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Emerging strategies in the diagnosis, prevention and treatment of *Chlamydophila pneumoniae* infections

Enrique Villegas, Ana Camacho, Antonio Sorlózano, José Rojas & José Gutiérrez[†]

[†]*University of Granada, School of Medicine, Department of Microbiology, Avda de Madrid 11, E-18012 Granada, Spain*

Background: *Chlamydophila pneumoniae* infections are a common cause of acute respiratory diseases, including upper respiratory tract infections and pneumonia. Over the past few years, *C. pneumoniae* infections have been strongly related to atherosclerotic cardiovascular diseases. *Objective*: The aim of this review is to offer an update and overview of recent advances in the diagnosis, prevention and treatment of these infections. *Methods*: Diagnostic systems have improved but further progress is required to allow a reliable diagnosis to be made. This is especially true for atherosclerotic diseases, for which standard criteria need to be established. *Results/conclusion*: Polymerase chain reaction and serological methods need to be standardized and made better to improve the diagnosis of *C. pneumoniae* infections. It seems to be crucial to obtain new and more selective antigens associated with persistent infections to explain the participation of *C. pneumoniae* in coronary artery disease.

Keywords: *Chlamydophila pneumoniae*, diagnosis, prevention, treatment

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1. Introduction: cell cycle

Chlamydophila (Chlamydia) pneumoniae is an obligate intracellular human pathogen with a unique biphasic development cycle, which causes acute respiratory disease, including pneumonia, bronchitis, sinusitis and pharyngitis. It has also been associated with atherosclerosis [1-3], among other diseases. This organism was first isolated in 1965 from the conjunctival tissue of a Taiwanese child being vaccinated against trachoma [4].

C. pneumoniae has two well-differentiated morphologies during its cell cycle [5]: the elementary body (EB), the extracellular and infectious form; and the reticulate body (RB), the intracellular and replicative form. The latter morphology develops after infection of the host eukaryotic cell by the EB, which then differentiates to RB in membrane-associated vacuoles, known as chlamydial inclusions.

The cell cycle may occasionally be temporarily or permanently arrested in the RB phase, as has been observed after treatment with cytokines, for example, IFN-γ, or antibiotics, after restriction of certain nutrients, or spontaneously under certain culture conditions, when they are designated persistent bodies (PBs). PBs are characterized as large non-infective aberrant forms.

Persistent *C. pneumoniae* infection seems to be related to the continuous expression of genes associated with DNA replication but not to genes involved in cell division. The cell division is very slow and the metabolic activity is complex. Thus, whereas the expression of some genes is downregulated, such as those

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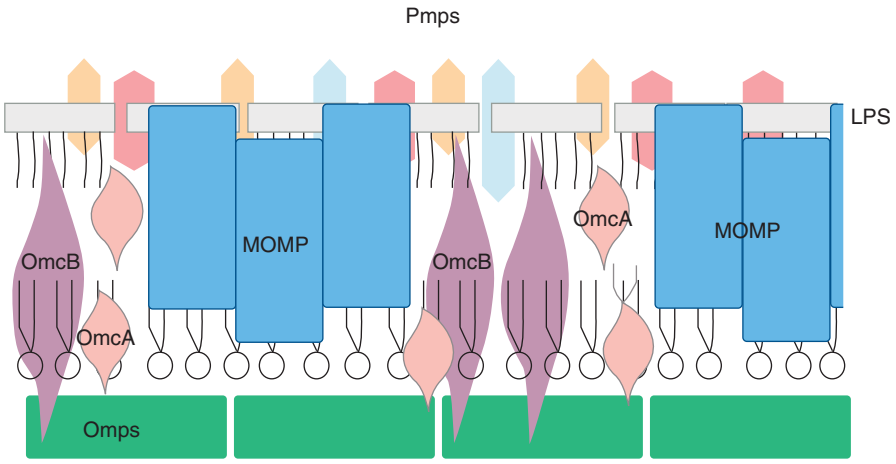

Figure 1. Cellular envelope proteins of *Chlamydophila pneumoniae* EBs.
LPS: Lipopolysaccharide; MOMP: Major outer membrane protein; Omc: Outer membrane complex protein; Omps: Outer membrane protein; Pmps: Polymorphic outer membrane protein.

related to cell division (*ftsK*, *ftsW*), others are upregulated (*pgk*, *groEL*), depending on the restricting conditions and the time after infection [6]. They have fewer porins compared with RBs and a lower expression of most structural constituents of the organism, including lipopolysaccharide (LPS), outer membrane complex protein B (OmcB) and major outer membrane protein (MOMP). However, synthesis of heat-shock protein 60 kDa (Hsp60) is maintained [7], suggesting that PBs may play an important role in the pathogenesis of chronic infection. In general, a lower MOMP:Hsp60 ratio has been found in cell culture studies on the persistence of *Chlamydia trachomatis*. However, recent studies of *C. pneumoniae* reported an upregulation of MOMP, increasing the MOMP:Hsp60 ratio, indicating that this ratio cannot be used as a universal marker of persistence, as was first thought [6].

C. pneumoniae has been implicated in the pathogenesis of various chronic diseases, including asthma and atherosclerosis. It has been proposed that this state is an adaptation of the bacteria to the host to avoid the immune system or remain viable under adverse conditions. Its presence may have diagnostic repercussions because it can generate false negatives owing to the scant antigen expression or low viability of PBs. It may also have therapeutic repercussions, because porin loss is frequently associated with resistance to treatment [8].

2. *C. pneumoniae* antigens

The outer membrane of *C. pneumoniae* is highly complex (**Figure 1**). Its antigens have been widely reported in numerous studies, and the more important are listed in **Table 1** [9-14]. Although the type and composition of its antigenic proteins are known, further knowledge of its immunogenic antigens is required to improve the diagnosis and prevention of this disease.

