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

Running title: Optimization of ELISA using ROC analysis

Olfa FRIKHA-GARGOURI¹, Radhouane GDOURA¹, Abir ZNAZEN¹, Nozha BEN ARAB², Jalel GARGOURI³, Mounir BEN JEMAA² and Adnane HAMMAMI¹*

1: Department of Microbiology and research laboratory "Microorganismes et Pathologie Humaine", Habib Bourguiba hospital of Sfax, Tunisia.
2: Department of infectious diseases, Hedi Chaker hospital of Sfax, Tunisia.
3: Department of blood bank, Sfax, Tunisia.

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E-mail addresses:

Olfa FRIKHA-GARGOURI: Olfafrikha2005@yahoo.fr; Radhouane GDOURA: gdourar@yahoo.com; Abir ZNAZEN: abirznazen2001@yahoo.fr; Nozha BEN ARAB: nozhabenarab@yahoo.fr, Jalel GARGOURI: jalelgargouri@yahoo.fr; Mounir BEN JEMAA: mounir.benjemaa@rns.tn; Adnane HAMMAMI: adnene.hammami@rns.tn

*Corresponding author: Adnane HAMMAMI

Mailing adress: Laboratory of microbiology, university school of medecine of Sfax, avenue Magida Boulila, 3027, Sfax, Tunisia.

Phone: 216 74 241 888
Fax: 216 74 246 217

E-mail: adnane.hammami@rns.tn
Abstract

**Background:** Serology is the method of choice in routine clinical laboratories for diagnosis of *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 ≥ 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 $\geq 256$ by the in house MIF test were selected for the detection of anti Cpn IgA antibodies. Prior approval by ethic committee (Association d'Enregistrement et de Lutte Contre le Cancer du Sud Tunisien) was obtained.

Control group
During the same period of this study, 100 sera specimens were collected from healthy blood donors. The mean age of this population was 34 years (range, 19-56 years), most of them were men (90/100). All healthy subjects had given informed consent prior to the collection of sera.

Techniques

MIF test
Cpn species specific IgG and IgA antibodies were measured by our in house MIF test using purified elementary bodies of Cpn, IOL-207 strain, *Chlamydia psittaci* Loth strain and *Chlamydia trachomatis* (Ct) L2 strain, as antigens. These antigens were produced in yolk sac membranes of infected eggs. The sac of uninfected eggs was used as negative control. No further treatment of purified elementary bodies was made such as removal of the lipopolysaccharide. Slides were prepared as acetone fixed preparations of the purified antigens by experienced laboratory technicians able to maintain all conditions equal between test runs. The antigen densities for all experiments were guaranteed by an optimal concentration of elementary bodies. Sera were tested in serial twofold dilutions for IgG from 1/16 to the end point in order to determine their IgG antibodies titers and at a single dilution for IgA (1/12). Prior to IgA testing, all sera were absorbed using the rheumatoid factor absorbent (Dade Behring Marburg GmbH) in order to remove IgG and rheumatoid factor interactions according to the manufacturer's instructions. All MIF series included a positive and a negative serum. Cut off titre for positive sera were 1/12 for IgA antibodies. Incubation time was 30 minutes with diluted sera and 30 minutes with 1:300 fluorescein isothiocyanate (FITC) conjugated anti human immunoglobulin (biorad) in a moisture chamber at 37 ºC. After each incubation, the slides were washed twice for 5 minutes with PBS. The mounting fluid for setting coverslips on the slides contained glycerol in PBS buffer. Two experienced and independent readers viewed all the slides using a fluorescent microscope (Zeiss AxioStar Plus) with x 40 objective on the next day after the preparation of slides. In case of discordant reading, a third reader was needed. The interpretation of the results was done under the same microscope and by the same experienced readers in the same period. In the reading of the MIF test, only fluorescence with evenly distributed elementary bodies is an acceptable positive reaction.

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\text{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\text{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.

Reproducibility of the ELISA test

Reproducibility of the ELISA test was essayed by conducting on different days, the same serum
samples, run in the same conditions. The differences between the OD values for sera; with low,
medium and high reactivity to the SeroCP-IgA test; were lower than 20% (figure 1).

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 2. 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). The sensitivity and the specificity of the SeroCP-IgA test according
to the MIF test before optimization were 97.4 % and 62.4 % respectively (table 3).

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 3) 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. After optimization, the sensitivity and the specificity of the SeroCP-IgA test according to the MIF test were 84.4 % and 87.2 % respectively (table 3).

