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16-Aug-2016

Dear Dr. Lagier,

I would like to thank you for submitting your revised manuscript entitled "P35 and P22 Toxoplasma gondii antigens abbreviate regions to diagnose acquired toxoplasmosis during pregnancy: towards single-sample assays." to Clinical Chemistry and Laboratory Medicine (CCLM). I have read the revised manuscript and the cover letter. In my opinion you have satisfactorily responded to the comments that were raised by the reviewers.

Therefore, I am pleased to inform you that the manuscript has now been accepted for publication in CCLM.

The CCLM production office will contact you for proof reading as soon as possible.

Your article will be published ahead of print soon, at least 3-4 weeks upon acceptance.

Thank you for your fine contribution. On behalf of the Editors of Clinical Chemistry and Laboratory Medicine we look forward to your continued contributions to the Journal.

Kind regards
Dr. Mario Plebani
Editor in Chief, Clinical Chemistry and Laboratory Medicine

DE GRUYTER Clinical Chemistry and Laboratory Medicine

P35 and P22 *Toxoplasma gondii* antigens abbreviate regions to diagnose acquired toxoplasmosis during pregnancy: towards single-sample assays.

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Section/Category:	General Clinical Chemistry and Laboratory Medicine
Classifications:	10.214 Immunoassay < 10.200 Plasma enzymes < 10 Proteins and Enzymes, 20.711 Prenatal diagnosis < 20.700 Other diseases and conditions < 20 Pathologies, 30.110 Enzyme immunoassay < 30.100 Methods < 30 Methods, 60.201 Infection < 60.200 General < 60 Bacterilogy/Parasitology/Virology, 90.202 Immunoassay < 90.200 Immunology < 90 Immuno/hematology
Keywords:	Toxoplasma gondii, P35&P22 recombinant proteins, Immunochemical reagents, Pregnancy control, Epitope prediction, Acute toxoplasmosis diagnosis

Responses to Reviewer #1's comments

(i) Quote- ….. The work is of interest, however, to my point of view, the paper, as it stands now, requires improvements before being published. Below are my major comments.
MATERIALS AND METHODS
All laboratory procedures were selected and used properly, however authors should add some important information in this section.
1/ Ethical approval of the human sera used during the study should be included to the main text.- **Unquote.**

Changes introduced:
As requested by Reviewer#1 and the Editor, a piece of text was included in Materials and methods section.
The new text appears in page 5, line 24 reads:

"The studies were made in the frame of the project "Development of analytical methods for determination in complex media: Application to clinical infection diagnosis", which counts with Ethical Committee approval, that contemplates the informed consent (Resolution N° 1070/2014, Expedient 6060/059, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario)."

(ii) Quote- 2/ Authors should placed the information that sera were collected for pregnant women.- **Unquote.**

Changes introduced:
The complete information on the sample's source has now been provided in the corrected sentence which now reads (page 6, line 4):

"The serum panel contained 72 pregnant-woman samples which were collected at the Central Laboratory of the Santa Fe province, Argentina. Samples were analyzed by reference techniques and classified according to their results."

(iii) Quote- 3/ The determination of the TC group of serum samples only on the presence of specific IgG and the lack of IgM antibodies, seem to be incomplete. Why the authors have not determined avidity of specific IgG?- **Unquote.**

Authors' comments and changes introduced:

We classified TC group based on the absence of specific IgM and the presence of specific IgG based on the usual protocols internationally accepted and routinely followed to typify *T. gondii* infection. (a) Liesenfeld O, Montoya JG, Tathineni NJ, Davis M, Brown BW Jr., Cobb KL, Parsonnet J, and Remington JS. Am J Obstet Gynecol, 2001, 184:2, 140-145. (b)Wong SY, Remington JS. Clin Infect Dis 1994,18:853-62. (c) Remington JS, McLeod R, Thulliez P, Desmont G. Toxoplasmosis. In: Remington

JS, Klein JO, editors. Infectious diseases of the fetus and newborn infant. 5th ed. Philadelphia: WB Saunders; 2001. p. 205-346. (d) Sensini A. Toxoplasma gondii infection in pregnancy: opportunities and pitfalls of serological diagnosis. Clin Microbiol Infect 2006;12:504-12.

However, following Reviewer #1 suggestion, we determined IgG avidity for samples on TC group. The avidity test results of samples from TC group ranged between 62% and 100%. This information was commented in the experimental section at page 6, line 24 and says:

"18 samples showed negative reaction for specific IgM, positive reaction for IgG and high AI% (typical chronic, TC)."

(iv) Quote- 4/ Page 7 line 41-43: What was the final concentration of IPTG?- **Unquote.**

Changes introduced:

The concentration of IPTG was added at page 7, line 16, and the new sentence now reads:

"Protein expression was induced for 3 h with 1 mM IPTG (final concentration)."

(v) Quote- 5/ Page 8 line 25-30: What the concentration of sulfuric acid was used?- **Unquote.**

Changes introduced:

The concentration of sulfuric acid was added at page 7, line 59, and the new sentence now reads:

"The colorimetric reaction was developed using 100 µL of tetramethyl benzidine (Zymed) in H_2O_2 (Sigma, St. Louis, USA), and stopped with 100 µL of 1000 mM H_2SO_4 (Cicarelli, San Lorenzo, Argentina)."

(vi) Quote- RESULTS
1/ Page 9 line 43-47: Authors should add information which P22 protein fragment was selected.- **Unquote.**

Changes introduced:

The requested information on the P22 selected fragment was now included in page 9, line 20, stating:

"Considering the uniform distribution of the predicted epitopes, we used the AA segment 27-173 of P22 sequence, excluding both the N-terminal signal peptide- and the carboxylic hydrophobic regions (Fig. 1B)."

(vii) Quote- 2/ In this section, there is no information concerning the expression and purification of the fragments of P35 and P22 recombinant proteins. In the Materials and Methods section was described

construction of recombinant plasmids and the procedure of gene expression and production of recombinant antigens, however, the reader could not see the results on these issues. Authors, among other things, should add information about expression yield, purity of antigen preparations and their molecular weight. Please include the photography of the SDS-PAGE stained by Coomassie Blue of the induced and non induced bacteria culture, as well as the picture of the purified protein. This will allow the reader to judge about the purity, the apparent MW, as well as the rate of production and purification of these recombinant proteins. These results should also be the basis for part of the discussion.- **Unquote.**

Changes introduced:

As suggested, we added information on the purified proteins through new text and the new Fig. 2. Thus, the *3.2. rP35a and rP22a general features* section was included in page 9, line 34:

"The recombinant proteins were obtained and purified according to the standard procedures described in 2.5. (see Fig. 2). rP22a and rP35a yields were typically above 3 mg per 100 mL of induced culture."

We also highlight the appropriate yielding we obtained in the *Discussion* section through incorporating the following sentence (page 12, line 47):

"The good yielding obtained when expressing and purifying both antigens indicate that rP35a and rP22a are good candidates to be used with diagnostic purposes. Moreover, yields can certainly be increased by tuning-up experimental conditions."

