* was found in a coronary artery, carotid artery, and aortic atheroma plaques[37]. In studies conducted with animal models, it has been revealed that the atheroma plaque of *C. pneumoniae* also plays a role[38].

2.1.8.5. Neurological Diseases

2.1.8.5.1. Cerebrovascular Disease

After explaining the suspected relationship of *C. pneumoniae* with coronary artery disease and atherosclerosis, it is deemed appropriate to examine its connection with cerebrovascular events with many similar pathological features with these diseases. However, there are far fewer details on these reviews.

Pneumoniae antibodies and cerebrovascular events. As a result of the research, *C. pneumoniae* was associated with chronic infection, increased risk of cerebrovascular events, and transient ischaemic events. Cook ve diğ[39]. reported a stronger association between cerebrovascular accident and transient cerebral ischemia and previous *C. pneumoniae* infection and relapse. In another study, the connection between circulating chlamydial lipopolysaccharide or cytomegalovirus, and the cerebrovascular event was determined[40]. Bucurescu and Stieritz[41] It has been stated that there is a correlation between IgG and IgA antibodies to *C. pneumoniae* and peripheral vascular disease, independent of other diseases such as hypercholesterolemia and diabetes.

2.1.8.5.2. Multiple Sclerosis

A chronic inflammatory condition characterized by demyelination of the central nervous system known as multiple sclerosis (MS). While all signs and symptoms concerning CNS damage can happen in patients with MS, some of them occur very frequently and several are uncommon enough to be considered a red flag. Viruses are often seen as potential agents of MS, but Since 1983, the assumption that C. pneumoniae may play a role in this disease has been emphasized[42]. In a case report, it was reported that *C. pneumoniae* was detected using culture and PCR in the cerebrospinal fluid of an MS patient whose condition improved with antibiotics[12]. The researchers concluded that although the microorganism has the property of

stimulating multiple sclerosis, it may only be a secondary infection of damaged central nervous system tissues.

The presence of chlamydia is not simply explained by the presence of *C.pneumoniae* infected monocytes crossing the blood-brain barrier only. Gieffers Diğ[43]. *chlamydia* were cleared from the blood but continued to exist in the central nervous system.

Although several studies could not detect *C. pneumoniae* in brain tissue or cerebrospinal fluid, *C. pneumoniae* is present in patients with neurological disorders and this is not limited to MS patients[44].

2.9. Microbiological Diagnosis

Diagnosis of *chlamydia pneumoniae* depends on several tests, which are serological examinations, methods of searching for DNA, isolation of organisms, and immunochemical methods of antigen in bacteria. These technologies are mainly used in research and are applied in specialized laboratories. Therefore, the routine diagnosis of *Chlamydia pneumoniae* in the clinic is based on serological tests that detect immunoglobulin G (IgG), IgA and IgM antibodies.

2.9.1. Sample Collection, Transport, Storage and Processing

Since *chlamydias* are obligate intracellular pathogens, the purpose of patient sampling is; It should be the collection of host cells containing microorganisms. when extracting bacteria from the host must be diagnosed immediately because short-lived without a host.

C. pneumoniae can be isolated from respiratory tract specimens (throat culture and nasopharyngeal, bronchoalveolar lavage [BAL] and tissue (blood-vessel and lung) biopsies. The microorganism can also be isolated from sputum, but sputum is toxic to cell culture and is often contaminated with other bacterial and fungal species. The nasopharynx is the optimal site for microorganism isolation.

Samples collected should be stored in a suitable transport medium optimized for chlamydia. 2-sucrose-phosphate (2-SP) transport medium should be used for swab samples. Samples must be delivered to the laboratory within 24 hours. Antibiotics such

as tetracycline, macrolide, and penicillin, which are effective against chlamydia, should not be added to the transport media.

For the successful culture of chlamydia, the time between sampling and processing should be very short. Samples to be processed within 24 hours should be kept in the refrigerator at 4 ° C and transported in wet ice. Samples that will not be processed within 24 hours must be frozen at -70 ° C after being kept at 4 ° C. It is not suitable to store the cultured sample at -20 C or in the freezer part of no-frost refrigerators.

In the processing of samples for culture; The swab samples are voided for 15-20 seconds in the transport medium and the tip of the swab is pressed against the side of the tube and squeezed in order to obtain all the liquid. An average of 200-1000 µl of sample is used for inoculation. This amount of sample is resuspended in the culture medium after being centrifuged at 8000-10,000g. In addition, prior to centrifugation and inoculation, samples must be treated with sterile glass beads or sonication to disrupt potential chlamydia-containing cells within them.

For the polymerase chain reaction, the specific recommendations for collecting, handling, and processing clinical specimens are the same as those described for culture. 1 mL sample taken from transport medium at 18,000g for 15 minutes. Centrifuge and the precipitate are used for DNA purification. Tissue fragments should be cut into small pieces (25 mg) before DNA purification. Samples, controls, and materials used in the PCR mix should be handled in different environments and with reliable pipettes to avoid contamination. The use of barrier pipette tips, lab coats, and gloves is highly recommended. Work areas and instruments should be checked routinely to monitor DNA contamination.

Historically, chlamydia was cultured in the yolk. The yolk method is still used to prepare antigens in the application of the MIF method.

In all of the cultural practices accepted in recent studies, for example, centrifugation, inoculation into a single layer human cell line is performed. Respiratory specimens need to be cultured by them twice more after primary isolation procedures. In tissue samples, it would be appropriate to add an extra 4-6 passages.

2.9.2. Serology

Many types of serological tests are commercially available today for use in detecting antibodies against *C. pneumoniae*. The most commonly used serological tests can be listed as MIF test and EIA (Enzyme Immune Assays) that detect IgM, IgA, IgG or total antibodies with family, species or serotype specificity, and complement fixation (CF). Serological tests are one of the most useful methods of determining the cause of an epidemic and the extent of infection in epidemiological studies. Among the serological tests used for the diagnosis of *C. pneumoniae*, although it has not been adequately standardized, the most widely used test used by the CDC is the MIF test . MIF is a specific and sensitive test that does not contain genus-specific antigens, but only antigens belonging to the species and serovars between the species (2). The specificity of the MIF test may be related to the use of purified MOMPs of all three types of chlamydia, rather than LPSs containing predominantly genus-specific epitopes. In this test, EBs of *C. pneumoniae*, *C. trachomatis*, and C.psittaci that are purified, formalin-treated, and fixed as antigens on separate points on glass slides are used.