All antigens depicted in **Table 1** were demonstrated in several immunogenetic analyses. Key studies in the identification of *C. pneumoniae* proteins include the reports by Vandahl *et al.* [11] and Molestina *et al.* [15], based on bidimensional electrophoresis analysis and subsequent identification by matrix-assisted laser desorption/ionization-mass spectrometry of a large number of *C. pneumoniae* proteins. Among studies describing immunogenic proteins, we highlight the identification by Montigiani *et al.* [10] of immunogenic EB surface proteins. They used a genomic and proteomic approach based on *in silico* prediction, followed by the expression and purification of *C. pneumoniae* surface antigens, production of mouse immune sera to be used in western blotting and fluorescence-activated cell sorter analyses, confirmed by mass spectrometry analysis of 2D electrophoresis. They obtained 53 antigens, of which 28 were surface antigens, and their study helped to clarify the organization of *C. pneumoniae* surface proteins.

3. *C. pneumoniae* and atherosclerosis

The possible influence of vascular infection with *C. pneumoniae* on the pathogenesis of atherosclerotic cardiovascular disease was first suggested in 1988 by the results of a seroepidemiological study in Finland [3]. This issue has been addressed by hundreds of publications since the presence of *C. pneumoniae* was first identified in coronary atheromas by electron microscopy and verified by polymerase chain reaction (PCR) and immunohistochemistry [16]. Nevertheless, other studies did not find this association, either by direct detection of the pathogen in atheromatous plaques in coronary arteries [17] or in seroepidemiological studies [18,19]. The possible causal role of *C. pneumoniae* in the pathogenesis of atherosclerosis is controversial because of the several obstacles in establishing this association, including the difficulty of avoiding contagion

Table 1. Some characteristics of important *Chlamydophila pneumoniae* antigens.

Antigens	Size	Function	Presence	Immunogenicity	pI*
LPS [9]	NS	Structural integrity	EBs and RBs	Genus-specific immunogenic	NS
Associated with outer membrane					
MOMP [10]	39.5 kDa	Porin structural integrity	EBs and RBs	No immunodominant	6.1
OmcA [10]	9 kDa	Adhesin Structural integrity	EBs	NS	6.1
OmcB [10]	2 × 60 kDa	Periplasmic adhesin	EBs	Genus-specific immunogenic	5.6
Pmps 1 – 21 [11]	180 – 94 kDa	Autotransporter proteins	EBs and RBs	Immunogenics	5 – 7
OmpH [10]	17.3 kDa	Unknown	Unknown	Unknown	4.7
Omp85 [11]	74 kDa	Unknown	Unknown	Immunogenic	8.3
76 kDa protein [10,12]	68.2 kDa	Unknown	EBs	Species-specific immunogenic	4.9
Associated with cellular processes					
IncA [13]	NS	Developed and mediators of the inclusion membrane	IM	NS	NS
IncB [13]	NS	"	IM	NS	NS
IncC [13]	NS	"	IM	NS	NS
Hsp60 [11]	58 kDa	Chaperone	EBs and RBs	Immunogenic	5.3
Hsp70 [10]	71 kDa	Chaperone	EBs and RBs	Immunogenic	5.6
Low calcium response E protein [10,14]	43 kDa	Regulator type III secretion system	Unknown	Immunogenic	5
Associated with metabolism					
Enolase [10]	46 kDa	Tissue invasion Coagulation	Unknown	Immunogenic	4.7

*Theoretic pI [10,11].

EB: Elementary body; IM: Inclusion membrane; NS: Not specified; pI: Isoelectric point; RB: Reticulate body.

by this pathogen and the wide inter-individual variability in the immune response.

Clinical manifestations of atherosclerosis vary from coronary artery disease (CAD) to cerebrovascular disease. Several infectious agents have been related to atherosclerosis so far, including cytomegalovirus, herpes simplex virus, *Helicobacter pylori* and *C. pneumoniae*, which is the most widely studied pathogen in this context [20-22]. These relationships may help to explain cases that cannot be attributed to conventional risk factors, including tobacco, hypertension and high serum lipid levels.

C. pneumoniae has been detected using electron microscopy, immunohistochemistry or PCR in several atheromatous vessels [1,2,23] but not in healthy arteries [24].

The presence of viable bacteria in atheromas was demonstrated by reverse transcriptase PCR and confirmed by culture of isolates from coronary and carotid artery samples [25,26]. The culture of *C. pneumoniae* from atheromatous tissue has only rarely been achieved [25-27], which may be because it is in a latent phase with low metabolic activity, generating forms that are difficult to culture.

The effect of *C. pneumoniae* infection in atherosclerosis seems to derive from the persistent presence of the pathogen in cardiovascular tissue, inducing an inflammatory response that initiates or exacerbates the disease. The inflammation of vessel walls plays an essential role in the initiation and progress of atherosclerosis, although there are several routes by which the infectious agent could influence the pathogenesis of this disease.

Serological, immunohistochemical and nucleic acid amplification techniques have been used to explore the relationship between infection with *C. pneumoniae* and atherosclerosis, and numerous studies have yielded inconsistent findings. These methods are not standardized, and results vary widely according to the diagnostic method used and even among

laboratories using the same methods. There is a need for a gold-standard method to be established the link between *C. pneumoniae* and atherosclerosis.

4. *C. pneumoniae* and chronic respiratory disease

Several studies have suggested that there is a direct association between *C. pneumoniae* infection and the pathogenesis of chronic respiratory disease, such as chronic obstructive pulmonary disease or asthma [28-30]. Many of the results in this field have been inconsistent, dividing specialists. This may be due to differences in study populations, epidemiologic variations, and limitations of the main diagnostic techniques, for example, serology and PCR. Thus, the possible role of *C. pneumoniae* in the pathogenesis of chronic obstructive pulmonary disease remains controversial, and the consequences of chronic tissue infection with *C. pneumoniae* are not clear [31].