(viii) Quote- 4/ Using 18 sera form each group is a rather small number. I have some concerns that the use of a larger pool of sera could change the results. Moreover, the main limitation of this paper is the lack of collection of sera from pregnant women with ongoing or recent seroconversion and for which times of infection are known.- **Unquote.**

Authors comments:

We do agree with Reviewer #1 on that it would be desirable to count with a larger number of samples than 18 in our presumably acute group. That is why in the very last sentences of both the abstract and the discussion sections we do emphasize the need of validating our findings with larger longitudinal and transversal studies. Nevertheless, we point out that it is very difficult to find samples from acute *T. gondii*-infected pregnant women, and 18 is a reasonable number of samples to be evaluated, as an initial step for the proof of concept we intend to emphasize in our work. Indeed, most of the works on acute toxoplasmosis diagnosis report having assayed sera from acute infected pregnant women where the panels contained about the same number of samples than our. Moreover, the number of works and samples where seroconversion-time point is known are generally fewer than those mentioned previously. As examples, we resume here below the number of samples from acute infected pregnant-women reported in works referenced in our Ms:

1- Ref. 2 (De Paschale et al. 2008): 10 samples.

2- Ref. 8 (Golkar et al 2008): 20 samples.

3- Ref. 11 (Li et al. 2000): 20 samples.

4- Ref. 15 (Belá et al. 2008): 20 samples.

5- Ref.16 (Pfrepper et al. 2005): 25 samples from which 22 were followed in time.

6- Ref-.20 (Elyasi et al. 2010): 19 and 35 samples (collaborative work between two groups from different countries, therefore they used two different panels)

7- Ref. 21 (Holec-Gasior et. al 2014): 16 samples.

8- Ref. 22 (Desphande 2013): 19 samples.

9- Ref. 30 (Drapala et al. 2014): 22 samples from which 17 were followed in time.

(ix) Quote- 3/ Figure 3: TC and NI group of sera should be removed from the description of this figure.- **Unquote.**

Changes introduced:

The requested text was removed from the original Figure 3 legend, which is now Figure 4 legend.

(x) Quote- DISCUSSION

1/ Authors should discuss the relatively low specificity of tests based on P22 and P35 recombinant antigens.- **Unquote.**

Comments and Changes introduced:

We have to point that the specificity calculated via the ROC curves, detail those values obtained when one group is compared only with another one, having used a particular cut-off value with which we reached the best discrimination between both of the groups studied. Thus, our specificity values are different for each group analyzed, reaching even a value of 100%. We now intend to emphasize this, as requested by Reviewer#1, by including a new row in Table 1 where rP35a and rP22a discrimination power between acute and not-infected samples is highlighted. We also rephrased and included new text in the description of the *Results* (a) and the *Discussion* (b) sections, at pages 10 and 13, lines 6 and 7, respectively, which read:

a) "When comparing results of all the infected groups (A+RC+TC) *vs.* those from NI group, specificity of rP22a turned out to be 94.4%, whereas for rP35a was 83.3%. Nevertheless, rP35a performed better than rP22a to distinguish between A and RC, the AUCs being 0.911 *vs.* 0.818; although both sensitivities were 83.3%, specificities were 83.3% *vs.* 72.2%, respectively. Regarding the proteins ability to distinguish between A and RC+TC groups, rP35a displayed again a better performance than rP22a, with the specificity being 83.3% vs. 77.8%, and the sensitivity being 83.3% *vs.* 80.6%, respectively. However, AUCs turned out to be similar, indicating analogous aptitude to discriminate acute from chronic infection.

Both rP35a and rP22a rendered positive results for all A group samples. Therefore, both antigens displayed 100% of equally sensitivity and specificity when comparing A with NI group, having the AUC reached a value of 1. The $ELISA_{index}$ obtained for rP35a was lower than that for rP22a, 4.2 *vs.* 6.2, respectively (see Fig. 3)."

b) "When discriminating among *T. gondii*-infection stages, rP35a reacted with all A but only a few of RC samples reaching 83.3% of specificity, as calculated having used ROC curves. This specificity value indicates that a positive result with rP35a would lead to unnecessary treat only 17 out of 100 chronic-infected patients, as compared with the alternative of treating 100 out 100 patients who present positive specific IgM."

Responses to Reviewer #2's comments

(i) Quote- Comments to the Author
good bibliography , topic of interest , perhaps give more evidence to the possible diagnostic applications.- **Unquote.**

Changes introduced:

As requested, we included two pieces of new text discussing the possibilities that opens this approach. The pieces of text were inserted in (a) the *Introduction* section page X7, line Y7, and in (b) the *Discussion* section, page 3, line 14, and are reproduced here below:

a) "Considering that symptoms during the acute-phase of the infection are typically unspecific or unobserved, acute toxoplasmosis diagnosis depends on serological tests that indicate the presence of specific antibodies. The undoubting evidence of recently acquired infection is seroconversion and/or an important increment of the specific immunoglobulin titer. Nonetheless, this diagnostic possibility is restricted to places where a prenatal screening, evaluated by analysis of samples serially collected at a 3-weeks interval is mandatory, not being the case for most of the countries. Even though, the chances of finding any of the mentioned events are very exceptional because they limit to cases of a carefully planned pregnancy or to the infrequent case of a first sample collection during the earliest weeks of titer augmentation after the parasite enters the host [1]."

b) "Bearing in mind the inconveniences described to diagnose acute toxoplasmosis in pregnant women, it has to be remarked the importance of the still up-to-date search for reliable tests. In this work, we highlight rP35a and rP22a features that make them

appealing antigens for an accurate acute-toxoplasmosis diagnosis using the very first sample taken, a fact that could accelerate treatment. We present a proof of concept on rP35a and rP22a usefulness when used in complementary tests. The diagnostic potentiality of our proposed antigens is supported by the following facts: *i*) indirect ELISA with rP35a allowed overcoming the inconvenience of the frequent presence of long-lasting IgM by rendering an appropriate sensitivity to discriminate between samples from acute and chronic infected patients; *ii*) indirect ELISA with rP22a showed quite higher $ELISA_{indexes}$ for samples from A than for samples from (RC+TC) groups, thus being a good antigen for the mentioned purpose, and an excellent protein to discriminate between not-infected and infected individuals (acute + chronic); *iii*) avidity tests with both recombinant proteins complemented each other, so as to replace the parasite homogenate. Furthermore, these antigens might avoid the disadvantage that appears when the patient has low IgG maturation rate and display low AI%, even when occurring chronic infection (1, 6, 20). Our results should be confirmed using a larger number of longitudinal and transversal samples."

Responses to the Editor's comments

(i) Quote- Editorial comments:
1. The abstract should be structured into Background, Methods, Results, Conclusions.- **Unquote**

Changes introduced:
The Abstract section has now been structured as requested.

(ii) Quote- 2. Please comment on ethical approval and informed consent (in the Materials and methods section) .- **Unquote**

Changes introduced:
We have now commented on the ethical approval and the informed consent, as described above in point (i) of responses to Reviewer's #1 comments.

We thank the Reviewers for their careful reviews of the manuscript. We hope it has now been improved enough by having amended the work according to their suggestions and requirements.

P35 and P22 *Toxoplasma gondii* antigens abbreviate regions to diagnose acquired toxoplasmosis during pregnancy: towards single-sample assays

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Full length research article.

Counting: Words, 4044/Abstract, 248/keywords, 6/Figures, 5/Tables, 2/References, 30.

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Abstract

Background: P35 and P22 *Toxoplasma gondii* proteins are recognized by specific IgG at the early infection stage, making them ideal for acute-toxoplasmosis pregnancy control. Both proteins have been studied to discriminate between acute and chronic toxoplasmosis. However, results were hardly comparable because different protein obtainment procedures led to different antigens, the reference panels used were not optimally typified, and avidity tests were either not performed or narrowly examined.

