Author's response to reviews

Title: Evaluation and optimization of commercial Enzyme Linked Immunosorbent Assay kit for detection of Chlamyphila pneumoniae IgA antibodies

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Author's response to reviews: see over
Dear Editor,

We are very grateful to you for your response to our manuscript entitled "Evaluation and optimization of commercial Enzyme Linked Immunosorbent Assay kit for detection of Chlamydia pneumoniae IgA antibodies" MS: 1140674523185246

Would you please find enclosed the revised form of our manuscript.

I- Responses to editor's notes

● Ethics

Details of ethical approval and consent from healthy blood donors were added in page 5 lines: 90-91 and in lanes 94-95 respectively.

Our revised manuscript was also formatted according to the Journal style (http://www.biomedcentral.com/info/ifora/medicine_journals).

II- Responses to reviewer’s comments and recommendations

We have listed below all revisions to the manuscript according to the reviewer’s comments and recommendations.
1- Responses to reviewer I:

- Responses to major Compulsory Revisions

1) The MIF test is still considered the gold standard for the diagnosis of C. pneumoniae infection, despite its limitations in terms of sensitivity and specificity. In this study, the authors use an in house MIF as the gold standard but they dont describe how the method was assessed. The antigen nature (purification, dot quantization, presence of LPS) of the MIF is very important to the aim of this paper. The MIF needs of a proficiency study (intra and inter laboratory variability or concordance). In the discussion section is mentioned that their MIF was validated using a commercial kit of bioMerieux, but not data were presented. Please, describe your in house MIF assay and the relative statistic tests. Alternatively, a commercial MIF test can be used.

Our in house MIF assay was described in the material session in pages 6 and 7 from lane 98 to line 120.

Our MIF test was introduced in our laboratory since 1992 and it was translated from the reference centre of Chlamydia in Amien France (Pr. Jeanne ORFILA). Three persons were trained at the time to perform all experiments from culture in yolk sac membranes of infected eggs until reading slides. These persons were able to reproduce serological results performed in France with antigens “made in Tunisia” and prepared in our laboratory. Sera used were our sera obtained from clinic departments and others were reference sera from France that were used in the two laboratories. Furthermore, in order to control the quality of our MIF test, we perform periodically a comparison between our in house MIF and the bioMerieux MIF kit. The positive serum included in each run is also titrated so that no titre variation occurs between runs. The intrinsic variation in our MIF test is minimal as at least two experienced and independent reader read the slides and results of repeated test are always reproducible as in determinations.
of endpoint titres, at maximum a difference of only one dilution step could be found. Furthermore, the inter observer agreement of our MIF test for detection of seropositivity at various cutoff levels shows good results for IgG and IgA testing.

2) The results of ELISA test need to be assessed for inter and intra run variability, specificity, sensitivity, positive and negative predictive values, according to standard definitions, before and after optimization. Insert a table.

**A table was inserted in page 25.**

Comments on result part in the manuscript have been added in page 8 (lanes: 149-152 and 162-163) and in page 9 (lines: 176-178).

A figure was also added (figure 1).

3) Why IgG ≥ 256? Explain this decision.

**Explanation was inserted in the discussion session page in page 13 from line 275 to lane 280.**

- **Responses to Minor Essential Revisions**

  Line 25. Delete acute

  **Acute has been deleted.**

  Figure 1 is unclear. Differentiate the positive and the negative results.

  **Figure 1 in the old version became figure 2 in the new version.**

  In figure 2, <1/12 and >1/12 were replaced by MIF IgA- and MIF IgA+ respectively.

- **Responses to Discretionary Revisions**

  4) In the methods section the IgM antibodies were tested in MIF, but no data were reported in the results. Include IgM results or delete IgM detection in methods.

  **IgM antibodies were deleted from the methods section.**
5) Why IgA > 1/12 and not 1/8 or 1/16 as usually dilution? Explain this decision.

There are no accepted criteria for positivity in the MIF test for IgA antibodies. Titres used in the literature were 1/8, 1/16 and 1/40. According to the literature as well as in our laboratory, the 1/12 dilution is exclusively used for MIF IgM positivity. In our lab, MIF IgA and IgM tests are generally run in parallel with the same serum absorption and so the dilution 1/12 was maintained as cut off.
2- Responses to reviewer II:

- Responses to major Compulsory Revisions

What was the incubation times in micro-IF?

**The incubation times in the MIF have been inserted in the material session in page 6 lane 111-112.**

What was the exact method by which the rheumatoid factor was moved?

**The exact method by which the rheumatoid factor was moved has been inserted in the material session in page 6 from lane 107 to 110.**

The age and sex distribution of blood donors are lacking.

**The age and sex distribution of blood donors have been inserted in the material session in page 5 from lane 94 to 95.**

Why bioMerieux MIF kit, not Savyon MIFA kit, as a reference?

**Biomerieux was used instead of Savyon MIFA because it is more easy and rapid to get the bioMerieux test.**

In discussion, one should concentrate in the possibilities, why ELISA and micro-IF are discrepant and what this means, if single cut-off points, as usual, are used to differentiate between patients and controls. The difference between antigens and reading of their reactivity should be discussed.

