Evaluation and optimization of commercial Enzyme Linked Immunosorbent
Assay kit for detection of *Chlamydophila pneumoniae* IgA antibodies

Running title: Optimization of ELISA using ROC analysis

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**Key words:** *Chlamydophila pneumoniae*, MIF, ELISA, IgA, TG-ROC.

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Abstract

Background: Serology is the method of choice in routine clinical laboratories for diagnosis of acute *Chlamydia pneumoniae* (Cpn) infection. IgG and IgM antibodies to Cpn are routinely analyzed. However, the significance of the detection of chlamydial IgA antibodies for serological diagnosis of chlamydial infections remains unclear. IgA antibodies have been found to be of interest in diagnosis of chronic Cpn infections. At present, the microimmunofluorescence (MIF) test is the recommended method for the measurement of Cpn antibodies. Some Enzyme Linked ImmunoSorbent Assays (ELISA) has been developed and is commercially available. However, these ELISA have not been fully validated. The purpose of this study was to evaluate and to optimize a commercial ELISA, SeroCP IgA test for the detection of Cpn IgA antibodies.

Methods: Ninety four sera from patients with IgG antibodies titers to Cpn $\geq 256$ (study group) and 100 sera from healthy blood donors (control group) were tested for the presence of IgA antibodies to Cpn by our in house MIF and the SeroCP IgA test. Two Graph Receiver Operating Characteristic (TG-ROC) curves were created to optimize the cut off given by the manufacturer.

Results: By the in house MIF test and the SeroCP IgA test, Cpn IgA antibodies were detected in 72 % and 89 % of sera from study group, and in 9 % and 35 % of sera from control group, respectively. Using MIF test as the reference method, the characteristics of the ELISA test calculated with the advised cut off value correlated in 76 % with an agreement of 0.54. Applying the optimized cut-off value using the TG-ROC analysis which is 1.65, a better concordance (86%) and a good agreement (0.72) were found between the MIF and the SeroCP IgA tests.

Conclusion: The use of the TG-ROC analysis described in this work may provide an approach to the standardization and optimization of ELISAs which are simpler, more objective and less time consuming than the MIF test. Commercial ELISA kits should be standardized and optimized and so might give better performance.
Background

*Chlamydia pneumoniae* (Cpn) is a common cause of acute respiratory infections, mainly pneumonia and other acute upper and lower respiratory tract infections (bronchitis, sinusitis, otitis and pharyngitis). About 5-20% of the community acquired pneumonia cases in adults and children have been associated with Cpn infection [1,2]. Up to now, there is no totally satisfactory serological method for the diagnosis of Cpn infection. However, during a workshop dealing with the standardization of Cpn diagnosis methods, the Centers for Disease Control and Prevention (CDC) recommended the use of the MIF test as the reference serological test, despite the poor predictive value of a single high IgG titer [3]. Diagnosis of acute infection is based on paired serum samples obtained within a period which varies from 4 to 8 weeks apart that show four fold increases in IgG antibody titers or in IgM antibody positivity in a single serum sample. IgM is helpful for rapid diagnosis of acute Cpn infections because of its early appearance compared to the IgG antibodies. The significance of the detection of chlamydial IgA antibodies for serological diagnosis of chlamydial infections remains unclear. The persistence of these short lived [4] specific Ig A antibodies to Cpn has been suggested as a marker of persistent infection [5], and has been used as criterion for the definition of chronic Cpn infection [6-10]. In fact, several studies have demonstrated an association between specific Cpn Ig A antibodies and several chronic diseases such as chronic obstructive pulmonary disease [11], cardiovascular disease [12,13], chronic pharingitis [14] and both upper and lower chronic respiratory tract infections [15].

Several methods were used for serological diagnosis of chlamydial infections such as the MIF test which represents the reference method for the diagnosis of Cpn infection. This test requires a highly experienced reader and has significant subjective components. It can be difficult to interpret and it usually requires both an acute and convalescent specimen to demonstrate an antibody titre rise. Furthermore, it lacks standardization [16]. Because of these problems related
to the MIF test, some partially automated commercial Enzyme Linked Immuno Sorbent Assay (ELISA) have been developed and are at present commercially available. They are relatively simple to perform, less time consuming, more objective and easier to standardize. However, these commercial ELISA have not been fully validated. They seem to be less specific but more sensitive than the MIF test [3].