Developed by Wang and Grayston in the early 1970s, the MIF test allows quantitative determination of IgG and IgM antibodies that can help in the differentiation of current and past infections. Grayston and diğ. suggested a number of criteria for the diagnosis of *C. pneumoniae* infection with the MIF test used by many clinicians and laboratories. For acute infection, the patient should have a four-fold increase in IgG titer, a single IgM titer should be 1/16 and above, or a single IgG titer should be 1/512 and above.

One of the most controversial issues regarding the serological diagnosis of pneumoniae is the definition of patients with persistent and chronic infections. Usually, the presence of constantly increasing IgG or IgA antibodies is used for this. As an indicator of chronic *C. pneumoniae* infection, it is recommended to use a high IgA titer instead of an IgG titer, since the half-life of IgA is 2-7 weeks and the half-life of IgG is months. However, there is still no approved serological indicator of persistent or chronic infection.

2.9.3. Polymerase Chain Reaction

The polymerase chain reaction (PCR) method; is a method that allows early and rapid identification of *C. pneumoniae* infections. Numerous PCR protocols targeting different genes and using different formats have been developed in many research laboratories for the diagnosis of *C. pneumoniae* in both respiratory and non-respiratory samples.

Nucleic acid-based amplification techniques can detect *C. pneumoniae* in many clinical samples, from respiratory tract samples to vascular tissue, serum, and peripheral blood mononuclear cell samples. The performance of these tests in identification varies between different laboratories. The reason for this variation may be related to differences between the clinical samples used, sample processing methods, primer design, nucleic acid purification methods, detection of the amplification product, or prevention and detection procedures for false-positive and false-negative results. Each PCR type has its own advantages and disadvantages, and these should be taken into account when developing or evaluating new tests.

Purification of *C. pn*eumoniae DNA from clinical samples before PCR should be done using reliable and efficient protocols. Positive and negative controls should also be used in parallel with clinical samples during all procedures from extraction to detection. Positive controls should contain a very small amount of DNA (less than 10 ng) and should be kept aliquoted in small amounts.

2.10. Treatment

Tetracyclines, macrolides, fluoroquinolones, and rifampin are antibiotics commonly used in the treatment of *chlamydia* infections. New macrolides such as doxycycline, azithromycin, erythromycin, levofloxacin and clarithromycin, and roxithromycin are recommended in the treatment of *C. pneumoniae*, but their use has been limited due to the few clinical studies supporting the efficacy of these antibiotics Successful results obtained with empirically selected antibiotics such as oral telithromycin and moxifloxacin have been reported in CAP cases including *C. pneumoniae.*

Drug resistance to recommended antimicrobial agents is rare in chlamydia but is also of concern. Because a few cases have been reported for *C. pneumoniae*.

In vitro, *chlamydial* resistance to fluoroquinolones, macrolides, tetracyclines, and rifampin can be induced by a large number of organisms cultured in the presence of antimicrobials. In an animal model, *C. pneumonia*e persistence has been demonstrated after antimicrobial therapy.

3. MATERIAL and METHOD

3.1. Material

Several chemicals and kits are used throughout the experiment. Kits, tools, and chemicals are purchased from different resources and different manufactures. Sterilized tips and tubes were used for sample preparation, antibiotic susceptibility tests, and PCR work.

Table 1. List of chemicals and kits

Name of chemicals and kits	Manufacturer	Aim of use
DNasey PowerSoil kit handbook	**Qiagen® Germany**	**For DNA extraction**
***C. pneumoniae* primers**	**Sentebiolab® Germany**	**For amplifying target genes**
PCR Master Mix		**PCR**
Yellow pipette tip		**PCR**
Protedinase K	**SIGMA® USA**	**Digestion of protein**
IFAT Kit		**To detect IgG and IgM**
Agarose	**Genaxxon bioseience GmbH® Germany**	**Used to separate DNA, RNA, or protein**
***H. pylori* primers**	**Sentebiolab Türkiye**	**For amplifying target genes**

3.2. Method

The aim of this study was to investigate the possible relationship of *C. pneumoniae* and *H. pylori* with atherosclerosis seen in the cardiovascular system. In this study, endarterectomy specimens of 11 patients, 4 of whom were female, who applied to cardiovascular surgery outpatient clinics with clinical complaints such as acute

myocardial infarction and coronary heart disease, and who received surgical indication, were included (Table.1)

Table 2. Age and gender distribution of patients.

Number	Gender	Age	Samples
1	Female	60	Endarterectomy specimens
2	Female	55	Endarterectomy specimens
3	Female	54	Endarterectomy specimens
4	Male	62	Endarterectomy specimens
5	Male	58	Endarterectomy specimens
6	Male	57	Endarterectomy specimens
7	Male	54	Endarterectomy specimens
8	Male	59	Endarterectomy specimens
9	Male	63	Endarterectomy specimens
10	Male	56	Endarterectomy specimens
11	Female	60	Endarterectomy specimens

3.3. DNA Extraction for *C. pneumoniae*

1. Add 0.25 g of soil sample to the PowerBead Tube provided. Gently vortex to mix.
2. Add 60 µl of Solution C1 and invert several times or vortex briefly.
3. Secure PowerBead Tubes horizontally using a Vortex Adapter for 24 (1.5–2.0 ml) tubes.
4. Vortex at maximum speed for 10 min.
5. Centrifuge tubes at 10,000 x g for 30 s.
6. Add 250 µl of Solution C2 and vortex for 5 s. Incubate at 2–8°C for 5 min.
7. Centrifuge the tubes for 1 min at 10,000 x g.
8. Avoiding the pellet, transfer up to 600 µl of supernatant to a clean 2 ml Collection Tube.
9. Add 200 µl of Solution C3 and vortex briefly. Incubate at 2–8°C for 5 min.
10. Centrifuge the tubes for 1 min at 10,000 x g.

11. Avoiding the pellet, transfer up to 750 µl of supernatant to a clean 2 ml Collection Tube.
12. Shake to mix Solution C4 and add 1200 µl to the supernatant. Vortex for 5 s.
13. Load 675 µl onto an MB Spin Column and centrifuge at 10,000 x g for 1 min. Discard flow-through.
14. Repeat step 14 twice, until all of the sample has been processed.
15. Add 500 µl of Solution C5. Centrifuge for 30 s at 10,000 x g.
16. Discard the flow-through. Centrifuge again for 1 min at 10,000 x g.
17. Carefully place the MB Spin Column into a clean 2 ml Collection Tube. Avoid splashing any Solution C5 onto the column.
18. Add 100 µl of Solution C6 to the center of the white filter membrane. Alternatively, you can use sterile DNA-free PCR-grade water for this step.
19. Centrifuge at room temperature for 30 s at 10,000 x g. Discard the MB Spin Column. The DNA is now ready for downstream applications.
20. Centrifuge at room temperature for 30 s at 10,000 x g. Discard the MB Spin Column. The DNA is now ready for downstream applications.