5. Diagnosis

With respect to serology techniques, the detection of current disease is hampered by the high prevalence of IgG in adults owing to repeated asymptomatic infections, and by the absence of IgM in some cases of primary infection [32]. Continuous cell culture remains the prevailing gold standard for demonstrating current infection by *C. pneumoniae* and for establishing viability and infectivity, but this technique is complex and has limited sensitivity [33]. According to the Centers for Disease Control (CDC), a positive result should only be declared if the strain is propagated by subsequent passage or is confirmed by another technique, for example, PCR [32]. Nucleic acid amplification techniques are the most sensitive and have the potential to improve *C. pneumoniae* detection [34]. However, although they are in wide use to diagnose *C. pneumoniae* infection, some validation and standardization issues remain unresolved [35,36].

5.1 Cell culture

Cell culture used to be the most widely used technique. However, the use of culture for the detection of *C. pneumoniae* is problematic because of the difficulty of its growth in cell cultures, especially from tissue samples. Despite the methodological difficulties, it remains in use to test for current infection by *C. pneumoniae*, characterize clinical isolates, and establish viability and infectivity.

The results of vascular tissue cultures are not always reproducible. Some researchers regularly recovered viable *C. pneumoniae* [20,27], whereas other groups reported low isolation indices [37,38]. The use of culture to detect *C. pneumoniae* is problematic due to the difficulty of *C. pneumoniae* growth in cell culture, especially from vascular tissue samples [32].

A highly sensitive culture system was developed by Maass *et al.* [27], who examined 70 samples of coronary arteries to determine the presence of viable *C. pneumoniae* and *C. pneumoniae* DNA by cell culture and nested PCR, respectively. Among patients with positive culture, 82% were positive for PCR in the three independently tested segments of coronary artery and 18% were positive in two of these three segments. All culture-positive patients were PCR-positive and six isolates could be permanently propagated by serial subcultures. The study showed that viable *C. pneumoniae* can be isolated from samples of atherosclerotic coronary arteries by using a sensitive culture system with several passages and serum-free medium [39].

5.2 PCR

Numerous molecular amplification techniques based on genomic sequences have been applied to detect and differentiate *Chlamydia* species, including DNA hybridization with genomic DNA probes, polymorphic analysis of restriction fragments of PCR-amplified products and nested PCR, among others. These techniques have shown a higher sensitivity in comparison to culture and other diagnostic tests for the detection of *Chlamydia* infection in certain tissues and respiratory samples [40].

Various in-house PCR assays using different *C. pneumoniae*-specific oligonucleotides directed against a specific sequence of the bacteria have been developed for its detection in respiratory, vascular tissue, serum and peripheral blood mononuclear cell samples [32]. The first tests to emerge were the single-step PCR and the nested PCR. A multiplex format was recently introduced to detect promoters of community acquired pneumonia, along with different techniques for visualizing the amplicons [41]. Nevertheless, there is no commercially available standardized assay that has been approved by the FDA for the detection in respiratory or other specimens.

Real-time PCR (RT-PCR) was recently used as a quantitative gene amplification technique to detect bacteria and viruses associated with respiratory tract diseases [42-46]. The results obtained have been promising, and the sensitivity and specificity has proved to be higher than those of other assays. In the future, RT-PCR may become a standard diagnostic method for the quantitative detection of respiratory infections and perhaps of other infections by *C. pneumoniae*. Kuoppa *et al.* [46] reported that RT-PCR appeared to be a more sensitive detection method than nested PCR. Although nested PCR was previously validated as a sensitive and specific detection method for *C. pneumoniae* compared with other methods, such as cell culture [46], it does not have the capacity to be a quantitative measure. Moreover, there is a high risk of contamination, which can be impossible to avoid [35,36].

Nucleic acid amplification is used to achieve a higher sensitivity of *C. pneumoniae* detection in vascular tissue than that offered by conventional diagnostic methods. PCR can detect the DNA or RNA of microorganisms that are present in small numbers or are not viable or show a low growth rate, and it can be used in tissues not available for culture.

Several difficulties remain to be overcome, however, mostly in relation to tissue sensitivity and specificity and to the validation of new tests. The main reason for this variation may be the use of different DNA extraction processes and PCR protocols. DNA extraction varies considerably according to the type of sample, with differences between respiratory samples (sputum, nasopharyngeal, throat) and serum or vascular tissue samples. With regard to respiratory samples, some authors obtained larger amounts of DNA of *C. pneumoniae* from sputum specimens than from nasopharyngeal and throat specimens [46] but there is no consensus on the type of sample to be used [35]. Protocols also vary widely, with different target genes, distinct types of PCR for the amplification and diverse detection methods. Consequently, even if samples are from the same patient and are analyzed by different laboratories, the variation in results can be significant [36].

Apfalter *et al.* [36] conducted a multi-center study on different PCR methods used for *C. pneumoniae* detection in vascular tissue. All laboratories received aliquots of 20 samples with the same composition. The maximum concordance was 25% for one carotid artery sample. There was no consistent pattern of positive results among laboratories, and the positivity index for individual tests was not correlated with their sensitivity values. These findings suggest that the variability of PCR-detected *C. pneumoniae* prevalence in vascular tissue can largely be explained by the different methodologies used rather than by distinct quantities of microorganisms in the tissue.

Only two multi-center studies have compared different PCR tests in respiratory samples for the detection of *C. pneumoniae*. The first compared an industrial PCR kit with 5 in-house PCR assays [47] and the second performed quality controls in two different years on 16 and 18 in-house PCR tests, respectively [48]. Both studies reported wide variations.

