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Research paper

Chlamydia pneumoniae sero-prevalence in Moroccan patients with cardiovascular diseases

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Abstract *Background:* Chlamydia pneumoniae is a pathogen associated with human respiratory tract infection, its viable presence in atherosclerotic plaques is also assumed to play significant function in cardiac diseases. Our study's main objective is to evaluate Chlamydia pneumoniae sero-prevalence in Moroccan patients with cardiovascular diseases using and comparing two serological methods.
Methods: Two hundred eighteen patients were enrolled; serums were tested by microimmunofluorescence to explore the sero-prevalence. Simultaneously 74 serums were analyzed by both immunoblot and micro-immunofluorescence to evaluate recombinant proteins diagnosis value.
Results: MIF results revealed 81% male and 84.5% female positive cases. The comparative study among 74 patients showed 78% men and 89% women positive cases by immunoblot, whereas MIF showed respectively 80% and 72%, a significant concordance between these methods was revealed. However, this comparison showed also two types of discrepancies, which may be related to difficulties in antigens detection by micro-immunofluorescence resulting from their structure complexity, or the antibodies reactivity with species' common antigens.
Conclusions: The study revealed a high sero-prevalence of Chlamydia pneumoniae in the studied population, a big interest of recombinant protein was also revealed in the diagnosis accuracy. We suggest therefore using immunoblot for diagnosis confirmation because it provides additional useful information.
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Highlights

- The study revealed a high sero-prevalence of *Chlamydia pneumoniae* in the studied population.
- The comparative study between the two methods revealed a significant correlation.
- We suggest therefore using immunoblot for diagnosis confirmation because it provides additional useful information.

Introduction

Chlamydia pneumoniae (*C. pneumoniae*) is a common respiratory pathogen that causes a chronic and persistent airway infection [1,2]; a potential link of *C. pneumoniae* to cardiovascular diseases was also reported in numerous studies [3,4] and most of these studies have shown that *C. pneumoniae* chronic infection represents a risk factor for atherosclerosis, for myocardial infarction and stroke [5,6], the link of *C. pneumoniae* to stroke was explained by the detection of many elementary bodies related to *C. pneumoniae* in atherosclerotic plaques and fatty streaks in the aorta, coronary artery cases autopsy samples, coronary arterectomy, and carotid endarterectomy samples [7,8].

In Morocco, cardiovascular diseases constitute also a major public health issue. However, *C. pneumoniae* prevalence and data regarding its association with cardiovascular diseases are sparse. In our previous study, we investigated *C. pneumoniae* infection in Moroccan patients suffering from cardiovascular diseases by molecular detection, and results revealed 61% and 86% positive cases in peripheral blood mononuclear Cells and atheroma plaques respectively [9]. The bacterium isolation is difficult and molecular tools are specific, but often limited to few laboratories [10]. Therefore, serologic methods are the most commonly used in *C. pneumoniae* diagnosis [11].

The main objective of this study is to evaluate the seroprevalence of *C. pneumoniae* infection among Moroccan patients with cardiovascular diseases and evaluate the recombinant proteins diagnosis value by the comparison between microimmunofluorescence (MIF) and immunoblot methods.

Methods

A total of 218 patients, including 147 males and 71 females (mean age 65 years), consulting in cardiovascular surgery department, CHU Ibn Rochd Casablanca Morocco were enrolled. A complete clinical examination was performed to confirm the cardiovascular diseases and risk factors including: age, diabetes, blood pressure, alcohol, and tobacco use were assessed.

Patients' informed consent was obtained before clinical and behavioural data recording and blood samples collection. Sera were frozen at −20 °C until analysis.

The study was approved by an institutional/local ethics committee and funded by Institut Pasteur du Maroc, Morocco.

Micro-immunofluorescence

IgG anti chlamydiae were measured in two hundred eighteen patients' serums by MIF test according to Wang et al., the antigens used consisted of formalin-fixed elementary bodies (EB) grown in an egg yolk sac and suspended in a saline buffer. The whole elementary body suspensions of *Chlamydia trachomatis*, *C. pneumoniae* and *Chlamydia psittaci* were fixed separately in the slides' wells. Serums were diluted in a geometrical progression and classical immunofluorescence test steps were performed for screening [12]. Slides were then read by visualisation under an UV-illuminated microscope. Positive test was revealed by a bright−apple green fluorescence and dilution 1:16 was considered as cut-off.