3.4. *C. pneumoniae* HL-1 ~ HR-1 gene amplification

The isolated DNA was subsequently analyzed by a two-phase PCR procedure. the objective is to support the content of a specific template. The DNA was amplified in 25-ml volumes containing 0.5mM HL-1 ~ HR-1 primer, 2.5 mM MgCl2, 100 mM nucleotides, standard PCR buffer, 1 U of Taq polymerase, and 2.5 ml of the sample.

Table 3. Primers used to amplify and sequence

Gene	Primer	Sequence (5′→3′)	Amplicon size (bp)
HL-1 ~ HR-1	F	TGC ATA ACC TAG GTG T	455.0
	R	TGCATAACCTACGGT GTG TT	438.7

Nested-PCR: is a technique that reduces nonspecific amplification of the DNA template. It is performed by two successive PCRs. The first reaction is performed with primers that cover the target sequence and some additional sequence flanking both ends

of the target sequence. After the first reaction, a second reaction is performed on the products of the first PCR with primers that bind to the target sequence and are within the amplified sequence of the first PCR. This reduces the amount of nonspecific binding because, in the second reaction, most of the amplicons of the first reaction only contain the target sequence and its surrounding sequences**.**

Table 4. **The first phase in the PCR for *C. pneumoniae***

Primer	Stages		Cycles
HL-1 ~ HR-1	**Initial Denaturation**	**94 °C for 2 min**	**1**
	Denaturation	**94 °C for 1 min**	**15**
	Annealing	**64 °C for 1.5 min**	
	Extension	**72 °C for 1 min**	
	Extension	**72 °C for 5 min**	**1**

Table 5. **The second phase in the PCR for *C. pneumoniae***

Primer	Stages		Cycles
HL-1 ~ HR-1	**Initial denaturation**	**94 °C for 2 min**	**1**
	Denaturation	**94 °C for 1 min**	**25**
	Annealing	**64 °C for 1.5 min**	
	Extension	**72 °C for 1 min**	
	Extension	**72 °C for 5 min**	**1**

Figure 3. Applied Biosystems Thermal cycler PCR

The Applied Biosystems StepOne ™ real-time PCR system (StepOne ™ system) is based on the principle of polymerase chain reaction (PCR).
PCR reagents based on the principle of fluorescence to ensure:

- Quantitative detection of target nucleic acid sequences (targets) by real-time analysis.
- Qualitative detection of target nucleic acid sequences (targets) through

end-point analysis and fusion curve analysis(biocompare.com).

3.5. DNA Extraction for *H. pylori*

The following method was used to extract DNA from endarterectomy biopsies. The biopsies samples were crushed and were added to a test tube containing 240µl D/W. Followed by the addition of 20-30µl 20%SDS, then 80µl PK buffer, and then 40µl proteinase K was added to each test tube. All the contents were mixed well. After mixing all the test tubes were incubated at 55 overnight if it was a biopsy and for 1 hour if it was a culture. After incubation, all the test tubes were placed at room temperature. Then to each test tube 100µl, 6M NaCl were added and mixed well. After mixing all the

test tubes were centrifuged for 1 minute at 14,000 g. After centrifugation, the supernatant was separated into a separate tube. To the supernatant 1ml, 100% chilled ethanol was added and centrifuged as mentioned above. The supernatant was discarded. Then 1ml 70% ethanol was added and centrifuged as mentioned above. The supernatant was discarded and the mixture was suspended in 100µl TE buffer.

3.6. *H. pylori* UreC gene amplification

Preparation of PCR Mix PCR mixture was prepared as follows. To each test tube 14.7µl Nuclease free water was added. Followed by the addition of 2.5µl PCR Buffer, 1.0µl MgCl2 and 0.5µl dNTPs. Than to each test tube 0.5µl forward primer and 0.5µl reversed primer were added. Finally 0.3µl Taq pol and 3.5µl of DNA sample were added to each test tube.

Table 6. Primers for UreC (glmM) gene amplification of *H. Pylori.*

Gene	Primer	Sequence (5′→3′)	Amplicon size (bp)
UreC (glmM) gene	F	59-AAGCTTTTAGGGGTGTTAGGGGTTT-39	294 bp
	R	59-AAGCTTACTTTCTAACACTAACGC-39	

Table 7. The PCR for *H. pylori*

Primer	Stages	Cycles
UreC (glmM) gene	**Initial denaturation 95 °C for 5 min**	**1**
	Denaturation 95 °C for 30 min **Annealing 55 °C for 30 min** **Extension 72 °C for 30 min**	**35**
	Extension 72 °C for 10 min	**1**

3.7. Gel Electrophoresis

Agarose electrophoresis is an important technique in genomic research. It separates biological molecules based on their size and charge. Influence of the negatively charged DNA molecules migrate towards the positive charge under the

constant current, also the separation depends on the mass and charge of DNA. The DNA molecules are forced to move through the agarose gel pores (geneticeducation.co.in).

- 10X TAE 48.4g of Tris Base and 3.72g of EDTA (disodium salt) was dissolved in sterile distilled water, and 11.4 ml of Glacial acetic acid was added and completed to a final volume of 1 liter. The buffer was diluted 10 times and 1X TAE buffer was used for running gel.
- 10X TBE buffer 108g of Tris base, 55g of Boric Acid and 7.5 g of EDTA was dissolved in 800 ml of sterile distilled water and completed to 1 liter. The pH was adjusted to 8.0 and the solution was sterilized. The buffer was diluted 10 times and 1X TBE buffer was used for running gel.

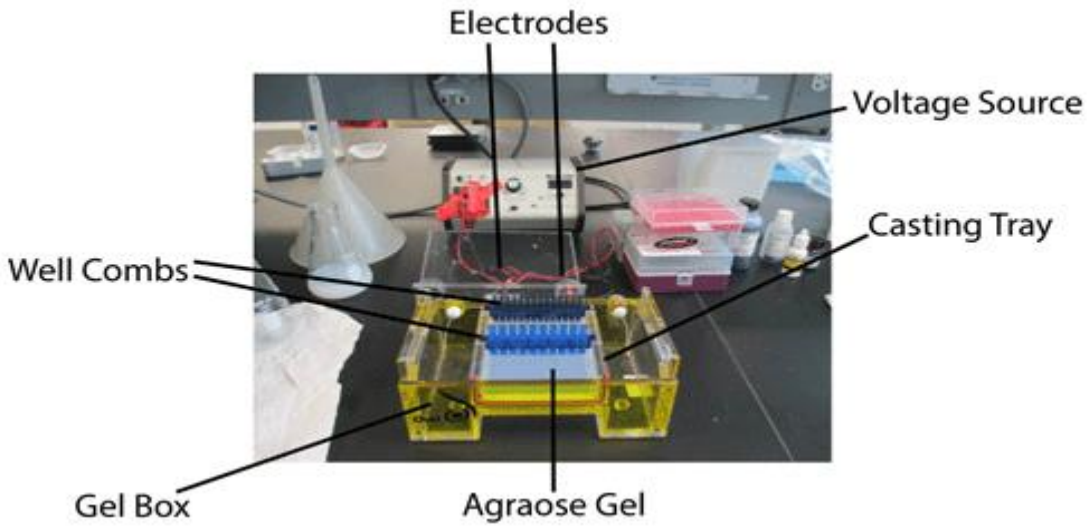

Figure 4. Agarose Electrophoresis (www.Labcompare.com)