The aim of this study was to evaluate and optimize a commercially available ELISA, the SeroCP IgA test, for the detection of specific Cpn IgA antibodies in comparison to our in house MIF. This study is not a diagnostic one, but an assay evaluation since no convalescent-phase sera has been used.

Methods

Sera

Study group

Serum samples were obtained from patients attending the department of infectious diseases and suspected to have chlamydial infections from January 2002 to November 2004. Serology was performed in the laboratory of microbiology in the university hospital of Sfax, Tunisia. Ninety four sera with IgG anti Cpn ≥ 256 by the in house MIF test were selected for the detection of anti Cpn IgA antibodies.

Control group

During the same period of this study, 100 sera specimens were collected from healthy blood donors.

Techniques

MIF test
Chlamydial IgG, IgM and IgA antibodies were determined using simplified method of the MIF test as described by Wang and Grayston [17]. The antigens of three species of *Chlamydiae* have been used: the L2 *Chlamydia trachomatis* strain, the Loth *Chlamydophila psittaci* strain and the IOL-207 Cpn strain. These antigens were produced in yolk sac membranes of infected eggs. The sac of uninfected eggs was used as negative control. Sera were diluted from 1/16 to 1/4096 in order to determine their IgG antibodies titers. Prior to IgM and IgA testing, all sera were absorbed by using the rheumatoid factor adsorbent in order to remove IgG and rheumatoid factor interactions. Cut off titres for positive sera were 1/12 for IgM and IgA antibodies respectively.

SeroCP-IgA test

All sera were tested for the presence of IgA antibodies to Cpn by a commercial ELISA: the SeroCP™-IgA® test (SeroCP) (Savyon Diagnostics Ltd, Germany) according to the manufacturer’s instruction. The IgA cut off value (COV) was calculated as twice the mean absorbance value at 450 nm (A$_{450}$) of the two negative controls tested in each run. The cut off index for each sample is calculated by the formula: cut off index = sample A$_{450}$/COV in order to normalize results of different runs. The threshold index for a positive test was 1 as recommended by the manufacturer.

Statistics

All data were collected using standardized forms and were analyzed using Epi-Info version 6. To assess the agreement between MIF and the SeroCP-IgA test, we used K (nominal scale variables) as proposed by Landis and Koch [18]. Guidelines for the interpretation of K were as follows: K<0.20, poor agreement; K=0.21 to 0.40, fair agreement; K=0.41 to 0.60, moderate agreement; K=0.61 to 0.80, good agreement; K=0.81 to 1.00, very good agreement. Two-Graph Receiver
Operating Characteristic analysis (TG-ROC) [19] was used to optimize the cut off index of the SeroCP-IgA test.

Results

Seroprevalence of Cpn IgA antibodies

IgA antibodies to Cpn were found in 72% (68/94) and in 89% (84/94) of sera from study group by the in house MIF and the SeroCP-IgA test using manufacturer cut off respectively. Furthermore, the positivity to Cpn IgA antibody was found to be increased with the increase of IgG antibodies titers (table 1).

The seroprevalence of Cpn IgA antibodies in the control group obtained by the in house MIF and the SeroCP-IgA test using manufacturer cut off was 9% (9/100) and 35% (35/100) respectively. Sixty of 100 sera from healthy blood donors (60%) had IgG antibodies titers to Cpn above 1/16 using the MIF test. Five of these sera had IgG antibodies titers to Cpn above 1/256 and were positive to Cpn IgA antibodies by MIF and ELISA. For the other 95 sera that had IgG antibodies titers less than 1/256, 4 were IgA positive by MIF and 30 were IgA positive by ELISA.

Correlation between detection of Cpn IgA antibodies by MIF and ELISA before optimization

When applying the cut off advised by the manufacturer on the total sera tested, a concordance of 76% and a moderate agreement (k = 0.54) were seen between the MIF and the SeroCP-IgA test (table 2). The correlations between the SeroCP-IgA test index and MIF seropositivity in sera from study and control groups are shown in figure 1. The concordance between the positivity of the in house MIF and that the SeroCP-IgA test is 78% in sera from the study group and 74% in sera from the control group as shown in table 2. According to the cut off value given by the manufacturer, the agreement between the two tests was fair in the study group (k = 0.34) and in the control group (k= 0.31).
Optimization of the SeroCP-IgA test

TG-ROC analysis was performed to determine an optimized cut off value for the SeroCP-IgA test. The sensitivity and specificity were plotted in comparison to the MIF results when different scores were used for cut off index (figure 2) and the one which gave the highest sensitivity and specificity was determined to be the cut off value to be used. TG-ROC analysis indicated that an optimal cut off index for the SeroCP-IgA test applied on the total sera tested would be 1.65 when compared to the MIF test.