Immunoblot

A total of 74 serums previously tested by MIF, randomly selected were simultaneously analyzed by immunoblot test for serological comparison.

Using the recombinant proteins as antigens, the immunoblot test's advantages lie in both possibility of simultaneous detection of multiple antigens (good sensitivity) and different immunologic profiles revelation (good specificity).

The recombinant proteins used as highly purified antigens of the three chlamydiae species were: *C. trachomatis* (MOMP, OMP2, HSP60, and MIP), *C. pneumoniae* (MOMP, OMP2, OMP4, and OMP5) and *C. psittaci* (MOMP).

The MOMP or OMP1, (Outer Membrane Protein 1) is a 40-kDa porin, with constant and variable immunogenic domains, MOMP is associated to lipo-polysaccharide on the chlamydial outer membrane [10,11].

The MIP (Macrophage Infectivity Potentiator) is a 27-kDa lipoprotein, homologous to a protein from Legionella pneumophila, and could play a role in *C. trachomatis* pathogenicity [13]. The HSP60 (Heat Shock Protein 60) is a 60-kDa heat shock protein, its sequence is conserved among procaryotic and eukaryotic species, the regulation of its expression is variable upon external conditions, HSP60 is a very immunogenic protein and is considered as a marker associated to the severity of *C. trachomatis* infection [14].

The OMP2 (Outer Membrane Protein 2) is a 60-kDa cystein-rich protein, which does not seem to be exposed on the surface of chlamydial Elementary Bodies (EB), but could be associated to recent infections [15,16]. The OMP4 (Outer Membrane Protein 4) and OMP5 (Outer Membrane Protein 5) are rather unstable 100-kDa proteins, with

exposed epitopes. These proteins belong to the POMP (Polymorphic Outer Membrane Protein) family of *C. pneumoniae* [17].

These recombinant proteins are demonstrating western blot multivalent, sensitive, specific, and able to detect antigenic reactivity that appear to be genus and species specific, these antigens are coated on a nitrocellulose membrane.

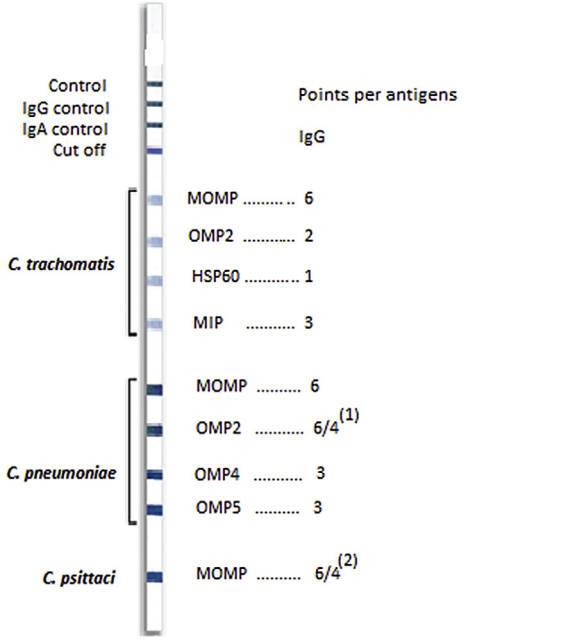
Immunoblot assay was performed and results interpretation was done following the manufacturer's instructions (Micrgen, Germany; distributor in France, AllDiag), antigens scoring are mentioned in Fig. 1. Scores $\geq$6 are considered positive.

Statistical analysis was performed using the EPI INFO of CDC, version 7. Categorical variables were analyzed by chi square (χ^2) and P-values $<$0.05 are considered statistically significant.