A further shortcoming of PCR is that it yields false negatives and false positives. False negatives may result from degradation of the DNA by the release of endonucleases [49] or the presence of inhibitors, for example, blood or mucus components [50] and some reagents [51]. The nature of most inhibitors in clinical samples remains unknown, hindering the development of techniques for their removal. Proposals have included the use of alternative methods of sample treatment and the introduction of positive internal controls [32]. False positives can result from contamination during laboratory processing and sampling or from deficiencies in technique. It is important to test for contamination by running negative controls subjected to the same handling as the samples, and a correct technique is vital. Ieven *et al.* [52] reviewed investigations of *C. pneumoniae* DNA in a total of 3551 samples, of which 2688 were found to be negative and 863 (24.3%) positive. The reported prevalence of *C. pneumoniae* in atherosclerotic vessels ranged extremely widely from 0 to 100%. However, > 6.5% of control arteries were also PCR-positive and this high positivity in control arteries has raised doubts about the technique and the selection of negative control samples.

There are major inter-laboratory variations and it is difficult to compare data with the results of other techniques because they detect different components (DNA, antibodies, bacteria viability). Moreover, PCR tests can detect DNA in patients without antibodies or with low titers and frequently fail to detect DNA in patients with high antibody titers [52]. For these reasons, among others, standardized tests are not yet available.

5.3 Serological methods

Infection by *C. pneumoniae* induces IgG, IgA and IgM responses that can be detected by serological methods. The CDC have established diagnostic criteria for acute infection by *C. pneumoniae* [32].

Serological testing most often provides only a retrospective diagnosis of acute infection because a convalescent serum specimen is needed to show a fourfold increase in titer. An accurate diagnosis requires paired serum samples, and single IgG titers lack clinical relevance. There is no reference test for validating persistent infection.

The methods used to measure *C. pneumoniae*-specific antibodies vary from laboratory to laboratory, and there is a need for the improvement, simplification and standardization of the methods to be used [53].

5.3.1 MIF test

According to the CDC microimmunofluorescence (MIF) is the serological method of choice for the diagnosis of acute *C. pneumoniae* infection [32], but it requires time, is difficult to perform and must be subjectively interpreted by an expert operator with a fluorescence microscope. Although many commercial MIF tests have shown a good concordance, there have been reports of significant inter-laboratory variations. Thus, Littman *et al.* [54] used a standard MIF protocol in two laboratories that are leaders in the development of MIF tests and compared specific *C. pneumoniae* IgG and IgA titers in 392 individuals. They reported a percentage exact agreement of 38 and 55% for IgG and IgA, respectively, and a percentage agreement for a twofold dilution of 66 and 75%, respectively. Peeling *et al.* [55] also performed an inter-laboratory study on specific titers against *C. pneumoniae* using MIF. Fourteen laboratories were sent 10 serum sets for the determination of IgG and IgM, with each set containing two or three samples from the same individual. Globally, the percentage agreement with reference standard titers was 80%. Paldanius *et al.* [53] measured specific *C. pneumoniae* IgA using one commercial and seven in-house tests and an enzyme immunoassay, reporting a wide variation in the detection of IgA antibodies in sera.

Finally, the absence of MIF antibodies in individuals with culture-confirmed infection has been reported. This is rare in adults but may be more common in young children [56].

5.3.2 *ELISA*

ELISAs were developed to overcome the shortcomings of MIF. Thus, they require less time, are more objective (photometric reading of results) and are easier to standardize because results are expressed in international units [57]. ELISAs can be prepared using *C. pneumoniae* EBs with LPS, *C. pneumoniae* EBs without LPS or *C. pneumoniae*-specific recombinant immune antigens [58]. Although some kits are commercially available, none of them have been approved by the CDC or the FDA.

In a study of sera from 80 apparently healthy individuals, Hermann *et al.* [59] found a high degree of variation in the sensitivity of serodiagnostic tests. However, in a subsequent investigation by the same group, they reported that ELISA tests had high specificity and sensitivity and showed a good correlation with MIF tests in sera from patients with respiratory tract diseases and from control children. Antibody titers determined by quantitative ELISA also showed an acceptable correlation with those determined by MIF [60].

ELISA is commonly used in seroepidemiological studies because it is less expensive and easier to use on a large scale. However, MIF continues in wide use for individual diagnoses, when there is no need to process numerous samples. The higher sensitivity and specificity of ELISA makes it useful for disease diagnosis when there is a major increase in IgG between two samples [61].

Both MIF and ELISA can produce false positives due to crossreactivity with other species of the *Chlamydiaceae* family. Thus, in *Chlamydia*, the major genus epitopes locate in the LPS and are responsible for most of the serological crossreactions. Although LPS has been used in ELISA tests to detect IgG, IgA or IgM, alone or in combination, there is no definitive consensus on its clinical utility [61]. Serological studies carried out with EB proteins have mainly employed MOMP. On the other hand, the 98 kDa protein, an abundant constituent of EB MOMP, shows an important species-specific reactivity in immunoblot. Thus, many of the EB antigens studied by western blot are essentially species-specific, and their application would present a negligible unspecific reactivity.

Conversely, Hermann *et al.* [60] obtained false negatives with ELISA in the samples with the lowest titers (1:64) in MIF, whereas true findings were recorded for all samples with medium (1:256 and 1:512) and high (1:1024) titers in MIF.

Findings on the relationship between seropositivity for *C. pneumoniae* and atherosclerotic diseases are influenced by the serological test used, as they vary widely in sensitivity and specificity. Hence, the selection of test has important implications in epidemiological investigations and in studies on *C. pneumoniae* seropositivity as a cardiovascular risk factor [57]. There are important inter- and intra-laboratory variations in this field and a poor relationship has been found among the different *C. pneumoniae* tests [62].

5.4 Diagnosis related patents

Several different diagnostic methods have been patented that use peptides for the *in vitro* detection of *C. pneumoniae*-specific infections. Thus, the patent application by Sayvon Diagnostics [63] describes a system with a peptide derived from the variable domain of *C. pneumoniae* MOMP protein. Another recent method patented by Sirs Lab GMBH [64] can detect antibodies against dnaJ protein and/or a hydrolase/phosphatase homologue protein of *C. pneumoniae*.