**Discussion**

In this study, we evaluated the detection of IgA antibodies to Cpn by our in house MIF and a commercial ELISA tests in patients suspected to have Cpn respiratory tract infections. Our MIF test was validated using reference sera and a commercial kit for Cpn (bioMerieux®, France) (data not shown). The seroprevalence of Cpn IgA and IgG antibodies in the tunisian healthy population was 9 % and 60 % respectively as determined by our MIF test. Our findings are generally in line with other studies where the seroprevalence of Cpn IgA and IgG antibodies ranged from 5 and 50 % and from 35 and 71 % respectively [1,10,12, 20-26]. Thus, 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 [25,26]. In our study, the positivity of Cpn IgA antibodies decreased with the decrease of IgG antibodies titers in study group. Vammen and colleagues [27] 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 [25,28,29,30]. In our study, we focused on the SeroCP ELISA as this test proved to be approaching more closely the results of MIF test than other commercial diagnostic kits [31]. In our study, the SeroCP-IgA test was found to be reproducible as reported previously by Ngeh and colleagues [32]. However, before optimization, the detection of Cpn IgA antibodies by MIF and ELISA tests was not sufficiently correlated and it showed a moderate agreement between the two tests. Our results are in line with those reported by Ciervo and colleagues who evaluated and optimized the SeroCP-IgA test for the detection of anti-Cpn IgA antibodies against a commercial MIF (Labsystems) used as a gold standard [31]. Furthermore, the presence of Cpn IgA antibodies was higher by SeroCP IgA ELISA test than by our in house MIF test. Paldanius and colleagues [33] reported also that the labsystems IgA ELISA test overestimated the prevalence of IgA antibodies (62% versus 26%) compared to their in house MIF. The same problem was previously reported with the detection of IgG antibodies by different serological methods [34].

The observed discrepancies between MIF and ELISA in the detection of Cpn IgA antibodies could be explained by the different experimental conditions used in the two serological tests such as Cpn strains, incubation time, antigen concentration, serum dilution, absorption experiment and the type of conjugates used.

The SeroCP test, as well as our MIF test, uses purified elementary bodies of Cpn as antigen. So, these two tests measures antibodies directed against antigens localized in the surface of Cpn elementary bodies. However, unlike the SeroCP test, which uses purified Cpn TWAR 183 as strain, our in house MIF uses the IOL-207 Cpn strain. Jantos and colleagues [35] reported antigenic differences between Cpn strains using immunoblot analysis. In another study, Black and colleagues [36] analyzed the antigenic profiles of six strains of Cpn by the MIF test and
found that serum samples from culture positive patients produce four to eightfold higher titers against autologous isolated strain than they were against TW-183 strain. Furthermore, the criteria for establishing the diagnosis of acute infection were met only with the use of the antigen from the local strain than against TW-183. Thus, the serologic diagnosis of Cpn infection may require the use of antigens from more than one Cpn strain. Contrarily to these two studies, Bennedsen and colleagues [37], compared the performance of three MIF tests (WRF, Labsystems and MRL MIF tests) for their ability to detect IgA antibodies to Cpn and found that although these assays use different Cpn strains as antigens, the detection rates of IgA endpoint titres were similar to each other.

The effect of incubation time and antigen concentration on the performance of the Labsystems MIF test for the detection of Cpn IgA antibodies was also studied. An overnight incubation of sera with antigen slides containing high concentrations of chlamydial elementary bodies was found to increase the sensitivity of the MIF test [38]. In our MIF test, and as the commercial ones, an incubation time of 30 minutes was used which differ from that used in the SeroCP IgA test using an incubation of 1 hour. Furthermore, our in house MIF test uses an optimal concentration of elementary bodies which was calibrated using the commercial MIF test and reference sera.

Other experimental conditions might explain discrepancies such as the higher dilution used in the SeroCP-IgA test (1/105) compared to that of the MIF test (1/12) as previously reported for Cpn IgA antibodies [31]. This prozone effect with higher dilution of antibodies is supported by the findings of Vainas and colleagues [39] who reported that the agreement between the MIF and ELISA tests was adequate at low but not at high titers and that the sensitivity of the ELISA could be increased by testing sera with elevated titers at higher predilutions. No data are available regarding the use of rheumatoid factor absorption by the SeroCP IgA test according to the
manufacturer instructions. If our sera were not pretreated before ELISA IgA testing, false
positive results could also be due to IgG interference in the detection of IgA antibodies.

It has been reported that the drawback in the measurement of Cpn IgA antibodies is the
considerable variation in commercial anti-IgA conjugates, a problem which hampers the accurate
detection of IgA antibodies [40,41]. In a comparative study of six fluorescein anti-human IgA
conjugates, Paldanius and colleagues [40] evaluated the IgA titers and seroconversions detected
and showed that only one out of the fourteen seroconversions was found by all conjugates and in
most of the cases, only one of the conjugates produced a significant titre increase.

Several authors reported that the discordance between MIF and ELISA is related also to the better
sensitivity of the ELISA tests compared to the MIF test [26,33]. In our study, the sensitivity of
the SeroCP-IgA test according to our in house MIF test was high before optimization (97.4%),
however its specificity was limited (62.4%). Using the same antigen for the detection of Cpn IgG
antibodies, we previously shown that the specificity of the SeroCP IgG antibodies was 38.5% and
that this test was not specific enough to distinguish between the presence of IgG antibodies to
Cpn and those of Ct [29]. Yetkin [42] reported that bearing in mind that the MIF test is the
international gold standard for determination of Chlamydia seropositivity, the high sensitivity of
ELISA tests could be interpreted in the reverse direction, instead, specificity of the ELISA may
be lower than that of the MIF test.