Results

Classical risk factors analysis

A total of 218 patients were enrolled, one hundred forty-seven males (67%) and seventy-one females (33%), M/F gender ratio 2%. Subjects' gender and age distribution showed a significant males' exposition to cardiovascular diseases, mostly in age group between 40 and 80 years old. The cardiovascular diseases most observed were myocardial infractus (49%), angora (29%), and stroke (14%). The behavioural risk factors (tobacco and alcohol use) and pathologic risk factors' (arterial hypertension and diabetes) distribution showed a dominance of tobacco (57%) and alcohol use (19%) among men. However, arterial hypertension was dominant among women (65%). The difference was significant between males and females in diabetes, arterial hypertension, alcohol and tobacco use ($p < 0.05$).



(1) = 6 points, if serology *C. trachomatis* being negative, otherwise 4 points.
(2) = 6 points if MOMP of *C. trachomatis* or *C. pneumoniae* is negative, resp., otherwise 4 points

TOTAL SCORE	RESULTS
≤ 3	Negative
4 - 5	Borderline
≥ 6	Positive

Figure 1 Positive immunoblot strip and antigens scoring. (Micrgen, Germany; distributor in France, AllDiag).

MIF and immunoblot results

MIF test revealed 82% positive cases in IgG anti *C. pneumoniae* among total patients, those with high titers equal or more than 512 UI represent 44% cases. Positivity distribution according to the gender didn't show significant difference between males and females ($p = 0.521$), with respectively 81% and 84,5%.

The comparison study between MIF and immunoblot tests concerned 74 patients. Immunoblot analysis showed 78% positive men and 89% positive women in IgG anti *C. pneumoniae*; whereas MIF results showed antibodies in 80% of men and 72% of women. No significant difference between MIF and immunoblot was observed when measuring anti *C. pneumoniae* ($p = 0.682$) and anti *C. trachomatis* ($p = 0.184$) antibodies in male and female patients (Table 1), the difference was however significant when researching anti *C. psittaci* antibodies ($p = 0.008$).

Furthermore, the results analysis let dividing the studied population into four groups. In the first group "G1" (n = 5), patients were negative in both MIF and immunoblot, in the second group "G2" (n = 54) patients were positive in both MIF and immunoblot, the third group "G3" (n = 11) patients were negative in MIF but positive in immunoblot. The last group "G4" (n = 4), MIF was positive but immunoblot was negative.

The analysis of immunologic profiles was restricted to immuoblot positive groups "G2" and "G3".

Immunologic profiles analysis in "G2" patients

Profile analysis in "G2" divides patients into two subgroups.

Subgroup1: *C. pneumoniae* MIF positive cases (n = 28)
In this subgroup, immunoblot results revealed 13 profiles with both *C. pneumoniae* and *C. trachomatis* markers (Fig. 2(c)), 12 profiles with *C. pneumoniae* markers (Fig. 2(b)), and 3 profiles revealing *C. trachomatis* markers (Fig. 2(a)). Following the manufacturer scoring, patients with *C. pneumoniae* markers only (43%) showed 8 diverse immunologic profiles, but only seven among these patients are *C. pneumoniae* positive. The patients with *C. trachomatis* markers (11%) revealed 3 immunologic profiles, according to the manufacturer scoring; only CtMOMP profile is *C. trachomatis* positive. Patients presenting both of *C. pneumoniae* and *C. trachomatis* markers representing 46% cases belong to 12 diverse profiles, among these patients, 67% are both *C.*

Table 1 Comparison of Chlamydia species prevalence obtained by MIF and Immunoblot according to the gender.

	Males (n = 56)		Females (n = 18)		Total patients (n = 74)	
	MIF	Immunoblot	MIF	Immunoblot	MIF	Immunoblot
C. pneumoniae	45 (80%)	44 (78%)	13 (72%)	16 (89%)	58 (78%)	60 (81%)
P value	0.815		0.206		0.682	
C. trachomatis	19 (34%)	28 (50%)	9 (50%)	(44%)	28 (38%)	36 (49%)
P value	0.0884		0.738		0.184	
C. psittaci	9 (16%)	1 (2%)	1 (6%)	0 (0%)	10 (14%)	1 (1%)
P value	0.0162		1		0.008	