Methods: We bioinformatically predicted P35 and P22 regions with the highest density of epitopes, and expressed them in pET32/BL21DE3 alternative expression system, obtaining the soluble proteins rP35a and rP22a. We assessed their diagnostic performance using pregnant woman serum samples typified as: not infected, NI (IgG-, IgM-), typical-chronic, TC (IgM-, IgG+), presumably-acute, A (IgM+, low-avidity IgG), and recently-chronic, RC (IgM+, high-avidity IgG).

Results: rP35a performed better than rP22a to differentiate A from RC, the areas under curve (AUC) being 0.911 and 0.818, respectively. They however performed similarly to differentiate A from TC+RC (AUC: 0.915 and 0.907, respectively). rP35a and rP22a evaluation by avidity ELISA to discriminate A from RC rendered AUC values of 0.974 and 0.921, respectively. The indirect ELISA and avidity ELISA results analyzed in tandem were consistent with those obtained using commercial kits.

Conclusions: rP35a and rP22a features suggest that, by using them complementary, they could replace parasite lysate for toxoplasmosis infection screening, and for acute toxoplasmosis diagnosis. Our proposal should be validated by a longitudinal study and may lead to a reliable toxoplasmosis pregnancy control, performing tests in only one serum sample.

Keywords: Acute toxoplasmosis diagnosis, *Toxoplasma gondii*, P35&P22 recombinant proteins, Immunochemical reagents, pregnancy control, Epitope prediction

1. Introduction

Toxoplasma gondii may be a harsh threat for the first time-infected pregnant women, because the parasite can cross placenta, originating congenital infection. *T. gondii* can cause severe damage to the offspring, including death. An early treatment can considerably reduce congenital transmission, but anti-parasite drugs may be harmful for both the mother and the foetus. Considering that symptoms during the acute-phase of the infection are typically unspecific or unobserved, acute toxoplasmosis diagnosis depends on serological tests that indicate the presence of specific antibodies. The undoubting evidence of recently acquired infection is seroconversion and/or an important increment of the specific immunoglobulin titer. Nonetheless, this diagnostic possibility is restricted to places where a prenatal screening, evaluated by analysis of samples serially collected at a 3-weeks interval is mandatory, not being the case for most of the countries. Even though, the chances of finding any of the mentioned events are very exceptional because they limit to cases of a carefully planned pregnancy or to the infrequent case of a first sample collection during the earliest weeks of titer augmentation after the parasite enters the host [1]. Consequently, the search for reliable methodologies to diagnose acute toxoplasmosis is an issue of continue research, particularly aimed to screen this condition in pregnant women so as to provide treatment only if necessary [2].

Classical criterion to diagnose acute-phase infections by finding specific IgM is not trustful for toxoplasmosis, because long-lasting IgM is detected even more than one year after the initial infection [2, 3]. It is then recommended to carry out serial tests which have to be carefully interpreted, a fact that delays the diagnosis in detriment of the patient's health, and increase diagnostic costs [2]. An easy-to-interpret test to rule out acute toxoplasmosis, to be performed in a single sample during pregnancy control would therefore be a very valuable tool.

One of the crucial issues to assess biological markers is the sample selection used as reference. Most of the works studying antigens to discriminate toxoplasmosis stages classify

their samples according to the specific IgM presence. However, specific IgM mostly persists after acute phase [2, 3], with genuine acute infection being less than 50% of those cases where specific IgM is present [4]. This should be critically considered when the acute screening efficiency of an assay is evaluated. Avidity assays are reliable to differentiate between distant and recently acquired toxoplasmosis [4-6], allowing to confirm the chronic stage. Therefore, they can be used to accurately typify the patient infection status [6], and then facilitate the comparison of the acute-*vs.*-chronic infection screening ability of new proteins. This was the criterion used in this work.

Recombinant DNA technology allowed for the individual evaluation of numerous *T. gondii* antigens. During the acute infection, some antigens generate high specific-antibodies levels, whereas these are either low or negligible during the chronic phase [7-9]. This feature could be used to develop immunoassays for differentiation between infection stages, based on identifying the antibody threshold levels [10, 11]. P35 (GRA8) and P22 (SAG2) proteins are antigens that notably elicit early specific IgG antibodies [10, 11]. They have been preliminary evaluated by assessing the specific IgG levels, having shown to be very sensitive during the acute phase [7, 12-16]. However, these previous studies are hardly comparable, because expressions systems, methodologies and solubilisation protocols were different in each work, probably determining dissimilar epitope display causing different antigenic performances [7, 11-17]. Furthermore, no systematic comparison has been published on P35 and P22 ability to screen between acute and recently chronic infection, both of which show positive results for specific IgM. Finally, these two antigens have been scarcely explored in avidity assays, though several alternative recombinant ones have been assayed to replace whole *T. gondii* lysate antigens [15, 16, 18-22]. Consequently, P35 and P22 proteins, early recognized in the acute phase with high sensitivity, should be studied in avidity assays pointing to replace antigens from total parasite homogenate, TPH, searching for more trustful tests that prevent unnecessary treatments.

In this work, we assessed the ability of both antigens to differentiate *T. gondii* infection stages that cannot be discriminated by specific IgM. Using bioinformatic tools we selected abbreviated regions bearing highly antigenic amino acids (AA), rP35a and rP22a, which we heterologously expressed as small soluble proteins that were easily purified; ensuring lower production cost and easier standardization procedures [23]. Their acute-*vs.*-chronic screening performances were evaluated by indirect- and avidity ELISA, with samples from patients coursing different infection stages, including the chronic one with residual specific anti-*T. gondii* IgM.

2. Materials and methods

The studies were made in the frame of the project "Development of analytical methods for determination in complex media: Application to clinical infection diagnosis", which counts with Ethical Committee approval, that contemplates the informed consent (Resolution N° 1070/2014, Expedient 6060/059, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario).

2.1. Epitope prediction program

We had previously studied the prediction ability of several free, on-line available prediction programs [24]. Based upon our results, we selected AAPPred [http://www.bioinf.ru/aappred/] as the better program to predict AA sequences of lineal epitopes. We used the optimum threshold suggested by default, and we considered 6 AA as the minimum sequence length to define an epitope [24]. DiscoTope program was used to predict B-cell structural epitopes [25].

2.2. Reagents

All chemicals were of analytical grade, from Sigma (St. Louis, MO, USA), and molecular biology reagents were from Promega (Madison, WI, USA), unless otherwise stated.

2.3. Serum panel

The serum panel contained 72 pregnant-woman samples which were collected at the Central Laboratory of the Santa Fe province, Argentina. Samples were analyzed by reference techniques and classified according to their results. IgG and IgM were detected by indirect immunofluorescence assay and ELISA-IgM-DS (Radim, Pomezia, Italy), respectively. The ELISA kit, VIDAS-TOXO (Biomerieux, Saint-Vulbas, France), was used to assess IgG avidity. 18 samples displayed negative reactions for both specific IgG and IgM antibodies (not infected group, NI). 36 samples displayed positive reaction for specific IgG and IgM, and were analyzed using IgG avidity test. From these latter samples, 18 turned out to have low avidity indexes, AI%, (presumably acute group, A), and 18 samples displayed high AI% (recently chronic group, RC). 18 samples showed negative reaction for specific IgM, positive reaction for IgG and high AI% (typical chronic, TC).