Correlation between detection of Cpn IgA antibodies by MIF and ELISA after optimization

When applying the new and optimized cut off on the total sera tested, a concordance of 86 % and a good agreement (k = 0.72) were seen between the MIF and the SeroCP-IgA test (table 2). A concordance of 80 % and a moderate agreement (k = 0.51) were seen between the MIF and the SeroCP-IgA test in sera from study group. Similarly for the control group, a concordance of 92 % and a good agreement (k = 0.62) were obtained between these two tests.

Discussion

We tested by MIF and ELISA the presence of IgA antibodies to Cpn. The MIF test used in this study is an in-house test which was validated using a commercial kit for Cpn (bioMerieux®, France) (data not shown). The seroprevalence of Cpn IgA antibodies in the tunisian healthy population was 9 %. Various studies reported that the seroprevalence of IgA antibodies to Cpn in the healthy population is ranging from 5 % to 55 % [10,12]. Our sera were also used to determine the prevalence of Cpn IgG antibodies that were, as expected, widespread among the tunisian healthy population (60 %). Our findings are generally in line with other studies where the seroprevalence of this common pathogen in the middle age is about 50 % [20-22] and is higher than 70 % in the adult population [1,23,24]. In Germany, The seroprevalences of Cpn IgA and
IgG antibodies in sera from German asymptomatic healthy blood donors were 50% and 71% respectively [25]. A lower prevalences of IgA and IgG antibodies have been reported (5% and 16.7%) in Norwegian healthy controls [26]. Ramano Carratelli and colleagues [27] reported that the prevalence of IgA and IgG antibodies in healthy Italian subjects was 5 and 35% respectively by the MIF test. Therefore, the detection of Cpn IgG antibodies seems to be a more sensitive method for the determination of the seroprevalence of anti-Cpn antibodies in a healthy population [25,27]. In our study, the positivity of Cpn IgA antibodies decreased with the decrease of IgG antibodies titers in study group. Vammen and colleagues [28] also demonstrated a correlation between IgA antibodies titers and IgG antibodies titers to Cpn by MIF test.

Various studies have compared the performance of ELISA in relation to the MIF test considered as the gold standard for the diagnosis of Cpn infection [25,29]. In our study, we focused on the SeroCP ELISA as this test proved to be more closely approach the results of MIF test than other commercial diagnostic kits [29]. The distribution of the SeroCP-IgA index in relation to the MIF IgA antibodies seropositivity to Cpn showed a good correlation. High ELISA indexes were seen only with seropositive MIF IgA antibodies in sera from study group. Our findings suggest that there is a moderate concordance between the detection of Cpn IgA antibodies by our in house MIF and the SeroCP-IgA test varying from 74 to 78% before optimization. This may be due to the higher dilution used in the SeroCP-IgA test (1/105) compared to that of the MIF test (1/12). Similar results were found by Ciervo and colleagues [30] who evaluated and optimized the SeroCP-IgA test for the detection of anti-Cpn IgG and anti-Cpn IgA antibodies against a commercial MIF (Labsystem) used as a gold standard in patients with coronary heart disease. In their study, they have also found that before optimization, the two tests used for the detection of Cpn IgA antibodies were not sufficiently correlated. In another study, Paldanuius and colleagues [31] compared the labsystem ELISA kit for the detection of Cpn IgA antibodies in relation to
their in house MIF in sera from healthy laboratory personnel in Finland. They found that the agreement between the two tests was moderate for IgA antibodies (K = 0.43) and that the prevalence of IgA antibodies detected by ELISA is about 30% higher than that by the in house MIF. Romano and colleagues [27] showed evidence of a relation between antibodies to Cpn and coronary heart disease, however, statistically significant differences were observed with different methods used. The same problem was previously reported with the detection of IgG antibodies by different serological methods. Hoyman and colleagues [32] compared five commercially available serological assays for the detection of Cpn IgG antibodies in sera from healthy individuals and in sera from patients with coronary artery disease and found important differences. Therefore, the choice of a serological test is of major importance in both diagnostic and seroepidemiological studies[26,31,32,33].