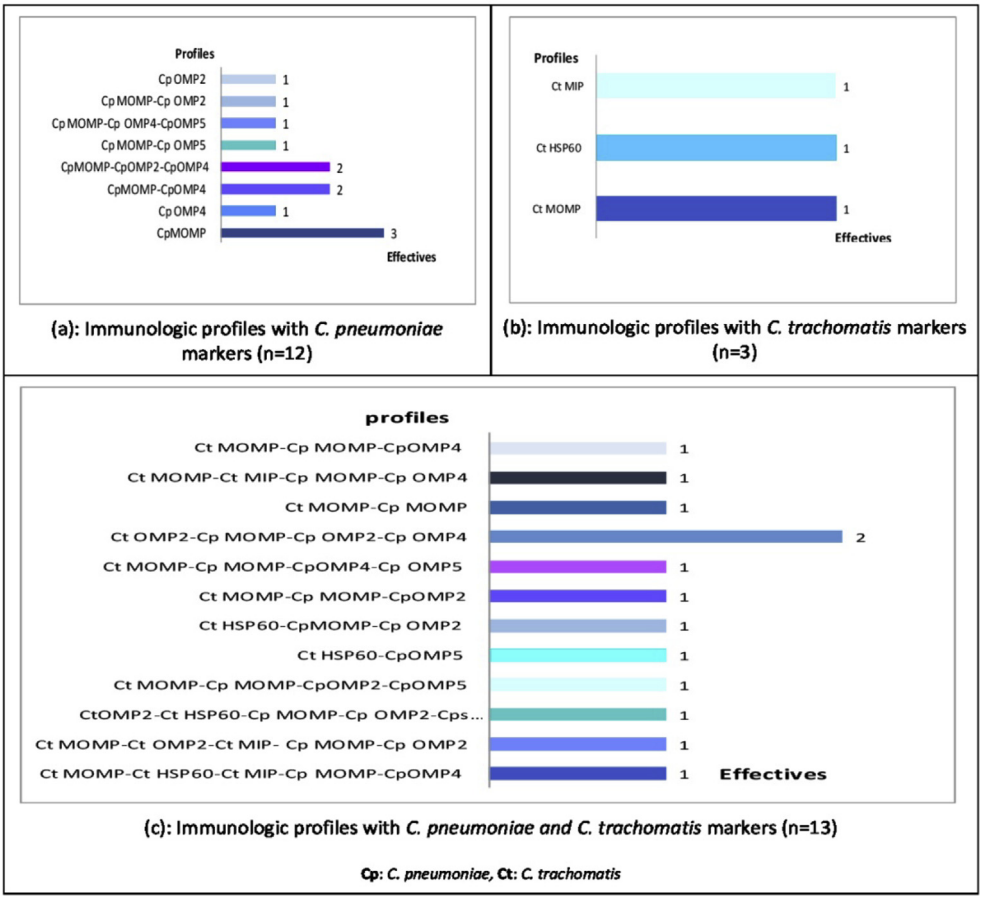


Figure 2 Immunologic Profiles of *C. pneumoniae* MIF positive cases "G2". Subgroup 1: MIF seropositive in *C. pneumoniae* (n = 28).

pneumoniae and *C. trachomatis* positive, and 33% are only *C. pneumoniae* positive.

Subgroup 2: *C. trachomatis* and *C. pneumoniae* MIF positive patients (n = 26)

Immunoblot results revealed 13 patients with both of *C. pneumoniae* and *C. trachomatis* markers (Fig. 3(f)), 11 patients with *C. pneumoniae* markers (Fig. 3(d)) only, and 2 profiles with *C. trachomatis* markers only (Fig. 3(e)).

Immunoblot profiles with *C. pneumoniae* markers only (n = 11), showed five immunologic profiles, four profiles (80%) are *C. pneumoniae* positive according to the manufacturer scoring. The fifth profile presents *C. pneumoniae* marker. Profiles showing *C. trachomatis* markers alone (n = 2) revealed two immunologic profiles and are *C. trachomatis* positive. Patients with both *C. pneumoniae* and *C. trachomatis* markers (n = 13) showed 12 different profiles, 83% are both *C. pneumoniae* and *C. trachomatis* positive and 15% are *C. pneumoniae* positive and present *C. trachomatis* markers.