2.4. Construction of expression plasmids

T. gondii nucleotide sequences were cloned using the pET-32a vector (Novagen, Itapira, Brazil). The Wizard Genomic DNA Purification Kit (Promega, Fitchburg, USA) was used to obtain the parasite genomic DNA, which was used as template for standard PCR amplification. The primers sequences were designed based on P35 and P22 *T. gondii* nucleotide sequences from GenBank database, accession numbers AF150729 and FJ825705, respectively. The sequences were:

P35f (5`-GAATTCGGAATGCCCAAGCCAGAG-3`), P35r (5`-AAGCTTTGGAGTGCCCACTGGATACG-3`), P22f (5`-GGATCCACCACCGAGACGCCAGC-3`), P22r (5`-GAATTCTTGCCCGTGAGAGACACAG-3`). Nucleotide sequence identity was confirmed by sequencing in each cloning step (Sequencing Service, GAD, Universidad Nacional de La Plata, Argentina). Codificant sequences were inserted in pET32a expression plasmids using restriction sites, and plasmidic DNA minipreparations were performed according to the

standard procedure. *Escherichia coli* cells bearing the plasmids were harvested overnight in Luria-Bertani (LB) medium with 0.1 mg/mL ampicillin (USB, Cleveland, USA), at 37°C. Competent bacteria were transformed by one-pulse electroporation (2.5 kV, 25 μF) using a Bio-Rad Gene Pulser (BioRad, Berkeley, USA).

2.5. Protein expression and purification

E. coli BL21 (DE3) cells bearing the plasmidic construction pET-32a/rP35a or pET-32a/rP22a were grown overnight, in LB medium with ampicillin, at 37 ºC. Protein expression was induced for 3 h with 1 mM IPTG (final concentration). Cells were washed with PBS, centrifuged, and resuspended in 50 mM NaH_2PO_4 (pH 8), 300 mM NaCl, and 20 mM imidazole buffer. Cells were lysed by sonication with a Sonics VibraCell Sonicator (Sonics&Materials Inc. Newtown, USA), and centrifuged 30 min at 13,000 rpm. rP35a and rP22a were obtained in soluble form, mostly in the supernatant fraction. Proteins were purified by Ni-nitrilotriacetic acid column (GE, Fairfield, USA) nickel affinity chromatography. Supernatants were applied into the columns, washed with the same buffer, and then eluted in different fractions, using the mentioned buffer plus 50, 100, and 250 mM imidazole, consecutively. Proteins purity and identity were analyzed by 15% polyacrylamide gel electrophoresis, and stained with Coomassie brilliant blue (Sigma, St. Louis, USA).

2.6. Protein antigenicity evaluation

Polystyrene microplates (GBO, Seattle, USA) were sensitized with 500 ng/well of antigen (in carbonate buffer, pH 9.6), incubated 1 h at 37°C, and then overnight at 4°C. Then, they were washed thrice with 0.05% Tween/PBS (Croda, Snaith, UK), and the unmodified surface was blocked with 5% skimmed milk, SM, (Molico, Buenos Aires, Argentina) in PBS, for 1 h at 37°C. Microplates thus sensitized were incubated with 1:100 dilutions of serum samples in 1% SM in PBS. After washing thrice with 0.01% Tween/PBS, microplates were incubated with a 1:2,000 dilution of the peroxidase-conjugated goat anti-human IgG, Fc_γ (Zymed, San Francisco, USA) in 1% SM in PBS. The colorimetric reaction was developed

using 100 µL of tetramethyl benzidine (Zymed) in H_2O_2 (Sigma, St. Louis, USA), and stopped with 100 µL of 1000 mM H_2SO_4 (Cicarelli, San Lorenzo, Argentina). IgG avidity ELISA was performed to samples at several dilutions, in two series of 3 repetitions. Each series was washed with PBS/Tween or 6 M urea in PBS/Tween, respectively. The results herein shown correspond to those obtained with 1:100-diluted samples, the one that allowed the best discrimination.

2.7. Data analysis

Indirect ELISA was performed in duplicate samples, determining their optical density, OD. Results were analyzed using the ELISA index, $ELISA_{index}$:

$$ELISA_{index} = \frac{\text{Mean OD}_{\text{sample}}}{\text{Mean OD}_{\text{NI group}} + 2\,SD_{OD\ NI}}$$

$ELISA_{index}$ was used to obtain the area under curve, AUC, the sensitivity, and the specificity percentages by ROC analysis, for the different cluster of groups compared. IgG avidity ELISA was performed in two series of 3 repetitions. Each series was washed with PBS/Tween or 6 M urea in PBS/Tween, respectively. AI% was calculated as:

$$AI\ \% = \frac{\text{Mean OD}_{\text{urea}}}{\text{Mean OD}_{\text{PBS}}}$$

ROC analysis was performed to obtain an AI% cut-off value corresponding to, at least 90% sensitivity and specificity respectively that allowed discriminating between A and RC. The AI% undetermined zone was defined as that cut-off value ± 10%. Graphics and statistical analysis were performed using GraphPad Prism version 6.00.

3. Results

3.1. Selection and obtainment of acute-phase antigenic regions

3.1.1. rP35a

AAPPpred predicted lineal epitopes at AAs 44-78, 86-91, and 190-196, with the two first displaying the highest scores. DiscoTope program predicted structural epitopes at AA regions 34-38, 41, 50, 57, 61, 62, 68-71, 73-79, 81-83, 88-98, 104-132, 135, 142-144, 150, 157, 158, 191-195, and 197-200. We identified two main antigenic regions, located at AA sequence 44-98, and 107-158. The AA segment 50-150 was selected as the abbreviated protein (Fig. 1A).

3.1.2. rP22a

AAPPred recognized 3 regions as epitopes, AA sequences 30-45, 93-99 and 115-145. P22 structural epitopes were envisaged at AA positions 34-40, 52, 63, 65, 66, 69, 77, 80, 83-86, 95, 96, 98, 162, 163, 165, 166, 169, and 182-186. Considering the uniform distribution of the predicted epitopes, we used the AA segment 27-173 of P22 sequence, excluding both the N-terminal signal peptide- and the carboxylic hydrophobic regions (Fig. 1B).

Figure 1

3.2. rP35a and rP22a general features

The recombinant proteins were obtained and purified according to the standard procedures described in 2.5. (see Fig. 2). rP22a and rP35a yields were typically above 3 mg per 100 mL of induced culture.

Figure 2

3.3. Evaluation of the rP35a and rP22a to differentiate between infection stages

3.3.1. Specific immunoglobulins by indirect ELISA

rP35a and rP22a ability to discriminate between samples from infected and non-infected individuals (A+RC+TC *vs.* NI) was studied by the analysis of the ROC curves. rP22a displayed the greatest area under the ROC curve (AUC), 0.998 *vs.* 0.939 for rP35a, its sensitivity being 98.2% *vs.* 83.3% for rP35a, see Table 1.

Table 1

When comparing results of all the infected groups (A+RC+TC) *vs.* those from NI group, specificity of rP22a turned out to be 94.4%, whereas for rP35a was 83.3%. Nevertheless, rP35a performed better than rP22a to distinguish between A and RC, the AUCs being 0.911 *vs.* 0.818; although both sensitivities were 83.3%, specificities were 83.3% *vs.* 72.2%, respectively. Regarding the proteins ability to distinguish between A and RC+TC groups, rP35a displayed again a better performance than rP22a, with the specificity being 83.3% *vs.* 77.8%, and the sensitivity being 83.3% *vs.* 80.6%, respectively. However, AUCs turned out to be similar, indicating analogous aptitude to discriminate acute from chronic infection.