At present time, the MIF test is the recommended method for the measurement of Cpn antibodies [3] and has been used in most clinical studies. However, this test is time-consuming and requires skilled personnel for the interpretation of the slides. Furthermore, the specificity of MIF has been questioned since cross-reactions between Chlamydial species occur [34-36]. Because of all these problems related to MIF, ELISAs were commercially developed; they are relatively simple to perform, are less time-consuming, more objective and easier to standardize. On the other hand, ELISA has been recognized as an unreliable method for Cpn identification compared to the MIF test [37]. In our study, TG-ROC analysis was used to evaluate and optimize the SeroCP IgA test in comparison to our MIF test used as the gold standard. In fact, test characteristics depend on the cut off value used. Instead of using the cut off value that was advised by the manufacturer, new cut off values were estimated from the TG-ROC to obtain the best discrimination between positive and negative test according to the MIF test [19]. The TG-ROC plots sensitivity and
specificity as a function of cut off and the one which gives the highest sensitivity and specificity was determined to be the cut off value to be used. In our study, the best sensitivity and specificity plot gave a cut off index of 1.65. After optimization of the cut off index of the SeroCP-IgA test using the optimized cut off index, we have found a better concordance and a good agreement between the MIF and the SeroCP-IgA tests. Hoymans and colleagues [32] reported that the use of different serological assays would be no problem if the agreement between the tests is high.

**Conclusions**

The results of our study indicate a high prevalence of Cpn IgA antibodies among the patients with IgG antibodies to Cpn ≥ 256. We have evaluated and optimized the SeroCP-IgA test in order to approach the results found by the gold standard: the MIF technique. Our results indicated that an optimal cut off index for the SeroCP-IgA test would be 1.65. The use of the TG-ROC analysis may provide an approach to the standardization and optimization of ELISAs which are simpler, more objective and less time consuming than the MIF test. Commercial ELISA tests would be standardized and optimized and so they might give better performance.

**Competing of interests**

The authors declare that they have no competing interests.

**Authors’ contributions**

Author O.F.G. carried out laboratory experiments, the evaluation and optimization of the ELISA, analyses of data, and drafted the manuscript. Author R.G. participated in the analysis of data and coordination of the study. Author A.Z. participated in the analysis of data and coordination of the study. Author N.B.A. participated in the collection of data. Author J.G. provided the healthy
blood sera and participated in the analysis of data. Author M.B.J. provided the patients sera and participated in the analysis of data. Author A.H. participated in design, data analyses, coordination of the manuscript and study.

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Reference List


Figure legends

**Figure 1.** Distribution of SeroCP IgA index in relation to the MIF IgA antibodies seropositivity.

a, Distribution of SeroCP IgA index in relation to the MIF IgA antibodies seropositivity in sera from study group.

b, Distribution of SeroCP IgA index in relation to the MIF IgA antibodies seropositivity in sera from control group.

The continuous lines in figure a and b represent the cut off index of SeroCP advised by the manufacturer.

The discontinuous lines in figure a and b represent the optimized cut off value.

**Figure 2:** TG-ROC analysis of the SeroCP-IgA test in sera from study and control groups.

Vertical line indicates the suggested cut off value (1.65).

se: sensitivity, sp: specificity.
### Table 1. Positivity of IgA antibodies in relation to MIF IgG antibodies titers in study group

<table>
<thead>
<tr>
<th>MIF IgG antibodies titers</th>
<th>256</th>
<th>512</th>
<th>1024</th>
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<tbody>
<tr>
<td>n = 62</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MIF IgA + (%)</td>
<td>41 (66)</td>
<td>12 (75)</td>
<td>6 (85)</td>
<td>9 (100)</td>
</tr>
<tr>
<td>ELISA IgA + (%)</td>
<td>57 (86)</td>
<td>15 (94)</td>
<td>7 (100)</td>
<td>9 (100)</td>
</tr>
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Table 2. Correlations between the detection of Cpn IgA antibodies by MIF and ELISA tests

<table>
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<th>MIF / ELISA</th>
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<th>After optimization</th>
</tr>
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<tr>
<td></td>
<td>Total</td>
<td>Study group</td>
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<tr>
<td></td>
<td>n=194</td>
<td>n=94</td>
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<tr>
<td>+ / +</td>
<td>75</td>
<td>66</td>
</tr>
<tr>
<td>- / -</td>
<td>73</td>
<td>8</td>
</tr>
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<td>+ / -</td>
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</tr>
<tr>
<td>- / +</td>
<td>44</td>
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</tr>
<tr>
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<td>K</td>
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Figure 1
Figure 2