3.8. IFAT of *C. pneumoniae* (IgM and IgG)

Principle of the test*:*

The IFA method is based upon the reaction of antibodies in the sample, tested with the antigen adsorbed on the slide surface. The specific antibodies present in the sample react with the antigen, and the immunoglobulins not bound to the antigen are removed in the washing step. In the next step, the antigen-antibody complexes react with the fluorescein-labeled antihuman globulin. It was examined using an immunofluorescence microscope. Positive and negative controls were included in each

test run. The observed fluorescence pattern was apple-green fluorescence of coco-bacillar morphology for positive samples and no fluorescence for negative ones.

Assay Procedure:

1. All reagents were brought to room temperature before use. The slides were allowed to reach room temperature before opening.
2. A 1/2 dilution of samples were prepared by adding 25 µl of sample to 25 µl of PBS. The control sera 3 and 4 were not diluted.
3. Samples were diluted with anti-human IgG sorbent 7, by adding 5 µl of diluted samples to 25 µl of sorbent and thoroughly mixed. Control sera 3 and 4 were not diluted nor sorbent treated. The treated samples used directly or centrifuged to remove the precipitate, which does not interfere with the test.
4. Totally 5 µl of the sorbent-treated sample were added in each of the 3 wells of every column of slide 1. The same was done with positive 3 and negative 4 control.
5. The slide was placed in a humid chamber and incubate at 37ºC for 90 minutes.
6. Slide 1 briefly rinsed with a gentle stream of PBS 2 (avoid directing PBS at wells) and immerse for ten minutes in PBS. Slides were dip washed briefly in distilled water.
7. Slide 1 was allowed to air dry.
8. Then 5 µl of anti-human IgM (anti-human IgG for IgG detection) FITC conjugate solution 5M were added to each well. (No dilution required).
9. The slide was incubated in a humid chamber for 30 minutes at 37ºC.
10. Steps 6 and 7 were repeated.
11. A small drop of mounting medium 6 added to each well and carefully cover with a coverslip.
12. The slide was read as soon as possible in a fluorescence microscope at 400x magnification. If this was not possible, stored in the dark at 2-8ºC up no more than 24 hours, until observation.

4. RESULTS

The atherosclerotic material is taken from 11 patients by surgical endarterectomy and examined by indirect immunofluorescence (IFA) test and polymerase chain reaction (PCR). *C. pneumoniae* positivity was 72.7 % (8/11) by IFA and 63.6 % (7/11) by PCR while the evaluation of the methods together yields a positivity of 87.5 % (7/8)

Table 8. ***C. pnuemoniae*** **IFAT Results**

Number	**Gender**	**Age**	***C. pneumoniae*** **PCR**	***C. pneumoniae IFAT (IgM)***	***C. pneumoniae IFAT (IgG)***
1	Female	60	(+)	(1/8)	(1/32)
2	Female	55	(+)	(1/4)	(1/8)
3	Female	54	(+)	(1/16)	(1/32)
4	Female	60	(-)	0	(1/64)
5	Male	62	(+)	(1/4)	(1/64)
6	Male	58	(+)	(1/4)	(1/128)
7	Male	57	(-)	(1/4)	(1/32)
8	Male	54	(+)	(1/8)	(1/32)
9	Male	59	(-)	(0)	(1/128)
10	Male	63	(+)	(1/8)	(1/32)
11	Male	56	(-)	0	(1/4)

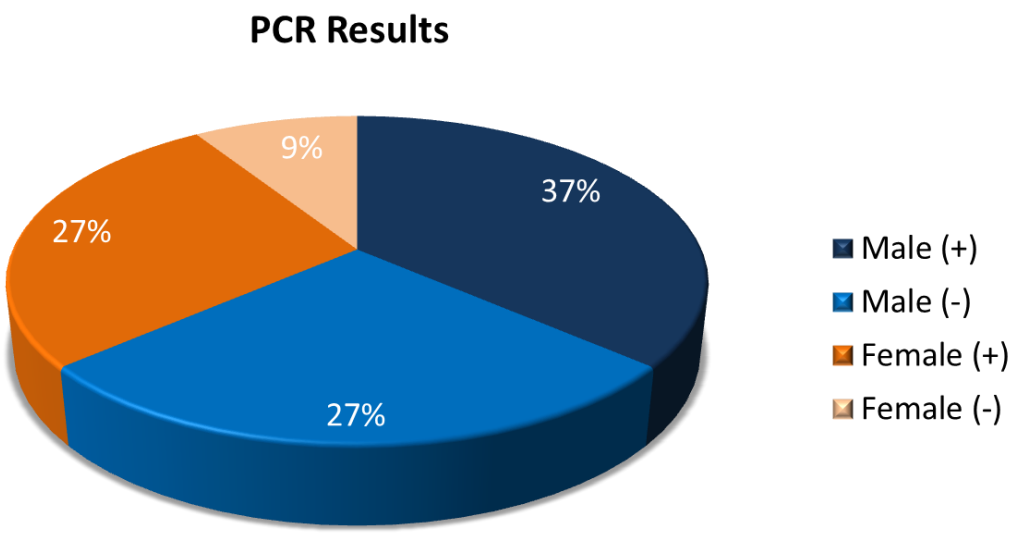

Figure 5. The percentage of PCR Result

As a result of the *H. pylori* ureC gene targeted PCR amplification, only 1 (9.1%) sample were found positive in atheroma plaques.