There are different patents for PCR-related methods based on the differential diagnosis of representatives of *Chlamydiaceae* family. Thus, Ehjdel *et al.* [65] used RT-PCR with a single pair of primers of *ompA* gene, which encodes for MOMP, and four probes for each of the *Chlamydia* species. Other methods are based on PCR differential diagnosis, using 10 types of the pathogenic bacteria that cause respiratory tract infection, and comprise primers of genes encoding the 16S rRNA of *C. pneumoniae* and the other bacteria [66]. More recently, Gen-Probe, Inc. [67] patented a primer set that can amplify a target sequence in the 16S rRNA without using a different primer set for each of the bacteria species. Other oligonucleotides that are useful for determining whether *C. pneumoniae* is present in a test sample are described in a patent application by Cantor Colburn LLP [68], which preferentially hybridizes to a target region present in nucleic acid derived from 23S ribosomal nucleic acid of *C. pneumoniae* and not to nucleic acid derived from *C. trachomatis* or *C. psittaci*.

6. Prevention

The first attempts to develop a vaccine for the *Chlamydia* genus were against *C. trachomatis*. These initial vaccines were tested with dead or inactivated organisms and, although some of them produced a reasonable degree of protection, the immunization sometimes led to a greater progression of the disease. Vaccines with the whole pathogen were abandoned for human vaccination, and a vaccine with components of the organism was developed thanks to a greater knowledge of the structure and pathogenesis of the *Chlamydia* genus. Most vaccines are immunogenic preparations that induce antibody production in animals but offer only partial or no protection. Numerous antigens have been proposed as possible candidate vaccines to offer partial or temporary immune protection, and it has also been suggested that several antigens be combined to induce an optimal protective response [69].

Some studies indicate that a vaccine capable of inducing a Th1 type immune response, characterized by the release of IFN-γ, would be effective to eliminate infection by *C. pneumoniae*. The role of humoral immunity in protection against *Chlamydia* infections is probably less important than the role of cell-mediated immunity [70].

However, the use of vaccines with live or attenuated microorganisms is not desirable because they may produce a pathological state. There have been attempts to develop

DNA-based vaccines against *C. pneumoniae*. The advantage of these vaccines is that the DNA will generate the expression of the recombining protein within the cell, and it can be presented by the cell for recognition in a class I MHC molecule response, thereby activating CD8 cells. This may generate the same type of response as live or attenuated vaccines but without the safety risk. It is increasingly clear that DNA vaccines can induce different immune responses (Th1 and Th2) according to the adjuvant used in the preparation [71].

The protection conferred by DNA vaccines is generally only partial. This is believed to be related to the weak *in vivo* capacity of genes to disseminate within the host, generating a poor antigenic expression in transfected cells [72].

An elevated expression of the antigen is necessary to improve the immunogenicity of DNA vaccines. This may be achieved by using stronger promoters or by stabilizing the mRNA. An improvement in the efficacy of DNA vaccines requires knowledge of the nature of the antigen, vaccine vector and/or adjuvant and how an effective immune response is generated [72].

6.1 Possible candidates as vaccines

6.1.1 MOMP

MOMP induces an immune response in the different *Chlamydia* species, leading to the production of neutralizing antibodies during infection. This antigen is not considered immunodominant, although there is evidence of the production of species-specific monoclonal antibodies that neutralize the infection *in vitro* [73]. This may be advantageous for the development of a vaccine against *C. pneumoniae* infection. Numerous efforts have been made to characterize immunodominant epitopes of MOMP but antibody production only seems to occur under native conditions, because they are conformational epitopes.

6.1.2 OmcB

The immune response to this protein is strong and genus-specific [74]. Although it presents specific epitopes that generate immunogenicity in its denaturalized form, it has not been studied in-depth in its conformational form.

The correct selection of adjuvant is essential to develop the immune response generated. Thus, when OmcB was used in DNA and protein vaccines with coadjuvants that induce a Th2 type response, for example, Freund's complete adjuvant (FCA), the susceptibility of mice to infection was higher compared with non-vaccination. This harmful effect of FCA–OmcB depended on the presence of both CD4 and CD8 cells. It seems reasonable to think that immunization with FCA–OmcB induces long-lasting memory immune responses that facilitate *C. pneumoniae* survival or growth. The enhanced bacterial load in FCA–OmcB-immunized mice was linked to a markedly worse outcome of infection, and no protection was offered by FCA as an adjuvant for OmcB [71].

However, a partial protection against *C. pneumoniae* was observed when FCA was replaced by oligodeoxynucleotides that contain CpG immunostimulatory motifs mixed with Freund's incomplete adjuvant. The distinct effects on the infection of the different coadjuvants seem to be related to the different responses they induce (Th1 or Th2) and their stimulation of different cytokines [71]. Similar findings were also observed after immunization with FCA-Hsp60 of *Chlamydia* [71].

6.1.3 Hsps

Heat-shock proteins (Hsps) are dominant antigens in numerous infections by pathogens, and their potential use as a vaccine has been proposed. Thus, various vaccination strategies have been used, inducing a significant protection against different infectious agents, including *H. pylori* [75], *Hitoplasma capsulatum* [76] and *Mycobacterium tuberculosis* [77]. However, the use of Hsps in DNA or protein vaccines should be approached with caution because they may induce a crossed immune response, generating autoantibodies, due to the similarity of sequences between Hsps of *C. pneumoniae* and humans [78].

6.1.4 Pmps

Polymorphic outer membrane proteins (Pmps) are antigens with considerable potential as vaccines because they are species-specific with a surface localization and are, therefore, accessible to antibodies and can induce a humoral response. Nonetheless, dominant epitopes of Pmps seem to be conformational [79,80]. Therefore, any vaccine based on Pmp must produce folded Pmps to obtain antibodies that will bind to native Pmps of *C. pneumoniae*.