Both rP35a and rP22a rendered positive results for all A group samples. Therefore, both antigens displayed 100% of equally sensitivity and specificity when comparing A with NI group, having the AUC reached a value of 1. The $ELISA_{index}$ obtained for rP35a was lower than that for rP22a, 4.2 *vs.* 6.2, respectively (see Fig. 3).

Figure 3

When assaying RC group with rP35a, only 50.0% of the samples exhibited positive results and the media index was quite similar to the cut-off value, see Fig. 3, whereas using rP22a, 94.4% were positive for the long-lasting IgM group.

3.3.2. IgG avidity by ELISA

rP35a and rP22a ability to discriminate between samples with low and high IgG AI% was studied by ROC curves analysis, for samples showing IgM+ (A *vs.* RC), see Table 2.

Table 2

Samples with AI% ranging between the cut-off values ± 10% were considered as belonging to the undetermined zone. The sample distribution analysis (Fig. 4) indicates AUC 0.974 and

0.921 for rP35a and rP22a, respectively. rP35a showed 100% sensitivity and 94% specificity, to discriminate between A and RC groups for samples out of the undetermined zone (Table 2).

Figure 4

On the other hand, rP22a displayed 94% sensitivity and 94% specificity to discriminate between both groups, for samples out of the undetermined zone (Table 2). Complementarity between both antigens was studied by plotting their respective AI% values, Fig. 5. Majority of samples from A (circles) are located in the left-inferior quadrant, whereas those from RC (the squares) are in the right-upper one.

Figure 5

rP35a displayed high AI% for most of RC group. Two samples typified as from A, according to the commercial test, rendered AI% one in the undetermined zone and the other one as RC. When assaying these 2 samples with rP22a, the one rendered AI% as from RC and the other sample in the undetermined zone. Two additional samples, with rP22a, showed AI% in the undetermined zone.

4. Discussion

Anti-*T. gondii* IgG determination is among the tests firstly required by obstetricians. In case of positive result, specific IgM, IgA and/or avidity additional tests will confirm or not acute toxoplasmosis. This diagnostic algorithm delays treatment initiation, thus augmenting the foetus infection risk [2]. Then, the finding of one single methodology reliable enough to discriminate late and recent *T. gondii* infection when performed in a sole serum sample is eagerly awaited.

Our objective was to obtain acute phase recombinant proteins to distinguish samples from acute and chronic infected individuals, particularly in cases where specific IgM anti-*T. gondii* is

present. We pointed to P35 and P22 because they had showed to be quite sensitive with sera from acute infected patients [7, 11, 14-16], and could be used in a single indirect ELISA, as previously proposed [10, 11]. Also, they could replace TPH antigens to determine avidity. We therefore heterologously expressed abbreviated regions of these antigens bearing the main antigenic sites, as bioinformatically predicted with prediction programs that we previously evaluated as the best [24, 25].

P35 had been expressed as the full-length protein [11, 12], and also the 1-135 [11, 13], and 23-169 [26] AAs N-terminal fractions, all the three rendering insoluble proteins. Only Hiszczynska-Sawicka *et al*. had expressed a P35 soluble fragment at AA 26-170 [14]. We obtained our soluble rP35a by expressing the selected 50-150 AA region, with the expression system pET32a/BL21DE3, which improves protein solubility, and it has been previously shown that leads to high yielding without immunochemical behaviour loss [17].

Regarding P22, we kept the whole AA region 27-172 described earlier [7] because we verified that the predicted epitopes were homogeneously distributed all along it. From Li *et al.*'s proposal onwards [11], most of the works used this region but with different diagnostic performances [12-15, 27, 28]. Apparently, the variety of expression systems used affected their respective antigenicity, as it had been stressed for other *T. gondii* recombinant antigens by Aubert *et al.* and Prepfer *et al.* [13, 16]. With pET32a/BL21DE3, we obtained P22 without denaturalizing steps for solubilisation, avoiding influencing the sensitivity and specificity of the final tests. Additionally, soluble proteins increase yielding lowering final assay costs and, more importantly, they may expedite a more accurate standardization during production. The good yielding obtained when expressing and purifying both antigens indicate that rP35a and rP22a are good candidates to be used with diagnostic purposes. Moreover, yields can certainly be increased by tuning-up experimental conditions.

Performances of rP35a and rP22a to discriminate among NI, A, and chronic toxoplasmosis were studied with pregnant women serum samples typified according to Section

2.3. Importantly, in our work, all samples were taken previously to treatment, if indicated, thus the delay in antibody maturation observed with treatment [29] has not influenced our study. When discriminating among *T. gondii*-infection stages, rP35a reacted with all A but only a few of RC samples reaching 83.3% of specificity, as calculated having used ROC curves. This specificity value indicates that a positive result with rP35a would lead to unnecessary treat only 17 out of 100 chronic-infected patients, as compared with the alternative of treating 100 out 100 patients who present positive specific IgM. When discriminating A *vs.* (RC+TC) groups, rP35a also presented the best performance. The whole P35 had been previously evaluated for discrimination between acute and chronic infection using an appropriate cut-off value, but low-avidity IgG and positive-IgM sera were not compared in that work [11]. Another study analyzed the full-length P35 with sequential samples from patients whose seroconversion *time-point* was known, describing that anti-P35 IgG levels decreased during the first 7 months of infection, becoming negative shortly later [12].

Interestingly, P35 was studied with sera classified as our A and RC in only one work, which reported 86.7% sensitivity with samples from positive IgM and low IgG avidity, and 54.5% sensitivity with positive IgM and high IgG avidity [14]. Contrarily with the mentioned report, Pfrepper *et al*. [16] showed sensitivities higher than 94% and strong reactivity with samples from recently infected patients, though no plus has been taken from this finding. In this line, our rP35a reacted with the whole A group (100% sensitivity) displaying notably high $ELISA_{index}$ values, and it showed low or negative reactivity with samples from either RC or TC groups. The same trend can be inferred when deeply examining previous data [16]. Therefore, we envisage rP35a as an excellent candidate to differentiate between these populations by using a suitable cut-off. This is apparent from the higher AUC value for A *vs.* (RC+TC) groups. Moreover, considering that avidity assay with TPH, confirms latent but not acute infection, anti-rP35a IgG detection together with a low IgG avidity may allow a more accurate diagnosis

of acute infection, although this finding should be confirm with a larger serum panel with known seroconversion *time-points*.

Unlike rP35a, rP22a sensitivity and specificity results indicate high capability to differentiate infected from uninfected patients (A+RC+TC *vs.* NI) though its performance is poorer than that of rP35a to discriminate A and RC groups. It is noteworthy that, if the ability to discriminate A from TC is analyzed, as other previous studies compared, the sensitivity calculated by AUC is 100%, whereas specificity is 94.4%. Interestingly, rP22a detect antibodies with high sensitivity in most of the populations evaluated, except in few samples where rP35a $ELISA_{indexes}$ were higher, suggesting that both antigens can be used as a set.

P22 high-quality ability to discriminate between samples from infected and uninfected individuals has already been reported [13, 15, 27, 28, 30], a property of this antigen that was confirmed in our work. The acute-*vs.*-chronic discrimination ability of both proteins was also previously evaluated [11]. Li *et al*. report that P22 performed better, since many of the acute-infected patient's sera did not react with P35 [11]. However, unlike here, A and RC groups were not typified as such. One study did so [14], but they found no sensitivity difference between both groups, thus merely pointing to P22 as a good candidate for *T. gondii* infection-*vs.*-not infection screening. Here, we confirmed that anti-rP22a IgG are present in both stages of the infection. Nonetheless, our results clearly show that $ELISA_{index}$ obtained with rP22a are quite higher for samples from acute than from chronically infected patients.