Immunologic profiles analysis in G3 patients

Immunologic profiles analysis in G3 showed a large heterogeneity and let dividing patients into two subgroups.

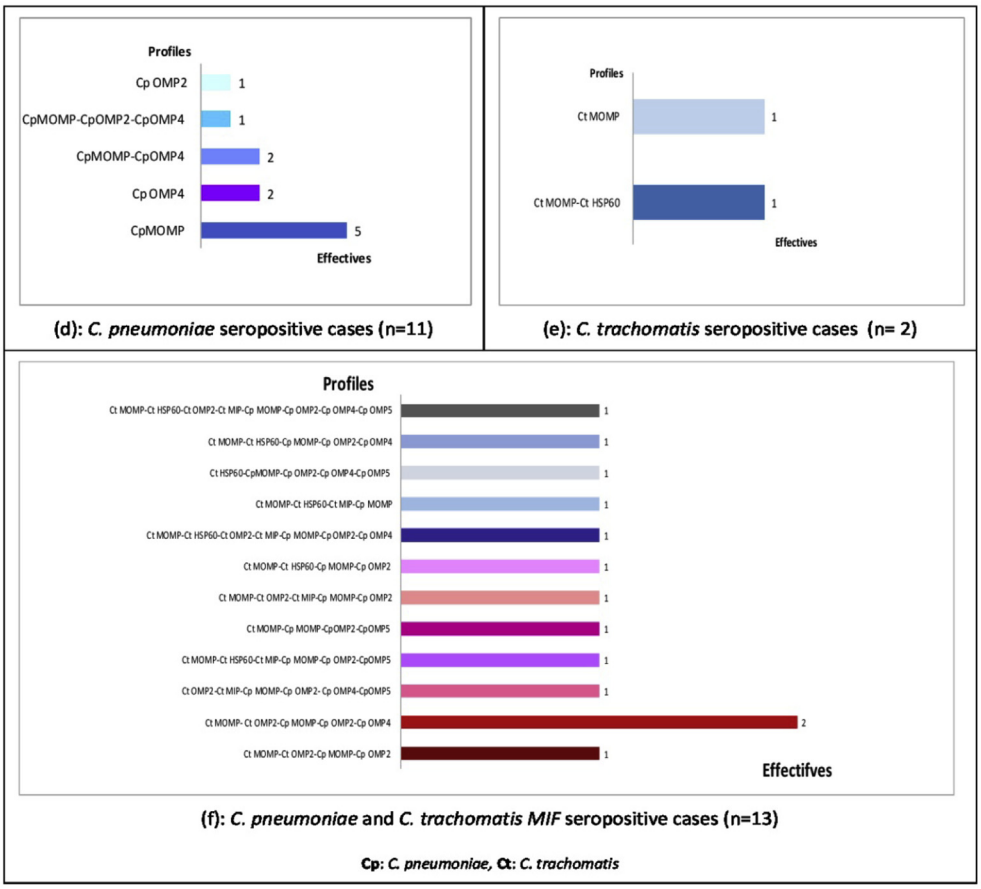


Figure 3 Immunologic profiles of *C. pneumoniae* and *C. trachomatis* MIF positive cases "G2". Subgroup 2: *C. pneumoniae* and *C. trachomatis* MIF seropositive (n = 26).

Subgroup 3: *C. pneumoniae* positive patients (n = 7)
Immunoblot analysis in these patients showed 5 profiles 80% (4/5) which are *C. pneumoniae* positive (Fig. 4(g)).

Subgroup 4: *C. trachomatis* and *C. pneumonia* positive patients (n = 4)
Immunoblot analysis and profiling showed 4 profiles, 75% (3/4) cases are positive in both *C. trachomatis* and *C. pneumoniae* and 25% (1/4) is *C. pneumoniae* positive only (Fig. 4(h)).