Although they are not yet well known and are inadequately characterized, Pmps stimulated both immune and innate defense during infection in mice with *C. pneumoniae* [81]. It has also been verified that Pmp2 and Pmp10 can induce neutralizing antibodies that eliminate infection by *C. pneumoniae* in hamsters [69].

A current research line supports the utilization of multi-antigen combinations to induce an optimal protective response.

6.1.5 Type III secretion system

Type III secretion system in Gram-negative bacteria is usually related to their pathogenesis, making it attractive for vaccine design [82]. Thus, another interesting candidate would be low-calcium-response protein E. This seems to be present on the surface of EBs and accessible to antibodies, according to Montigiani *et al.* [10], inducing activation of T CD4 and CD8 cells with cytokine secretion and antibody neutralization, proving completely effective in the elimination of the infection [14]. It is, therefore, a candidate as a vaccine because it is related to the type III secretion system, and an effective blockade of this system may permanently inhibit the *C. pneumoniae* infectious process [10].

Table 2. *Chlamydophila pneumoniae* candidate antigens for vaccines and corresponding DNA patented.

Candidate vaccines	Publication number	Applicant	Patents
Outer membrane protein	US2005220805	Aventis Pasteur	[115]
76 kDa protein	US2005202048	Aventis Pasteur	[116]
60 kDa cysteine-rich membrane protein (OmcB)	US2005186266	Aventis Pasteur	[117]
Inclusion membrane protein C	US2004228874	Murdin *et al.*	[118]
Pomp91B	US2004022801	Murdin *et al.*	[119]
ATPase	US2003225017	Aventis Pasteur	[120]
98 kDa putative outer membrane protein	US2003170259	Mintz Levin Cohen Ferris Glovs	[121]
Outer membrane protein P6 precursor	US2003161833	Aventis Pasteur	[122]
CPN100149 polypeptide	US2003147924	Aventis Pasteur	[123]
5′-truncated and 3′-truncated 76 kDa protein	US2003095973	Murdin *et al.*	[124]
CPN100605 polypeptide	US2002150591	Mintz Levin Cohen Ferris Glovs	[125]
Glutamate-binding protein	US2002094965	Murdin *et al.*	[126]
Lorf2 protein	US2002091096	Murdin *et al.*	[127]
Transmembrane protein	US2002082402	Murdin *et al.*	[128]
ATP/ADP translocase	US2002081682	Aventis Pasteur	[129]
89 – 101 kDa and 56.1 kDa proteins family	US7264941	Birkelund *et al.*	[130]
54 kDa protein	US2004029806	Neutec Pharma PLC	[131]
Proteins encoded by open reading frames	US6822071	California University	[83]
Combinations of immunogenic molecules	WO2005084306	Chiron Corp	[84]
Genomic sequence and nucleotide sequences	US2007053927	Serono Genetics Inst SA	[85]

OmcB: Outer membrane complex protein B.

6.1.6 Other components

In vitro, OmpH and enolase can induce neutralizing antibodies and have shown capacity to inhibit the spread of *C. pneumoniae* in hamsters. These proteins are, therefore, immunoaccessible in EBs and are of research interest as candidate components for vaccines [69].

6.2 Prevention related patents

Patents have recently been registered for the nucleic acids of these antigens proposed as possible candidate vaccines and other nucleic acids encoding immunogenic proteins (**Table 2**). These patents describe methods with nucleotide sequences for immunizing a host, including humans, against disease caused by *C. pneumoniae*.

C. pneumoniae-specific multi-antigen combinations have also been patented. Both polypeptides can be used to prepare pharmaceutical compositions for the prevention of disease, as reflected in patent applications by California University [83] and by Chiron Corp. [84]. The genomic sequence and nucleotide sequences encoding polypeptides of *C. pneumoniae* appear in a patent application by Serono Genetics Institute SA [85].

7. Treatment

C. pneumoniae is sensitive to macrolides, tetracyclines, quinolones and rifamycins in *in vitro* studies [86-90]. Macrolides are the most widely used antibiotics for *C. pneumoniae* infections. They are effective for acute *C. pneumoniae* infections of the upper respiratory tract owing to their anti-inflammatory and antimicrobial properties, which should be monitored. These antibiotics have an efficacy of around 80% in respiratory tract infections by *C. pneumoniae*. According to these studies, persistence does not seem to be related to the development of resistance to the antibiotics, because the isolates were susceptible to them. One possible explanation may be that the dosage, duration or pharmokinetics of the treatments were not optimal.

It has been demonstrated that the live respiratory pathogen can be disseminated by monocytes through the systemic circulation and cannot be eliminated from the monocytes by standard antichlamydial treatment. It seems that the persistent state can be spontaneously induced. This state seems to be typical of chlamydiae ingested by human monocytes under *in vitro* culture conditions and is not induced by antibiotics because

Table 3. Relevant prospective antibiotic trials performed in humans for CAD and ACS secondary prevention.