P22 and P35 usefulness for avidity assessment has been poorly explored, and has never been reported for discrimination between IgM+, and low or high AI% samples. Full-length P35 was evaluated in sera of only two patients over time. In turn, another work evaluated P22 for this purpose, and found lesser discrimination ability between acute and chronic phases with P22 than with homogenate [15]. However, the authors do not report having typified samples as A and RC separately, as we did in this work.

Avidity ELISA results show that rP35a performed better than rP22a, based on AUC values. The results obtained with rP35a in 2 samples showed high AI%, meanwhile the same sera had displayed low AI% using the VIDAS test. rP22a allowed classifying those same 2 sera as displaying intermediate or high AI%, together with other 2 sera that had showed low AI% using the VIDAS test. This denotes that antibodies which recognize rP35a and rP22a have high maturation rate (except for one sample assayed with rP22a). These results agree with those reported for other acute-phase recombinant antigens [16, 18, 20, 22]. Drapala *et al.* showed that infections shorter than 4 months were more certainly determined using a mixture of P22 and GRA1 than by using avidity tests with TPH [30]. It should be emphasized that the criterion of low AI% with TPH, with which we classified sera as belonging to the A group [6], did not allow to rule out unequivocally that some of those samples belonged to the RC group. Indeed, some A-group samples displaying low AI% with TPH, displayed high AI% with rP35a and/or rP22a. This suggests that rP22 and rP35 may be better antigens than TPH to typify sera as from individuals coursing acute infection. This feature should be deeper studied using sera where seroconversion time-point is known, to determine whether antibodies against these antigens are more trustful markers than those against TPH to infer recent chronic infection.

When comparing rP35a and rP22a ability to typify sera into A or RC groups, the avidity ELISA results indicated that they could complement each other (Fig. 5). While rP35a performed better with sera from A group in indirect ELISA, IgG determination was less sensitive for the chronic phase. Therefore, in those cases where no anti-rP35a antibodies are detected, avidity ELISA using rP22a could complete the study. This turns out to be crucial in cases where specific anti-*T. gondii* IgM is present.

Bearing in mind the inconveniences described to diagnose acute toxoplasmosis in pregnant women, it has to be remarked the importance of the still up-to-date search for reliable tests. In this work, we highlight rP35a and rP22a features that make them appealing antigens for an accurate acute-toxoplasmosis diagnosis using the very first sample taken, a fact that

could accelerate treatment. We present a proof of concept on rP35a and rP22a usefulness when used in complementary tests. The diagnostic potentiality of our proposed antigens is supported by the following facts: ***i*)** indirect ELISA with rP35a allowed overcoming the inconvenience of the frequent presence of long-lasting IgM by rendering an appropriate sensitivity to discriminate between samples from acute and chronic infected patients; ***ii*)** indirect ELISA with rP22a showed quite higher $ELISA_{indexes}$ for samples from A than for samples from (RC+TC) groups, thus being a good antigen for the mentioned purpose, and an excellent protein to discriminate between not-infected and infected individuals (acute + chronic); ***iii*)** avidity tests with both recombinant proteins complemented each other, so as to replace the parasite homogenate. Furthermore, these antigens might avoid the disadvantage that appears when the patient has low IgG maturation rate and display low AI%, even when occurring chronic infection [1, 6, 20]. Our results should be confirmed using a larger number of longitudinal and transversal samples.

Acknowledgments

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References

1. Sensini A. Toxoplasma gondii infection in pregnancy: opportunities and pitfalls of serological diagnosis. Clin Microbiol Infect 2006;12:504-12.

2. Montoya JG,Remington JS. Management of Toxoplasma gondii infection during pregnancy. Clin Infect Dis 2008;47:554-66.

3. De Paschale M., Agrappi C, Belvisi L, Cagnin D, Cerulli T, Clerici P, et al. Revision of the positive predictive value of IgM anti-Toxoplasma antibodies as an index of recent infection. New Microbiol 2008;31:105-11.

4. Candolfi E, Pastor R, Huber R, Filisetti D, Villard O. IgG avidity assay firms up the diagnosis of acute toxoplasmosis on the first serum sample in immunocompetent pregnant women. Diagn Microbiol Infect Dis 2007;58:83-8.

5. Pelloux H, Brun E, Vernet G, Marcillat S, Jolivet M, Guergour D, et al. Determination of anti-Toxoplasma gondii immunoglobulin G avidity: adaptation to the Vidas system (bioMerieux). Diagn Microbiol Infect Dis 1998;32:69-73.

6. Villard O, Breit L, Cimon B, Franck J, Fricker-Hidalgo H, Godineau N, et al. Comparison of four commercially available avidity tests for Toxoplasma gondii-specific IgG antibodies. Clin Vaccine Immunol 2013;20:197-204.

7. Parmley SF, Sgarlato GD, Mark J, Prince JB, Remington JS. Expression, characterization, and serologic reactivity of recombinant surface antigen P22 of Toxoplasma gondii. J Clin Microbiol 1992;30:1127-33.

8. Golkar M, Azadmanesh K, Khalili G, Khoshkholgh-Sima B, Babaie J, Mercier C, et al. Serodiagnosis of recently acquired Toxoplasma gondii infection in pregnant women using enzyme-linked immunosorbent assays with a recombinant dense granule GRA6 protein. Diagn Microbiol Infect Dis 2008;61:31-9.

9. Martin V, Supanitsky A, Echeverria PC, Litwin S, Tanos T, De Roodt AR, et al. Recombinant GRA4 or ROP2 protein combined with alum or the gra4 gene provides partial protection in chronic murine models of toxoplasmosis. Clin Diagn Lab Immunol 2004;11:704-10.

10. Redlich A,Muller WA. Serodiagnosis of acute toxoplasmosis using a recombinant form of the dense granule antigen GRA6 in an enzyme-linked immunosorbent assay. Parasitol Res 1998;84:700-6.

11. Li S, Galvan G, Araujo FG, Suzuki Y, Remington JS, Parmley S. Serodiagnosis of recently acquired Toxoplasma gondii infection using an enzyme-linked immunosorbent assay with a combination of recombinant antigens. Clin Diagn Lab Immunol 2000;7:781-7.

12. Lu B, Wu S, Shi Y, Zhang R, Zou L, Gao S, et al. Toxoplasma gondii: expression pattern and detection of infection using full-length recombinant P35 antigen. Exp Parasitol 2006;113:83-90.

13. Aubert D, Maine GT, Villena I, Hunt JC, Howard L, Sheu M, et al. Recombinant antigens to detect Toxoplasma gondii-specific immunoglobulin G and immunoglobulin M in human sera by enzyme immunoassay. J Clin Microbiol 2000;38:1144-50.

14. Hiszczyñska-Sawicka E, Kur J, Pietkiewicz H, Holec L, Gasior A, Myjak P. Efficient production of the *Toxoplasma gondii* GRA6, p35 and SAG2 recombinant antigens and their applications in the serodiagnosis of toxoplasmosis. Acta Parasitol 2005;50:249-54.

15. Bela SR, Oliveira Silva DA, Cunha-Junior JP, Pirovani CP, Chaves-Borges FA, Reis de CF, et al. Use of SAG2A recombinant Toxoplasma gondii surface antigen as a diagnostic marker for human acute toxoplasmosis: analysis of titers and avidity of IgG and IgG1 antibodies. Diagn Microbiol Infect Dis 2008;62:245-54.