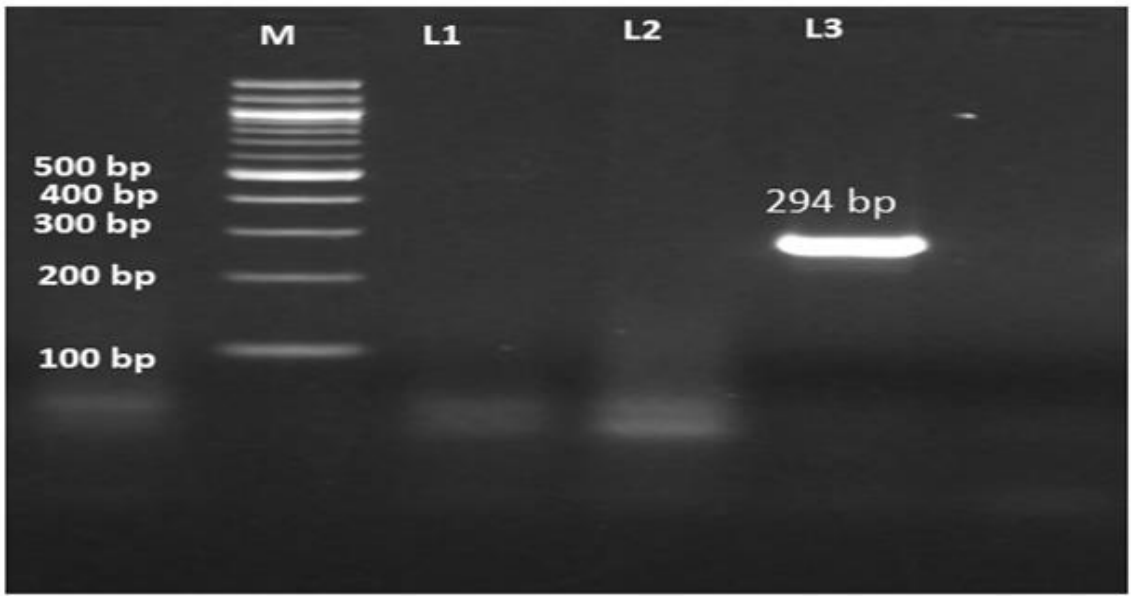

Figure 6. Detection of *H. pylori* UreCgene by PCR method

Gel photograph showing specific detection of *H. pylori* UreCgene by PCR method and examined by gel electrophoresis in biopsy specimens. Paraline (L3) 3 indicated amplify product of 294bp while the rest are negative.

As a result of the *C. pneumoniae* gene targeted PCR amplification, only 7 (63.6%) samples were found positive in atheroma plaques.

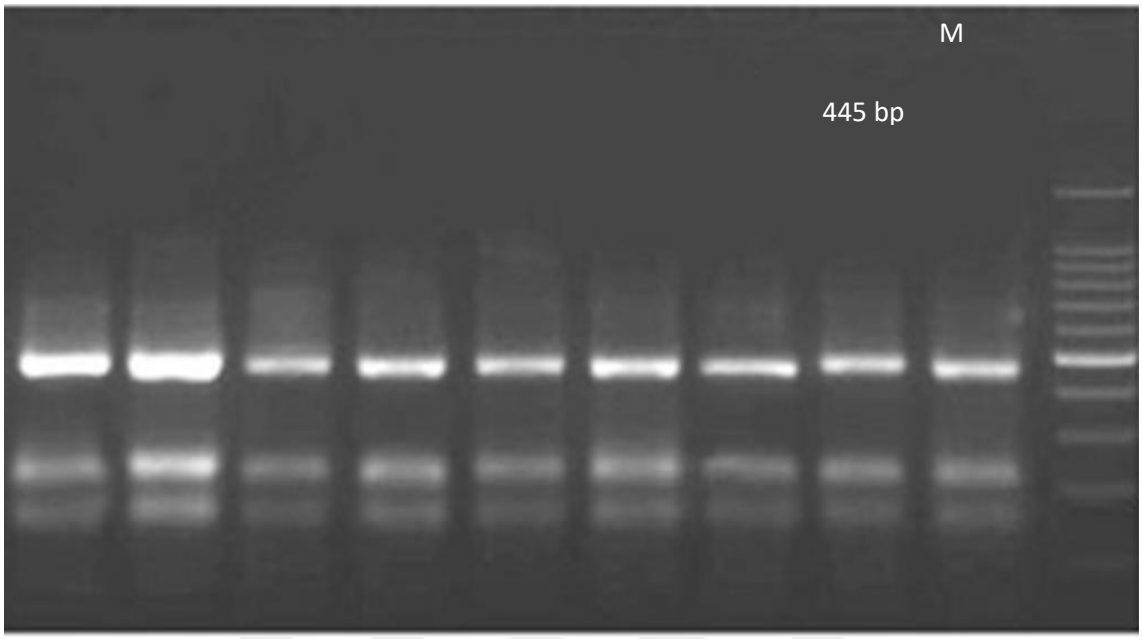

Figure 7. Detection of *C. pneumoniae* by PCR method

5. DİSCUSSİON

In the study of Melanıe C. et al.[45], In which they evaluation samples belonging to 18 normal persons with 29 atheroma in Avustria in order to determine the relationship between *C. pneumoniae*, and formation of atheroma plaques; While they detected PCR positivity in 2 '(6.9%) of the atheroma plaques, they could not detect PCR positivity in normal vessel samples. They detected positive antibody response to *C. pneumoniae* with IFAT in the serum samples of 15(51.7%) of 29 patients with atheroma plaques. reported that only one of the seropositive patients had PCR positivity. On the other hand, they reported that only 3 (20%) of people with normal tissue samples had seropositivity with IFAT. As a result; They stated that there is a relationship between *C. pneumoniae* and atheroma plaque formation, but the results obtained with IFAT are more important because PCR has low sensitivity in determining the relationship.

In the study of Francesco B. et al[46], They evaluated 51 samples for atherosclerosis in Milan, Italy in order to detect *Chlamydia pneumoniae* in Atherosclerotic Plaques.Their results showed 26(51%) of 51 samples were positive in molecular screening. Moreover, they did not detect *H. pylori* DNA that means it does not involve atherosclerotic plaques of the aortic. These findings suggested a possible role for *C. pneumoniae* chronic infection and the development of aneurysmal lesions and ruled out the possibility of a direct involvement of *H. pylori* infection in the pathogenesis of atherosclerosis.

Community-acquired pneumonia (CAP) is an acute infection of the pulmonary parenchyma that occurs during daily life. Community-acquired pneumoniae are responsible for a significant portion of physician applications, treatment expenses, work-school days, and deaths worldwide. These infections have become a major health problem worldwide. Pneumoniae ranks 6th among causes of death in England and USA; It ranks first among deaths due to infections. Pneumoniae is an infection of the lower respiratory tract that affects the great majority of people in Turkey. T.R. According to the final report published in December 2004 and explaining the results of the national disease burden and the cost-effective project carried out by the Ministry of Health, Refik Saydam Hygiene Center Presidency and Başkent University in recent years; lower respiratory tract infections in Turkey, it ranks 5th among the leading causes of

death[47]. In the past decade, admissions to hospitals for CAP have increased by 34%, especially among adults.