Exploration of the anti-recombinant protein antibodies reactivity

Simultaneous detection of antibodies against recombinant proteins indicated relative antigenicity of each individual protein. Immunologic profiles showed a dominant humoral immune response against *C. pneumoniae* MOMP. This reactivity occurred in 68% cases. The reactivity against OMP4 is secondary with 46% cases and is followed by reactivity against OMP2, which occurred in 39% cases. Finally, the reactivity against OMP5 represents 15% cases. The humoral immune response against *C. trachomatis* MOMP was revealed in 32% (24/74) cases, it is followed by reactivity against CtHSP60 in 20%, CtOMP2 in 14%, and CtMIP with 15% cases.

Discussion

Demographic data analysis showed 2% M/F gender ratio, reflecting cardiovascular diseases' dominance in male patients. Similar results were reported by Azzouzi N and Padmavati S studies [18,19]. The age-related distribution showed that cardiovascular diseases are dominant in patients' age group ranging from 40 to 80 years. The affection of the same age group was reported in Indian population [19].

Behavioral and pathologic risk factors analysis and comparison between the two genders showed that tobacco use and arterial hypertension are the primary risk factors in males. Whereas, diabetes and hypertension are the principal risk factors in females. These results are comparable to those found in Spanish population [20]. In addition, the difference is significant between males and females in diabetes, arterial hypertension, alcohol and tobacco use ($p < 0.05$).

Micro-immunofluorescence results showed a high IgG anti *C. pneumoniae* prevalence estimated at 82% (81% males and 84.5% females). Our results are comparable to those described in Spanish population [21], where the sero-prevalence was evaluated at 95%. Similar sero-prevalence studies were also carried out in Spain and showed identical results with respectively 78% and 71.6% [22,23]. The similarity of these studies' results with ours suggests that

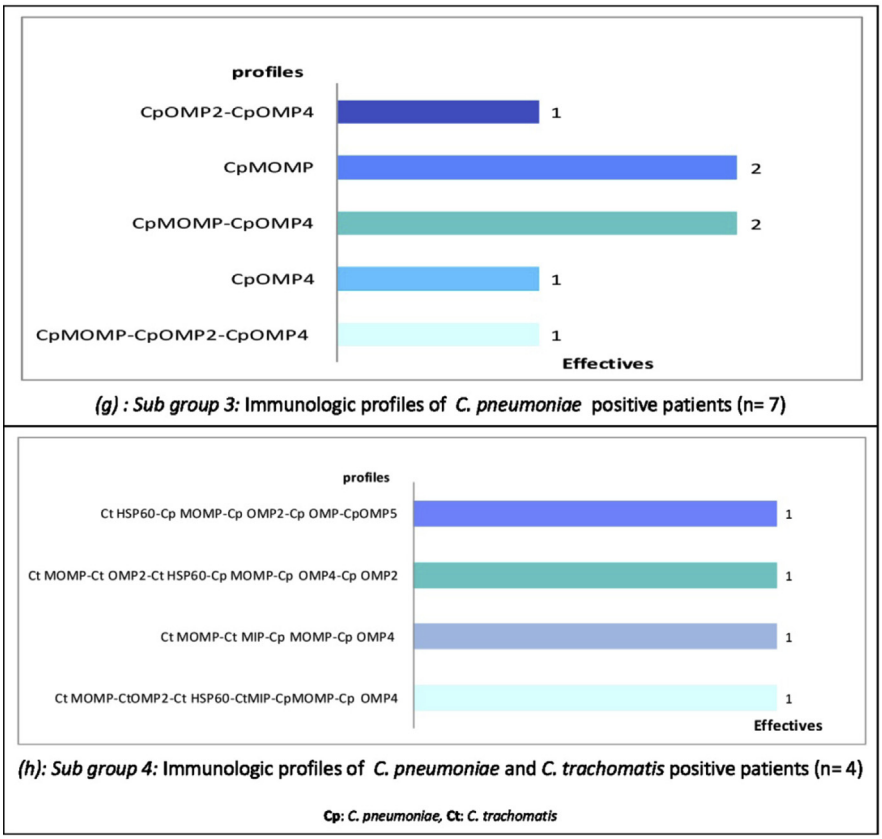


Figure 4 Immunologic profiles of MIF negative cases "G3" (n = 11).

the countries proximity and exchange between Moroccan and Spanish populations can be considered as factors of infection's transfer and spread between Moroccan and Spanish populations.