Study	Ref.	No. of cases	Clinical cases	Antibiotic	Duration (treatment/follow-up)	Results	
						Differences serologic markers	Differences clinical end points
Gupta *et al.* (1997)	[98]	60	Post-MI (CAD)	Azithromycin	3 – 6 days/18 months	NO	NO
ROXIS (1999)	[99]	202	Acute non-Q-wave coronary syndromes (ACS)	Roxithromycin	1 month/6 months	NO	NO
ACADEMIC (2000)	[101]	302	Post-MI > 50% stenosis coronary artery (CAD)	Azithromycin	3 months/2 years	NO	NO
STAMINA (2002)	[102]	325	Acute MI unstable angina (ACS)	Azithromycin or amoxicillin	1 week/1 year	NO	SI
CLARIFY (2002)	[103]	148	Acute non-Q-wave MI unstable angina (ACS)	Clarithromycin	3 months/1.5 years		SI
ANTIBIO (2003)	[104]	872	Acute MI (ACS)	Roxithromycin	6 weeks/1 year		NO
WIZARD (2003)	[105]	7747	Post-MI (CAD)	Azithromycin	3 months/3 years	NO	NO
ACES (2005)	[106]	4012	Stable CAD (CAD)	Azithromycin	12 months/4 years		NO
AZACS (2003)	[107]	1439	Acute MI unstable angina (ACS)	Azithromycin	5 days/6 months		NO
PROVE-IT (2005)	[108]	4162	ACS	Gatifloxacin	2 weeks + 10 days each month/2 years	NO	NO

ACS: Acute coronary syndrome; CAD: Coronary artery disease; MI: Myocardial infarction.

it occurs without any antibiotic supplementation. The inclusions and their content were morphologically different from what is found in the acute infection of epithelial cells.

Chlamydiae can survive an antichlamydial therapy within monocytes *in vitro* and *in vivo*. Optimal regimens for chlamydial eradication from monocytes are not known. In fact, this may seriously affect the current efforts made in large prospective trials to alleviate clinical CAD symptoms by antichlamydial treatment [91].

7.1 Studies in animal models

Numerous studies have suggested an association between various pathogens and CAD. These pathogens can stimulate inflammatory responses, and the pathogens and inflammation together may contribute to the pathogenesis of atherosclerosis. The presence of herpes simplex virus, cytomegalovirus, *C. pneumoniae*, *H. pylori* and dental pathogens has been reported in patients with this disease. These agents have been studied in animal models to explore this possible relationship but a positive association was only found with *C. pneumoniae* and cytomegalovirus.

A study on the interaction between *C. pneumoniae* and murine cytomegalovirus in normocholesterolemic mice indicated that further interacting mechanisms participate in the development of chronic arterial diseases [92].

C. pneumoniae pathogenicity in cardiovascular diseases was demonstrated by several studies in white New Zealand (NZ) rabbits intranasally injected with *C. pneumoniae* [93-95]. These rabbits can develop atherosclerosis if fed with a cholesterol-rich diet. Early atherosclerosis was observed in infected animals fed with a normal diet [93,94]. Among NZ rabbits fed a cholesterol-rich diet, infected animals showed a faster development of atherosclerosis and larger lesions in comparison to non-infected animals [95].

In studies on mice deficient in LDL and apolipoprotein E, a group infected with *C. pneumoniae* developed hypercholesterolemia, inducing atherosclerotic disease [96,97].

7.2 Trials with antibiotics

Based on *in vitro* and animal study results, numerous investigations have been conducted in humans to determine the benefits of antibiotic therapy in the treatment of cardiovascular events (**Table 3**).

The first small pilot trials on antibiotic therapy for secondary prevention of cardiovascular events seemed to show that the administration of macrolides was of benefit. Gupta *et al.* [98] reported lower IgG anti-C. pneumoniae titers in an azithromycin-treated group than in a control group at 6 months after the end of treatment. However, subsequent studies found no significant effects on cardiovascular mortality or serologic markers. The

ROXIS (the Roxithromycin in Ischemic Syndromes) study [99] used roxithromycin and found no changes in anti-C. pneumoniae titers between treated and control groups. The authors reported a clinical benefit in preventing death and reinfarction for at least 6 months after initial treatment, although significance was not reached.

In the light of the above findings, studies with larger sample sizes and longer follow-up periods were conducted. Thus, a randomized study of 302 *C. pneumoniae*-seropositive patients with CAD treated for 3 months with azithromycin showed no reduction in secondary cardiovascular events after a 6-month follow-up [100]. However, there was a reduction in four inflammatory markers (C-reactive protein, IL-1, IL-6 and TNF-α) in the treated group. After 2 years of follow-up, there remained no differences between the treated and non-treated groups [101].

Encouraging results were reported by the STAMINA (South Thames Trial of Antibiotics in Myocardial Infarction and Unstable Angina) study [102], which compared outcomes in acute coronary syndrome (ACS) patients receiving antibiotics against *C. pneumoniae* and *H. pylori* with those in ACS patients without this treatment. At 12 weeks, patients under antibiotic treatment showed 36% fewer major events (cardiac-related death or new ACS) compared with the placebo group. The effect of antibiotic therapy in the secondary prevention of ACS was also assessed in the CLARIFY (Clarithromycin in Acute Coronary Syndrome Patients in Finland) study [103], in which a group of 148 patients with acute non-Q-wave coronary syndrome or unstable angina were randomly assigned to clarithromycin or placebo treatment for 3 months. The results of these two studies were not sufficiently conclusive to confirm the benefits of clarithromycin treatment.

The ANTIBIO (Antibiotic Therapy in Acute Myocardial Infarction) study [104] found that a 6-week course of roxithromycin showed no benefits in 872 patients with acute myocardial infarction after a 1-year follow-up.

The WIZARD (Weekly Intervention with Zithromax for Atherosclerosis and its Related Disorders) study [105] was the first large randomized placebo-controlled trial to examine the role of antibiotics in the prevention of CAD. More than 7000 *C. pneumoniae*- seropositive patients with a history of stable myocardial infarction underwent a 3-month treatment with placebo or azithromycin. The drug treatment only produced a 7% reduction in myocardial infarction incidence, hospitalization for unstable angina or need for revascularization at 3 years. Nonetheless, a possible benefit was indicated during and up to 6 months after treatment, with a 33% reduction in death and myocardial infarction. These outcomes raised questions about the clinical benefits of prolonged antimicrobial therapy.

The ACES (Azithromycin and Coronary Events Study) trial [106] was conducted in 4000 adults with stable CAD. Patients were randomly treated with azithromycin or placebo for 1 – 12 months. After 4 years, no difference in cardiovascular end points was found between the azithromycin- and placebo-treated groups.