**The discrepancy between MIF and ELISA have been discussed in pages 10-12 from lane 206 to 257.**
Is there difference between different strains?

The impact of the use of different strains in the MIF test has been discussed as a possible cause of discrepancy between MIF and ELISA in pages 10 and 11 from line 210 to 224.

In our laboratory, we have studied two Cpn strains (IOL207 and AR39) for their reactivity in the MIF test. No difference was found between the two strains.

The material is too small for profound discussion on local prevalence.

**Discussion on local prevalence was shortened.**

- **Responses to Minor Essential Revisions**

  The English should be checked.

  **Language corrections were shacked with an English teacher.**

  Ref. 27. Romano CC should be Romano-Cavarelli C

  **Romano CC has been deleted in the text.**

  Ref. 34. ??

  **Reference 34 in the old version has been deleted.**

  All bright fluorescence is accepted?? in the article is explaining the numerous acclaims of cross-reactions in the micro-IF.

  **In the MIF test, only fluorescence with evenly distributed elementary bodies is an acceptable positive reaction. False positive results could be obtained when all clear bright fluorescence is accepted leading to an over reading of the MIF test. Cross-reactivity can thus be avoided by ignoring all but homogeneous fluorescence. Furthermore, the fluorescence without distinctive elementary bodies morphology is considered non specific on the chlamydial antigen spot. The positive reaction should be studied until negative reactions are seen in dilution.**
3- Responses to reviewer III:

- Responses to major Compulsory Revisions

1. The authors address the question of the significance of C. pneumoniae specific IgA antibodies as detected by immunofluorescence and ELISA assays and suggest resolving the discrepancies observed between these two techniques by adjusting the cut-off of the ELISA following ROC analysis. The question posed by the authors is how to evaluate and optimize a commercial ELISA assay, SeroCP for the detection of C. pneumoniae IgA antibodies. The answer brought by the authors is adjustment between the two techniques, ELISA and MIF. However, even though, as the authors state, the MIF is still considered to be the serological gold standard in chlamydial serology, we cannot make the assumption that such an adjustment is equivalent to ELISA ?optimization?. The observed discrepancies between MIF and ELISA are possibly due to differential antigen detection, the MIF detecting antibodies reacting against whole chlamydial elementary bodies (EB) and the ELISA detecting antibodies against a specific chlamydial antigen (a recombinant C. pneumoniae protein). The authors never ask the question as to the biological significance of the observed discrepancies. Therefore the question should not be ?evaluation and optimization? but rather ?harmonization? between the two serological techniques. Moreover, this harmonization attempted by the authors no way resolves the question of the biological significance of IgA antibodies. The conclusion stating that ?commercial ELISA kits should be standardized and optimized? to ?give better performance? is hazardous. If more than one ELISA kits detecting different types of chlamydial antigens gave good correlation between each other but not with MIF, may be both types of techniques should be maintained because providing complementary biological information.
Possible causes of discrepancy observed between MIF and ELISA have been inserted in pages 10-12 from lane 206 to 257.

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 (page 10 from lane 210-212).

The main objective of this article was not to study the biological significance of the presence of IgA antibodies but a harmonization of these tests and as we stated before “this study is not a diagnostic one, but an assay evaluation”.

Paldanius and colleagues reported that IgA ELISA test overestimate the prevalence (62% versus 26%) and the persistence (63% versus 17%) of IgA antibodies compared to their in-house MIF. The authors conclude that the difference in detection and kinetics of IgA antibodies between MIF and ELISA demonstrate that the MIF test should remain the gold standard in the measurement of Cpn IgG and IgA antibodies.

2. More precisely, although a good correlation can be observed between MIF and immunoenzymatic methods for the detection of C. trachomatis specific antibodies, no such correlation is ever observed for the detection of C. pneumoniae antibodies, independently of the type of immunoenzymatic assay used. Rather than struggling to harmonize the two types of serological methods, we should rather try to understand the biological significance behind the observed discrepancies. Moreover, the method used was ROC analysis. This type of analysis should be used when an unequivocal gold standard is available. In this case, if a reliable PCR method for detection of C. pneumoniae acute or chronic infection was available, ROC analysis could be used for calculation of the most appropriate cut-off. Unfortunately, such a PCR is not commercially available. Using ROC analysis to adjust the cut-off of serological method against the results of a different TYPE of serological method can be controversial: one could argue, why not adjust the other way round, and try to define a better
MIF cut-off so as to ensure correlation? The authors themselves state that the MIF "lacks standardization"; therefore why attempt standardization against a non-standard? If it is true that the ELISA methods have not been fully validated, the same stands for MIF, in the absence of comparison to direct detection methods.

**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 (page 10 from lane 210-212).**

Possible causes of discrepancy observed between MIF and ELISA have been inserted in pages 10-12 from lane 206 to 257.