Many types of microorganisms, including bacterial, fungal, viral, and parasitic agents, can cause community-acquired pneumonia. In studies conducted abroad with CAP cases since 2000, it has been observed that the etiological agent detection rates vary between 31-86%. When studies with a high rate of etiological agent detection are examined, it is noteworthy that unlike others, invasive and sterile samples were used for culture or PCR tests in these studies[48][49][50]. Saito et al[51]. detected an etiological agent at a rate of 73.3% by using culture and PCR methods in invasive samples taken from CAP patients[52]. However, there are many studies in which factors could not be detected at a high rate, and on average, the agent was detected in 50% of CAP cases. There are many factors affecting this ratio. These factors may be the effect of previous antimicrobial therapy, the presence of unidentified and rare pathogens or viral infections, or pathogens that have not yet been identified. Taken Studies involving patients with CAP performed in Turkey and an average detection rate of the etiologic agent has been shown that hovered around 21 to 62.8%[53]. Prospective eight university hospitals in Turkey with the participation of the general, in a multicenter study, the etiologic agent could be detected in 62.8% of cases. In community-acquired pneumoniae cases, even if the etiological agent can be identified in addition to the identification of such a low rate of etiological agents, culture, and agent determination studies require a certain amount of time. In addition, delay in pneumoniae treatment increases morbidity and mortality[54]. Therefore, the pathogen must be determined in a short period. In order to determine the most appropriate empirical treatment regimen for patients, it is very important to have data on etiological agents and diagnostic criteria throughout the country and to create specific diagnosis and treatment guidelines. This situation will both increase the success of the treatment of the clinician, reduce the hospitalization time of the patients, decrease the treatment cost and mortality rate. In addition, it will be avoided to select drug-resistant strains as a result of unnecessary use of antibiotics at unnecessarily high or low doses or in inappropriate time and combination[1].

It is important to divide community-acquired pneumonia patients into two as typical and atypical in terms of providing appropriate empirical treatment. Atypical

pneumonia is an infectious disease mostly characterized by prodromal symptoms such as fever, weakness, headache, cough with dry or mucoid sputum, and wheezing in young people. These are pneumoniae that usually show non-lobar involvement radiologically, often have an inconsistency between physical examination and radiological findings, and symptoms and signs of extrapulmonary-systemic organ involvement can be seen. The main atypical pneumonia agents are *Mycoplasma pneumoniae*, *Legionella pneumophila*, *Chlamydophila pneumoniae*, and viruses. There is no reliable clinical indication for clinicians to differentiate atypical pneumoniae from typical pneumoniae[55].

Many studies support that bacterial and viral atypical pneumoniae agents are etiological agents in a considerable part of CAP cases. In the studies of Ngeow et al. [56]published in 2005, 493 children and 1263 adult CAP patients were included. They investigated the prevalence of atypical pneumoniae agents with the serological and molecular methods they used. MIF and PCR tests were used for *C. pneumoniae*. As a result of the study, atypical pneumoniae agents were found with a rate of 23.5% [56]. Lui et al. [55] found this rate as 28.6% in their study with 1193 adult CAP patients.

Efforts to determine the causes of atypical pneumoniae have been made in Turkey. In the serological examination for atypical factors in community-acquired lower respiratory tract infections, positivity was found in 43.4% of the cases in the Chest Diseases Clinic of Karadeniz Technical University Faculty of Medicine. In this study, *C. pneumoniae* was detected in 17% and more than one agent was isolated in 7.5% of cases[57]. Upon thinking of an atypical pneumoniae epidemic in a private institution in Istanbul, 49 cases were diagnosed with pneumoniae in the examination of 408 personnel by Çapa Medical Faculty, and *M. pneumoniae*, *C. pneumoniae*, and Legionella serology were studied in these patients. As a result of the examination, *M. pneumoniae* was found in 70.6% of the cases, *C. pneumoniae* in 17.6%, and both (mixed infection) in 11.8%[57]. Erdem et al.[58] of the 2009 Infectious Diseases and clinic microbiyology Journal of the issued and they are 14 regional studies in their research to examine, atypical pneumoniae in Turkey has indicated that accounts for 29% of all pneumoniae. Another study conducted in Turkey on this issue published in Microbiology Spring ~ al in Bulletin in 2004 and his work. In this study, 24.6% of

atypical pneumoniae agents were found in 65 adult pneumoniae patients by serological methods (ELISA, IFA) [59].

This study, it is aimed to determine the frequency of detection of *C. pneumoniae*, which is one of the most common agents in atypical pneumoniae infections, which includes a significant rate in CAP. Serological (ELISA and MIF) and molecular (PCR) of this factor by methods adults by determining the prevalence of patients with CAP, arranging the empirical treatment in Turkey and aimed to contribute to the studies for the collection of epidemiological data. Due to continuing to be a major health problem in Turkiye and in the world of community-acquired pneumoniae, Turkey is expected to contribute significantly to the identification of data in general.

Zaki and Goda [48]reported that atypical 22 had diabetes mellitus, 20 had COPD, 10% had chronic liver disease and 10% had congestive heart failure. In the studies published by Özlü et al. [53]in the Journal of Tuberculosis and Thorax, it was stated that there were underlying diseases, especially COPD, at rates varying between 33.3-54% in CAP cases. Fidan et al. [60]stated in the study they published in the Thorax Journal in 2005 that 57.8% of CAP cases treated in an inpatient state had at least one risk factor. Risk factors in this study; 65 years and older (38.5%) and underlying disease (37.8%) [60]. In this study, 54% of 50 CAP patients are 65 years and older. In 58% of this patient group, at least one underlying disease was determined. COPD was defined in 38% of the patients, diabetes mellitus in 31%, hypertension in 45%, and congestive heart failure in 13.7%.

Wu et al., The role of *C. pneumoniae* was investigated in 209 CAP patients, 92% of the patients had fever and 52% had cough symptoms. 130 CAP patients were prospectively studied at Çukurova University and 75% of these patients had fever (37.8 ° C and above), cough in 75%, sputum in 50%, pleuritic pain in 21%, and headache in 15%.

C.pneumoniae is a pathogenic bacterium that was first isolated 60 years ago, are an obligate intracellular parasite and its life cycle consists of two different phases. *C. pneumoniae* is associated with many different acute and chronic diseases. Diseases have a wide clinical spectrum, ranging from asymptomatic infections to acute bronchitis, pneumoniae, atherosclerosis, asthma, cerebrovascular accident and Alzheimer's. Community-acquired pneumoniae is one of the leading infections caused by

C.pneumoniae. Saito et al. [51]determined *C. pneumoniae* as the 3rd most common factor in CAP patients over 65 years of age, as a result of their study.