16. Pfrepper KI, Enders G, Gohl M, Krczal D, Hlobil H, Wassenberg D, et al. Seroreactivity to and avidity for recombinant antigens in toxoplasmosis. Clin Diagn Lab Immunol 2005;12:977-82.

17. Marcipar IS, Olivares ML, Robles L, Dekanty A, Marcipar A, Silber AM. The diagnostic performance of recombinant *Trypanosoma cruzi* ribosomal P2beta protein is influenced by its expression system. Protein Expr Purif 2004;34:1-7.

18. Beghetto E, Buffolano W, Spadoni A, Del PM, Di CM, Minenkova O, et al. Use of an immunoglobulin G avidity assay based on recombinant antigens for diagnosis of primary Toxoplasma gondii infection during pregnancy. J Clin Microbiol 2003;41:5414-8.

19. Pietkiewicz H, Hiszczynska-Sawicka E, Kur J, Petersen E, Nielsen HV, Paul M, et al. Usefulness of Toxoplasma gondii recombinant antigens (GRA1, GRA7 and SAG1) in an immunoglobulin G avidity test for the serodiagnosis of toxoplasmosis. Parasitol Res 2007;100:333-7.

20. Elyasi H, Babaie J, Fricker-Hidalgo H, Brenier-Pinchart MP, Zare M, Sadeghiani G, et al. Use of dense granule antigen GRA6 in an immunoglobulin G avidity test to exclude acute Toxoplasma gondii infection during pregnancy. Clin Vaccine Immunol 2010;17:1349-55.

21. Holec-Gasior L, Ferra B, Hiszczynska-Sawicka E, Kur J. The optimal mixture of Toxoplasma gondii recombinant antigens (GRA1, P22, ROP1) for diagnosis of ovine toxoplasmosis. Vet Parasitol 2014;206:146-52.

22. Deshpande PS, Kotresha D, Noordin R, Yunus MH, Saadatnia G, Golkar M, et al. IgG avidity Western blot using Toxoplasma gondii rGRA-7 cloned from nucleotides 39-711 for serodiagnosis of acute toxoplasmosis. Rev Inst Med Trop Sao Paulo 2013;55:79-83.

23. Makino M, Uemura N, Moroda M, Kikumura A, Piao LX, Mohamed RM, et al. Innate immunity in DNA vaccine with Toxoplasma gondii-heat shock protein 70 gene that induces DC activation and Th1 polarization. Vaccine 2011;29:1899-905.

24. Costa JG, Faccendini PL, Sferco SJ, Lagier CM, Marcipar IS. Evaluation and comparison of the ability of online available prediction programs to predict true linear B-cell epitopes. Protein Pept Lett 2013;20:724-30.

25. Kringelum JV, Lundegaard C, Lund O, Nielsen M. Reliable B cell epitope predictions: impacts of method development and improved benchmarking. PLoS Comput Biol 2012;8:e1002829.

26. Babaie J, Miri M, Sadeghiani G, Zare M, Khalili G, Golkar M. Expression and Single-step Purification of GRA8 Antigen of Toxoplasma gondii in Escherichia coli. Avicenna J Med Biotechnol 2011;3:67-77.

27. Khanaliha K, Motazedian MH, Kazemi B, Shahriari B, Bandehpour M, Sharifniya Z. Evaluation of recombinant SAG1, SAG2, and SAG3 antigens for serodiagnosis of toxoplasmosis. Korean J Parasitol 2014;52:137-42.

28. Singh H, Tewari AK, Mishra AK, Maharana B, Sudan V, Raina OK, et al. Detection of antibodies to Toxoplasma gondii in domesticated ruminants by recombinant truncated SAG2 enzyme-linked immunosorbent assay. Trop Anim Health Prod 2015;47:171-8.

29. Roberts A, Hedman K, Luyasu V, Zufferey J, Bessieres MH, Blatz RM, et al. Multicenter evaluation of strategies for serodiagnosis of primary infection with Toxoplasma gondii. Eur J Clin Microbiol Infect Dis 2001;20:467-74.

30. Drapala D, Holec-Gasior L, Kur J, Ferra B, Hiszczynska-Sawicka E, Lautenbach D. A new human IgG avidity test, using mixtures of recombinant antigens (rROP1, rSAG2, rGRA6), for the diagnosis of difficult-to-identify phases of toxoplasmosis. Diagn Microbiol Infect Dis 2014;79:342-6.

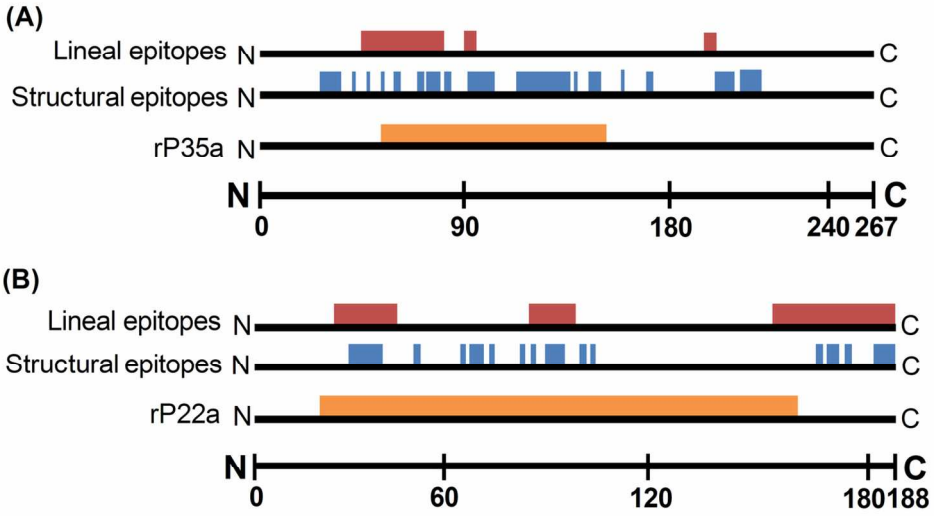

Figure 1: Epitopes predicted for: (A) P35 and (B) P22 proteins, together with abbreviate recombinant proteins rP35a and rP22a. First line displays the lineal epitopes predicted by AAPPred. Second line displays the structural epitopes predicted by DiscoTope. The third line shows the abbreviated region selected.
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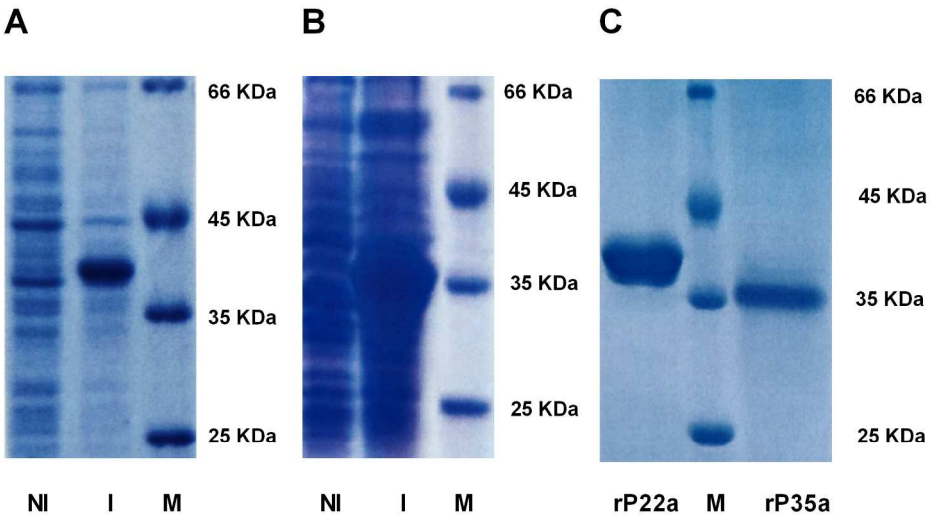

Figure 2: Expression and purification of the antigens. Coomassie blue-stained gels after SDS-PAGE of the antigens. A, expression of rP22a. B, expression of rP35a. C, rP22a and rP35a purification. Lanes: NI, not induced; I, induced; M molecular weight marker with their respective weights indicated aside.
207x114mm (300 x 300 DPI)

Table 1. rP35a and rP22a performances to discriminate among samples from different patient groups.