3. It seems surprising that MIF is less sensitive than ELISA, if MIF detects antibodies against all surface-exposed C. pneumoniae antigens while ELISA detects antibodies against only one antigen. The authors should state the antigen used by SeroCP and try to explain this surprising observation. It could be that SeroCP positive IgA antibodies are directed against an antigen which is "hidden" by LPS or LPS-protein complexes on the surface of whole EB used in the MIF assay. In that case, it would be an error trying to "eliminate" these positive results which could in fact reflect a low sensitivity of the MIF in detection of antibodies of certain specificities. From a technical point of view, the authors should state if rheumatoid factor was removed before the ELISA. If not, could this pre-analytical difference account for the higher sensitivity of ELISA?

**The impact of the use of different strains in the MIF test has been discussed as a possible cause of discrepancy between MIF and ELISA in pages 10 and 11 from line 210 to 224.**

Data concerning the use of rheumatoid factor absorption by the SeroCP IgA test are lacking. The effect of removal of rheumatoid factor by the ELISA has been inserted in the discussion session in page 12 (lane 238-241).
4. In the absence of commercial C. pneumoniae PCR methods, this manuscript would be valuable if more than one immunoenzymatic assays were compared to MIF for the detection of IgA antibodies.

**Responses to Minor Essential Revisions**

1. The population studied is poorly defined from a clinical point of view. On what criteria were the patients suspected to have chlamydial infection? The significance of a MIF titer \( \geq 256 \) in the absence of clinical symptoms is completely uncertain.

   **Explanation of the choice of the study group was inserted in the discussion session page in page 13 from line 275 to lane 280.**

   *We agree with you that the significance of a MIF titre \( \geq 256 \) in the absence of clinical symptoms is uncertain.*

   **Patients suspected to have chlamydial infection were collected from the Department of infectious diseases having MIF titer \( \geq 256 \) with atypical signs of atypical pneumonia, fever, pseudo-grippal syndrome, some of them were also IgM positive.**

2. The authors analyse IgA antibodies detection by MIF and ELISA. It would be interesting to know the corresponding results for IgG detection. Was the MIF equally less sensitive for IgG detection or was this observation limited to IgA? If the authors modify the cut-off for IgA, what happens with the cut-off for IgG? For coherence purposes, the IgG cut-off should also be adjusted against the MIF. And what happens in this case to the correlation between the two techniques for detection of C. pneumoniae IgG? Table 1 would be more informative if it contained the ELISA IgG data.

   **Several authors reported that the SeroCP IgG test has proved to be more closely approach the results of the MIF than other commercial kits. As reported earlier in our previous work dealing with cross reactivity in MIF test and the ability of two**
commercially available test (the SeroCP an the SeroCT IgG tests) to diminish the extent of cross reactions, we have shown that the SeroCP correlated in 92% of cases with our in house MIF test. In another study, Ciervo and colleagues have evaluated and optimized the SeroCP-IgG and the SeroCP-IgA tests 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 for IgG antibodies, the two tests correlated well and the optimized cut off value correspond to that recommended by the manufacturer.

In fact the observation that ELISA test was much more sensitive than the MIF test was limited to only IgA even in the literature. In deed, Paldaniu s and coll., 2005; studied the prevalence and the persistence of Cpn antibodies in healthy laboratory personnel in Finland using their in house MIF and a commercial ELISA (Labsystem) In this study, the authors reported that the IgA ELISA test overestimated the prevalence (62% versus 26%) and the persistence (63% versus 17%) of IgA antibodies compared to the in-house MIF.

3. In the discussion section, the authors state seroprevalence results in Germany and Norway; by which technique were these results obtained?

The seroprevalences in Germany and Norway have been deleted.

4. The higher dilution used in the SeroCP IgA test (1/105) compared with the MIF (1/12) cannot be used as an argument to explain observed discrepancies as these discrepancies were almost entirely due to SeroCP-positive/MIF-negative samples.

The effect of sera dilution has been dicussed in pages 11 and 12 from lane 233 to lane 238.
5. The authors state cross-reactivity between chlamydial species by the MIF assay; cross-reactivity between C. pneumoniae and other Gram negative bacteria should also be stated.

**Cross reactivity occurring between *C. pneumoniae* and other Gram negative bacteria has been added in page 13 from lane 261 to lane 264.**

6. Figure 1 is not clear: (a) and (b) do not appear. The data points are presented according to MIF Cpn IgA antibodies seropositivity? (on the abscissa), does that mean that data points on the left correspond to MIF negative and data points on the right correspond to MIF positive samples?

**Figure 1 in the old version became figure 2 in the new version.**

In figure 2, <1/12 and >1/12 were replaced by MIF IgA- and MIF IgA+ respectively. **Figure 2 has been clarified. In deed, data points on the left correspond to MIF negative and data points on the right correspond to MIF positive samples.**

7. Some English language corrections are needed.

**Language corrections have been shacked with an English teacher.**

The name of the the first author of reference 32 should be confirmed.

The name of the first author of reference 32 has been confirmed and omitted in the new version.
We will be ready for any suggestion or further modification that you need me to make.

Furthermore, could you please send us your response about our manuscript after revision to the two first authors.

Looking forward to having our manuscript published in your Journal, we would like to send you my highest respect.

Sincerely yours

Olfa FRIKHA GARGOURI