Since 2000, nine of 24 studies in which the rate of CAP cases responsible for *C. pneumoniae*. According to these studies, *C. pneumoniae* in children 0.9-12.4%[61], in adults 0.9-21.1%[62][63], and 2.2-22.5% in both groups[56]. The role of *C. pneumoniae* in respiratory tract infections were seen in Turkey investigated 15 studies. Five of these studies were conducted with patients with lower respiratory tract or respiratory tract infections, nine with CAP, and one study included 14 regional studies. Results of these studies.

Although many methods are used to detect *C. pneumoniae* in clinical samples, it is very difficult to detect due to the lack of a standardized and approved diagnostic method[39]. In addition, it is difficult to decide whether the infection is primary, reinfection, chronic persistent or past infection. Culture from the past, *C. pneumoniae* Although it is accepted as the gold standard in its diagnosis, the culture process is not used as a routine diagnostic method because the growth of this microorganism takes weeks and the method is very complex in technical terms. In addition, the sensitivity of the culture is considered to be low due to the death of many *chlamydia* during transport or storage of the sample. For these reasons, when it comes to the differential diagnosis of respiratory tract infections caused by *C. pneumoniae*, PCR and serological methods provide better performance and faster diagnosis.

Serological tests have been the most widely used methods in the diagnosis of infections caused by *C. pneumoniae*. Since reference standards of some serological tests used in the diagnosis of chronic or persistent *chlamydia* infections are not defined, it is difficult to estimate the diagnostic value of these tests. General problems encountered in *chlamydial* serological diagnosis; Difficulties in collecting duplicate serum samples include the high seroprevalence of *C. pneumoniae* in adults, the lack of good quality reagent, and the lack of standardized species-specific test methods. Large surface-associated macromolecules, including MOMP, OmcB, and LPS, can induce a strong antibody response in infected individuals. However, because these molecules share common epitopes in chlamydia species, they can induce a cross-reactive antibody response in infected individuals.

The microimmunofluorescence (MIF) test is the most preferred test for the serological diagnosis of *C. pneumoniae*. The results of this test differ between laboratories as it is evaluated subjectively, is technically difficult, and the reagents are not standardized. Littman et al[64]. and Peeling et al[65]. demonstrated this difference between laboratories with their studies. Microimmunofluorescence test results may vary depending on the reagents, antigens used, incubation time, temperature and the experience of the technicians. In addition, this test requires fluorescence microscopy experience[64].

MIF test was used in 12 (80%) of 15 studies published in the world since 2000 where *C. pneumoniae* was shown as an etiological agent in adult patients with CAP[66]. Chedid et al in Brazil[67]. investigated the frequencies of *C. pneumoniae* IgG and IgM antibodies using MIF method in 58 adult patients with CAP in their study. Detecting a four-fold increase in *C. pneumoni*ae IgG and IgM antibody titers in paired serum samples was accepted as the criteria for determining acute *C. pneumoniae* infection. Passed infection was defined by the IgG titer higher than 1/16. At the end of the study, 63.8% *C. pneumoniae* seropositivity was detected. 60.3% of the cases with seropositivity are patients with IgG antibodies and 3.5% with IgM antibodies. In 39.6% of seropositive patients, past *C. pneumoniae* infection was found. The study conducted by Wellinghausen et al. In Germany was conducted with 546 adult CAP patients. Microbiological determination of *C. pneumoniae* was made using MIF and PCR methods. IgG, IgM and IgA antibodies against the agent were investigated by microimmunofluorescence test. As a result of this study, the PCR test detected 4.6%, the MIF-IgG test showing the previous infection 70.8%, and the MIF-IgA test found the *C. pneumoniae* at the rate of 19.7%[67].

In the study conducted by Ngeow et al[62]. In which many countries in Asia were included, samples of 493 children and 1263 adult patients with CAP were examined. *C. pneumoniae* IgG antibody levels were determined by performing MIF test with acute and convalescent serum samples. A separate PCR test was performed to show the *C. pneumoniae* infection. As a result of the microimmunofluorescence IgG test, 4.3% *C. pneumoniae* infection was detected. When these results were combined with the PCR results, the incidence of *C. pneumoniae* was found to be 4.7%. For this

reason, the researchers pointed out that when only the PCR test results were taken into account, the rate of agent determination decreased.

C. pneumoniae in adult patients with CAP Turkey 's been found in a study of prevalence as active as determined by the MIF test. Somer et al. At Istanbul University. They detected the *C. pneumoniae* factor by studying MIF IgG and IgM tests in dual serum samples of pediatric patients with CAP. As a result of this study, they identified 5% acute *C. pneumoniae* infection[68].

At the end of our study, 9 (81%) of our patient group whose mean age was 58, IFAT, and anti-*C. pneumuniae* antibody response was> 1/16, 7 (63%) of these patients had *C. pneumoniae* in atheroma plaques. In our study, we were aimed to determine the frequency of detection of *C. pneumoniae*, which is one of the most common agents in atypical pneumonia infections, which covers a significant rate in CAP.

Although they still low and the reliability is not standardized for the diagnosis of *C. pneumoniae*, ELISA tests are often used in the determination of *C. pneumoniae* active in Turkey and the world. ELISA method was used in six of 23 studies published in the world since 2000, in which C.pneumoniae was shown as an etiological agent in adult patients with CAP. Behbehani et al. In Kuwait. [69], dual serum samples of 124 adult patients with CAP were studied. These samples were analyzed using the *C. pneumoniae* ELISA IgG and IgM tests. 70 of the patients are younger than 45, and 54 are older than 45. 71 C. pneumoniae was detected in 9% of patients younger than 45 years old and 4% of patients older than 45 years.

In Turkey, there is only one study in which *C. pneumoniae* was shown as an etiological agent in adult patients with CAP using the ELISA method. In Ankara, Gulhane Military Medical Academy (GATA) and published in the Turkish Armed Forces (TAF) Preventive Medicine Bulletin, Güneş et al. [70] in their study with 87 CAP and 21 healthy adults, a single ELISA kit that detects IgM antibody against four different atypical pneumonia agents (*L. pneumophila*, *M. pneumoniae*, Coxiella burnetii, *C. pneumoniae*) (Pneumobact ELISA IgM, Vircell, Spain) were used. At the end of this study, the *C. pneumoniae* seropositivity was found to be 56.3%. *C. pneumoniae* IgM was not detected in the sera of the control group. The reason for detecting such high *C. pneumoniae* seropositivity is that the transmission of *C. pneumoniae*, which is

transmitted by respiratory tract secretions and known to cause epidemics in military environments, maybe faster and easier in the closed and military environment [70].