The repartition of *C. pneumoniae* infection based on gender showed that males and females are similarly infected with no significant difference of exposure ($p > 0.05$).

High prevalence with high titers of *C. pneumoniae* suggests that this infection can represent a significant risk factor in cardio-vascular diseases [24,25]. Our results are in agreement with those reported in Tiran A et al 2004. In these studies, they examined whether serological tests are able to predict individual endovascular infection using MIF. The results revealed 77% positive cases and they concluded that serologic exams could be used as a marker to identify patients with cardiovascular diseases [26].

MIF is the recommended serological method for Chlamydiae infection diagnosis. It's sensitive, specific, and considered as reference test [27]. However, because of Chlamydia species antigenic structure complexity, MIF interpretation remains difficult [27,28]. Thus, immunoblot using recombinant proteins is proven to be multivalent, sensitive, specific, and able to detect antigenic reactivity that appears to be genus and species specific.

MIF and immunoblot results showed a significant concordance when measuring IgG anti *C. pneumoniae* and anti *C. trachomatis* ($p > 0.05$). However, two types of discrepancies were found. The comparison of results and profiles analysis in subgroup 1 (group 2) indicate that *C. pneumoniae* MIF positive patients have a reactivity with one or more *C. trachomatis* proteins in immunoblot (*C. trachomatis* false negative patients). In group 3 patients, MIF is negative in both of *C. pneumoniae* and *C. trachomatis* whereas immunoblot revealed also reactivity with one or more proteins. These results could be related to antibodies reactivity with recombinant proteins in these cases, the antigens could not be detected by MIF test because of their structures' complexity and their deep location in the bacterium membrane. In the other cases, MIF is positive in both of *C. trachomatis* and *C. pneumoniae*. However, immunoblot revealed positive cases in only one of two species. This could be related to cross-reactivity in MIF test, which be explained by antibodies' reactivity with species common antigens such as MOMP and LPS. These results are comparable to those reported in Biendo et al.1996 study [28].

The use of serological methods to assess the relationship between *C. pneumoniae* infection and cardiovascular diseases remains dependant on the serological methods and antigens used [29,30]. Chlamydiae's outer membrane surface antigens are very complex; some of them are specific but poorly immunodominant. Others have stronger immunogenicity but are cross-reactive among chlamydia species. Therefore, new and highly immunodominant species' specific antigens should be sought [31].

In the present study, we attempted to determine the recombinant proteins diagnostic value, because

simultaneous detection of antibodies directed against the recombinant proteins allowed the evaluation of relative antigenicity of each individual protein. The immunological profiles analysis showed that the humoral immune response directed against *C. pneumoniae* MOMP was dominant. It's considered to be the best immunogenic protein and is therefore the best candidate for vaccine development [32]. We also noticed that reactivity against *C. trachomatis* MOMP was prominent in immunological profiles. This demonstrates that the MOMP of *C. pneumoniae* and C. *trachomatis* are the most immunogenic antigens and are good indicators that chlamydia infections can trigger an autoimmune reaction that could be the primary cause of cardiovascular disease.

Conclusion

MIF is the recommended serological method for chlamydia infection diagnosis, it's still considered as the reference. However, because of interpretation's difficulties, western blot allows species specific antigen analysis and distinction between a cross-reaction and a double specific seropositivity. Hence, we propose its use as a confirmation test.

Ethics

The study was approved by the Institution's ethics committee and informed consent was taken at patients' recruitment.

Authorship statement

All authors certify that they have participated sufficiently in the work to take public responsibility for the content, including participation in the concept, design, analysis, writing, or revision of the manuscript.

Conflicts of interest

All authors approved the manuscript content and declare that no conflict of interest exists.

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Provenance and peer review

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