At the 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 [29,43]. The
detection of cross-reacting antibodies by this test is not surprising because it detects antibodies
against surface protein antigens of elementary bodies, which are shared between chlamydial
species and other Gram negative bacteria [44-46]. Because of all these problems related to MIF, ELISAs were commercially developed; they are relatively simple to perform, less time consuming, more objective as they rely on photometric reading and easier to standardize. However, ELISA has been recognized as an unreliable method for Cpn identification compared to the MIF test [47]. In a previous study, Ciervo and colleagues [31] evaluated and optimized; using ROC analysis; the SeroCP-IgG and the SeroCP-IgA tests for the detection of anti-Cpn IgG and anti-Cpn IgA antibodies against a commercial MIF (Labsystems) used as a gold standard in patients with coronary heart disease. In their study, they found that for IgG antibodies, the two tests correlated well and that the optimized cut off value correspond to that recommended by the manufacturer. In our study, we focused only on the SeroCP-IgA antibodies as they have been found to generate a moderate concordance when compared to the MIF test [29,31]. Sera from healthy blood donors were used in our study in order to determine the prevalence of Cpn IgG and IgA antibodies and also to optimize the SeroCP IgA test. Only 9 out of these 100 sera were positive for IgA antibodies against Cpn. In order to make an accurate optimization of the SeroCP IgA test, a homogenous distribution of IgA antibodies in the total sera was necessarily. In addition, since our patients had clinical suspicion of Cpn infection, we choose the IgG titres that were \( \geq 256 \) instead of \( \geq 512 \) to have the maximum of IgA positive cases. TG-ROC analysis was used to evaluate and optimize the SeroCP IgA test in comparison to our MIF test considered as the gold standard. In fact, test characteristics depend on the cut off value used. Instead of using the cut off value that was recommended by the manufacturer, new cut off values were estimated from the TG-ROC to obtain the best discrimination between positive and negative tests 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. Hoynans and colleagues [34] reported that the use of different serological assays would be no problem if the agreement between the tests is high. Furthermore, with the optimized cut off value, a sensitivity and a specificity of the SeroCP-IgA test of 84.4% and 87.2% were obtained respectively. When comparing the sensitivities and the specificities according to the tested groups, results were always better in the control than in the study group indicating that the correlation between MIF and SeroCP IgA test was better with Cpn IgA negative sera than with IgA positive sera in line with the results reported by Vainas and colleagues [39].

**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. However, the difference in the detection of IgA antibodies between MIF and ELISA supports the use of the MIF method as a gold standard in the measurement of Cpn IgA antibodies. Commercial ELISA tests would be standardized and optimized according to the MIF test 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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Figure legends

**Figure 1.** Reproducibility of the SeroCP IgA ELISA assay. The figure shows OD at 450 nm values obtained in two ELISA tests conducted on different days with the same serum samples. The differences between the OD at 450 nm values were less than 20%.

**Figure 2.** 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 3.** 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>
<th>≥2048</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</td>
<td>12</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>ELISA IgA + (%)</td>
<td>57</td>
<td>15</td>
<td>7</td>
<td>9</td>
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</tbody>
</table>
Table 2. Correlations between the detection of Cpn IgA antibodies by MIF and ELISA tests

<table>
<thead>
<tr>
<th>MIF / ELISA</th>
<th>Before optimization</th>
<th>After optimization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Study group</td>
</tr>
<tr>
<td></td>
<td>n=194</td>
<td>n=94</td>
</tr>
<tr>
<td>+ / +</td>
<td>75</td>
<td>66</td>
</tr>
<tr>
<td>- / -</td>
<td>73</td>
<td>8</td>
</tr>
<tr>
<td>+ / -</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>- / +</td>
<td>44</td>
<td>18</td>
</tr>
<tr>
<td><strong>Concordance (%)</strong></td>
<td>76</td>
<td>78</td>
</tr>
<tr>
<td><strong>K</strong></td>
<td>0.54</td>
<td>0.34</td>
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Table 3. Performance of the SeroCP IgA ELISA test before and after optimization

<table>
<thead>
<tr>
<th>MIF / ELISA</th>
<th>Before optimization</th>
<th>After optimization</th>
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<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Study group</td>
</tr>
<tr>
<td>Sensitivity (%)</td>
<td>97.4</td>
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<tr>
<td>Specificity (%)</td>
<td>62.4</td>
<td>30.8</td>
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<tr>
<td>PPV (%)</td>
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<tr>
<td>NPV (%)</td>
<td>97.3</td>
<td>80.0</td>
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</table>

PPV: Positive predictive value, NPV: Negative predictive value
Figure 1
Figure 2

- Left graph: MIF Cpn IgA antibodies seropositivity
- Right graph: SeroCP IgA index

The graphs illustrate the distribution of MIF Cpn IgA antibodies seropositivity and SeroCP IgA index.
Figure 3