The AZACS (Azithromycin in Acute Coronary Syndrome) study [107] of 1450 patients with ACS included patients who were serologically positive or negative for *C. pneumoniae*. They were treated with azithromycin for only 5 days and followed up for 6 months, finding no ischemic benefits. The AZACS study indicates with reasonable power and certainty that short-term azithromycin treatment is of no benefit and does not reduce the development of recurrent events in patients with ACS.

The PROVE-IT (Pravastatin or Atorvastatin Evaluation and Infection therapy) study [108] enrolled 4162 patients with ACS. Subjects received gatifloxacin or placebo daily during an initial 2-week course of therapy that began 2 weeks after randomization, followed by a 10-day course every month for the duration of the trial (mean duration, 2 years). No benefits were observed in secondary end points or in patients with elevated titers for *C. pneumoniae* or C-reactive protein.

The encouraging results of preliminary studies with antibiotics have not been repeated in large trials. Controversial questions include the selection of antibiotic (bacteriostatic or bactericide) and the need for a combination of antibiotics, as used in tuberculosis treatments. In a study of great interest, Gieffers *et al.* [91] reported that *C. pneumoniae* infects circulating monocytes and that antibiotics cannot inhibit its growth within them. The elimination of vascular infection with antibiotics may be difficult if the *C. pneumoniae* residing in monocytes, which can disseminate the pathogen, is resistant to antibiotics [109].

7.3 Treatment related patents

Antibiotics or combinations of antibiotics from antibiotic families commonly used to treat *C. pneumoniae* infection have been shown to combat atherosclerosis. These include azithromycin, a macrolide antibiotic, included in a patent application by Pfizer, Inc. [110], and azithromycin administered in combination with a glycogen phosphorylase inhibitor in synergistic effective amounts. This method was discovered by Pfizer, Inc. [111].

Alternative treatments against acute lower respiratory tract infections due to *C. pneumoniae* have been developed, such as thiamphenicol, patented by Zambon Group SPA [112], which represents an alternative to clarithromycin, and Rifalazil, a rifamycin, patented by Kaneka Corp. [113] and developed to replace rifampin. It has superior antimicrobial activity and high intracellular levels.

Recently, Innate Pharmaceuticals Ab [114] patented a method of treating respiratory infection and atherosclerosis that comprises a pharmacologically effective amount of a type III secretion blocker.

8. Conclusions

C. pneumoniae is a pathogen that infects most population throughout their lives. Although primary infection is usually

subclinical, it can cause pneumonia, bronchitis, pharyngitis and sinusitis. A rapid and unequivocal diagnosis is very important but hampered by the lack of standardized diagnostic methods for serology and PCR and by inter- and intra-laboratory variations.

PCR is a promising tool for the diagnosis of primary or chronic *C. pneumoniae* infections and other *C. pneumoniae*-associated infections. Although our knowledge of *C. pneumoniae* proteomics is at a very initial stage, its study will decisively improve our understanding of the final pathogenic mechanisms that produce the disease in humans and will assist the development of vaccines.

9. Expert opinion

The past decade has seen controversial reports on the relationship between *C. pneumoniae*, responsible for respiratory diseases, and atherosclerosis.

Numerous PCR tests have been developed to detect this pathogen in respiratory samples, providing improved and faster results. Each type of PCR offers different advantages and disadvantages that must be taken into account before their utilization in clinical practice. RT-PCR has shown high sensitivity and specificity, and multiplex yields savings in time and costs, and a combination of the two methods offers a promising approach. Work is already in progress on a real time multiplex polymerase chain reaction or duplex RT-PCR for *C. pneumoniae* and *Mycoplasma pneumoniae*, which cause the majority of atypical community pneumonias.

The main difficulty with the diagnosis of *C. pneumoniae* infection in atherosclerosis is the lack of standardized tests, reflected in the wide variation in results among studies. Further prospective studies on atherosclerosis are warranted because there is at present no serodiagnostic tool available to detect persistent infections. Serological tests that differentiate between past and persistent infections may offer the key to resolving its possible role in CAD. Proteomics has much to contribute in this respect, obtaining selective antigens or combinations of antigens associated with persistent infection and improving the serodiagnosis in these infected patients. Another research line of interest is the role in the genesis of CAD played by novel biomarkers such as neopterin or lactoferrin.

Although antimicrobial therapy is relatively effective in acute infections, clinical trials on CAD prevention have not found it to be effective in chronic infections. Because monocytes can be infected and antibiotics cannot fully inhibit chlamydial growth, antibiotic therapy may not be sufficient to prevent or eliminate infection of vascular tissue. For this reason, new approaches should be adopted in the design of assays and therapies against atherosclerosis associated with chronic infection.

The development of an effective vaccine is another challenge that has yet to be overcome, due to lack of knowledge on the immune host response and antigenic proteins. The ideal strategy would be to obtain a long-lasting protective immunity with a contribution from both humoral and cell immunity, although the latter seems to be more important. Proteomic studies have not yet identified a highly immunogenic and specific protein that could be a target for a vaccine, although novel candidates have recently been proposed, including Pmps. The use of preparations with several antigens may lead to the development of an effective vaccine.

Declaration of interest

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Affiliation

Enrique Villegas[1] PhD, Ana Camacho[2] PhD, Antonio Sorlózano[1] MD PhD, José Rojas[3] PhD & José Gutiérrez[†1] MD PhD
[†]Author for correspondence
[1]University of Granada,
School of Medicine,
Department of Microbiology,
Avda de Madrid 11,
E-18012 Granada, Spain
Tel: +34 958 24 20 71; Fax: +34 958 24 61 19;
E-mail: josegf@ugr.es
[2]Laboratory Vircell,
Department of Molecular Biology,
Granada, Spain
[3]Management D&R,
Laboratory Vircell,
Granada, Spain