There are also studies investigating the presence of IgM antibodies in addition to *C. pneumoniae* IgG by ELISA. *C. pneumoniae* IgM positivity rate was found in two studies, and this rate was found to be high in both studies. In the report of Wattanathum et al. (142), which is one of these studies, IgM positivity specific to *C. pneumoniae* was stated as 22.5%. In the second of these studies, Zaki and Goda [48] published in 2009, double serum samples of 100 adult patients with CAP were evaluated in terms of the *C. pneumoniae* agent using the ELISA IgM test. As a result of this study, Adenovirus and *C. pneumoniae* were serologically detected with the highest rate (30%). Mixed infection was detected in 30% of the patients. 50% of these are *C. pneumoniae-Pneumococcal* infection. The reason for such high *C. pneumoniae* IgM positivity detection; They attributed the presence of *C. trachomatis* in the working environment and the formation of cross-reaction accordingly [48].

CAP in adult patients with *C. pneumoniae* IgM ELISA in Turkey The study investigating the presence of antibodies is the work done by Güneş et al. [70] mentioned earlier. CAP in adult patients in Turkey IFA in four other studies investigating the presence of IgM antibodies to *C. pneumoniae* method has been used ([59], [50], [71], [72]. Results of these studies When examined, *C. pneumoniae* IgM antibody positivity was found to be 3.9% on average.

There are studies arguing that the ELISA IgM test can give false positivity. Miyashita et al. In the study published by [73], it is stated that false-positive ELISA IgM reaction frequently occurs against *C. pneumoniae*. As a result of their studies evaluating the false-positive results occurring in the ELISA IgM test, it was emphasized that every ELISA IgM positivity obtained would not be an indicator of acute *C. pneumoniae* infection even if it was detected in high titers [73]. Kishimoto et al. [74] suggested that non-specific binding that causes IgM false positivity specific to *C. pneumoniae* in ELISA can be prevented by increasing the IgM cut-off ID value.

ELISA IgM and IgG tests can give false positive results for *C. pneumoniae* agent, as well as false negative responses. In patients with primary infection, the IgM antibody appears approximately two to three weeks after the onset of the disease and is usually undetectable after two to six months. IgG antibodies may not reach high titers

until six to eight weeks after the onset of the disease. Serum samples were taken from patients early (for example, earlier than 3 weeks) due to the long process until the development of serological response to primary infection[75].

Otherwise, the antibody response may be missed. Thus, false negative results are obtained. Non-culture based tests such as PCR have been widely used in routine laboratories for the identification of *Chlamydia* infection due to the low yield and technically complex culture protocols, long-term results, and the need for special fluids while collecting, transporting and storing samples. Especially in some patients, because of the disease. In addition, this method is said to be much more sensitive than isolation . There are seven studies conducted in the world since 2000 in which the role of *C. pneumoniae* agent in adult patients with CAP was investigated using PCR method [62][56][76][52][77]. In the study conducted by Phares et al[76]. in Thailand, 208 children and 547 adult patients were included. MIF IgG / IgM and PCR tests were applied in order to determine the rate of *C. pneumoniae* in patients with CAP. It is not specified which PCR method is used. Nasopharyngeal swab samples were taken from patients for PCR testing. At the stage of They evaluated that it was not available. At the end of the study, two patients were evaluated as PCR positive and *C. pneumoniae* was detected serologically at a rate of 2.4%[76].

In the study by Wellinghausen et al[62]. Which was mentioned earlier, MIF and semi-nested PCR were applied as methods and the presence of the Pst1 gene was investigated. For this test, throat swelling water, sputum, tracheal aspirate, bronchial fluid, BAL and pleural aspirate samples were taken from the patients. As a result, 0.9% PCR positivity was detected. Again, in the study of Ngeow et al[56]. Mentioned earlier, *C. pneumoniae* agent was investigated in sputum, nasopharyngeal aspiration, BAL and pleural fluid samples by PCR method. Primers targeting the 16S rRNA gene were used for this method. The PCR method used is not specified. Only 2.5% *C. pneumoniae* infection was detected according to the PCR result. This rate increased to 4.3% when the result of the MIF test was added. The researchers stated that when the data obtained by the PCR method are evaluated together with the serological data, the diagnostic success will increase. Ruiz-Gonzalez et al[52]. In Spain. In their study, they tried to develop a predictive model and scoring system to determine etiological agents in patients with CAP. They tried to detect the *C. pneumoniae* agent using MIF and PCR

methods in 103 adult patients with CAP. Transthoracic needle aspiration samples were taken from the patients and in-house PCR method was applied. As a result, *C. pneumoniae* and *C. psittaci* were detected with a total rate of 20.4%[52]. the identification of etiological agents, for *C. pneumoniae*, in patients without dual serum samples, PCR negativity.

In our study, these results were obtained using the following methods, PCR, and IFAT, which confirmed the presence of *chlamydia* in cardiovascular plates. *Chlamydia* has been shown to be related to atherosclerotic disease. In addition to the high results we obtained, Helicobacter pylori bacteria were also detected in cardiovascular plates. After it is proved that the bacteria have a role in hardening the arteries, which leads to sudden death in the world, so care must be taken and an accurate diagnosis for the purpose of treatment and also to control the use of antibiotics.

6. CONCLUSION and SUGESTİONS

The isolates included in this study were collected from the Cardiovascular Department over a period of one year in Adana / Turkey. In this respect, the study aims to investigate the relationship between *C. pneumoniae* and atherosclerosis by PCR AND IFAT methods.

1. The presence of *C. pneumoniae* was confirmed in 7 samples out of a total of 11 as mentioned in the polymerase chain reaction in Figure (7).
2. As for the second method, in which indirect immunofluorescence was performed, antibodies indicating whether the infection was recent or previous were searched for, which is as shown in Table (8).

More research is needed to determine the prevalence of *chlamydia* bacteria in Turkey in order to limit its spread. It is known that this bacterium is an intracellular infection, so it is dangerous as it causes many diseases such as Alzheimer's, atherosclerosis, lower and upper respiratory tract diseases. In our study, from the samples collected from the cardiac arteries, it was proved that *chlamydia pneumoniae* is associated with atherosclerosis disease. According to the numerous studies that were conducted in Turkey on this bacterium, but until now the 16S rRNA method has not been used to find the sequence of nitrogenous bases to these bacteria. Therefore, this technique must be used to find out whether the *chlamydia* present in Turkey has mutations or not for the purpose of easy diagnosis and appropriate treatment for these bacteria.

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