ROC analysis	rP35a			rP22a		
	AUC	Sensitivity (%)	Specificity (%)	AUC	Sensitivity (%)	Specificity (%)
A+RC+TC *vs.* NI	0.939	83.3	83.3	0.998	98.2	94.4
A *vs.* RC	0.911	83.3	83.3	0.818	83.3	72.2
A *vs.* RC+TC	0.915	83.3	83.3	0.907	80.6	77.8
A *vs.* TC	0.920	83.3	83.3	0.997	100.0	94.4
A *vs.* NI	1	100	100	1	100	100

Serum groups: **A**, Presumably acute (*IgG+, IgM+, low avidity*); **RC,** recently chronic *(IgG+, IgM+, high avidity*); **TC**, typical chronic (*IgG+, IgM-*); **NI**, not infected (*IgG-*). Each group contained 18 samples.
AUC was determined for the groups or group clusters indicated in the first column. Sensitivity and specificity were determined using the cut-off value obtained by ROC analysis, for the best discrimination among the groups indicated in each row.

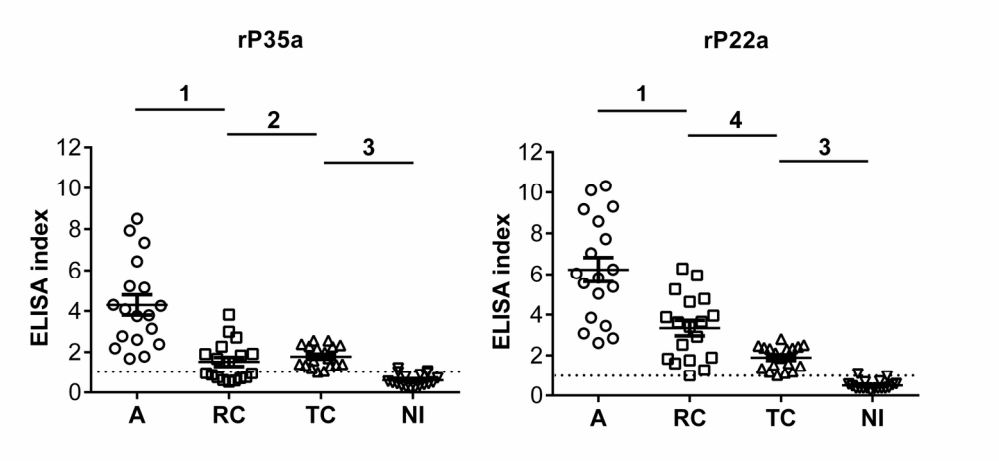

Figure 3: IgG antibody level against rP35a and rP22a in sera from the different groups. ELISA index for serum groups A, presumably acute (IgG+, IgM+, low-avidity IgG); RC, recently chronic (IgG+, IgM+, high avidity IgG); TC, typical chronic (IgG+, IgM-); NI, Not infected (IgG-), 18 samples per group. Horizontal lines indicate the mean, and error bars the SEM. Statistical significance was determined by ANOVA and Tukey's multiple comparisons post test. (1) A *vs.* RC, difference statistically significant (P<0.0001). (2) RC vs. TC, difference statistically not significant (P>0.05). (3) TC vs. NI, difference statistically significant (0.01<P<0.05). (4) RC vs. TC, difference statistically significant (0.01<P<0.05).
165x76mm (300 x 300 DPI)

Table 2. Comparison of rP35a and rP22a performances to discriminate between samples from **A** and **RC** groups by avidity ELISA.

ROC analysis	rP35a	rP22a
Undetermined zone (cut-off±10%)	50%<AI%<56%	56%<AI%<68%
AUC	0.974	0.921
Samples not unequivocally typified (%)	12	15
Sensitivity (%)	100	94
Specificity (%)	94	94

A, Presumably acute (*IgG+, IgM+, low avidity*); **RC,** recently chronic *(IgG+, IgM+, high avidity*).
Sensitivities and specificities were calculated taking into account samples displaying AI% values out of the undetermined zone.

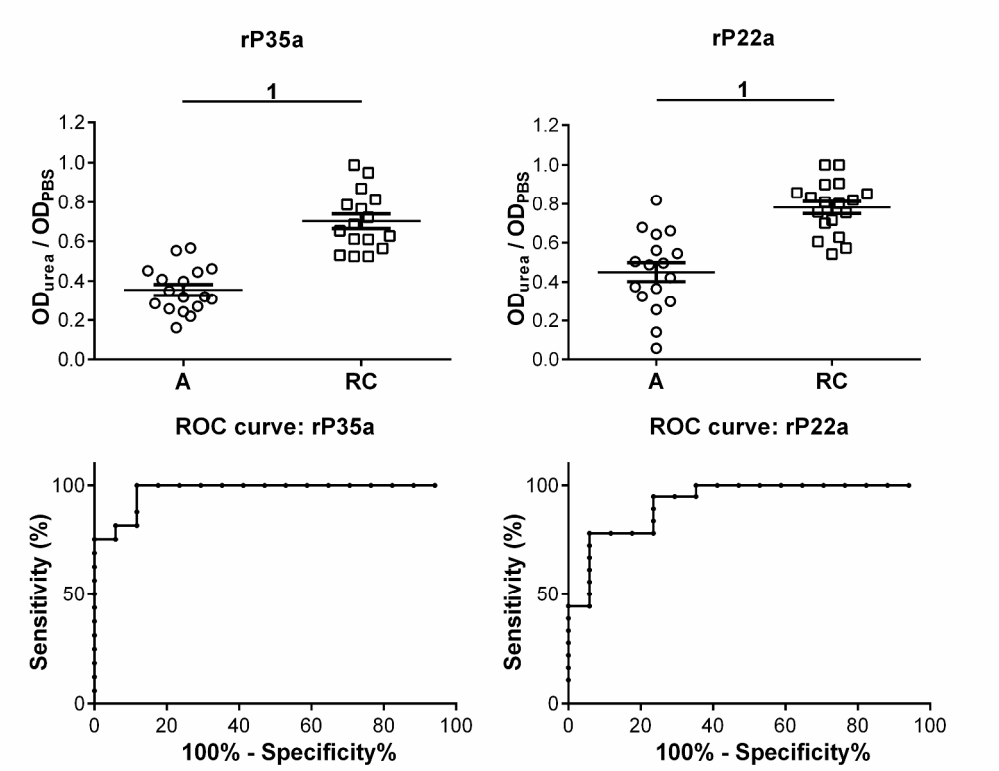

Figure 4: Avidity percentage of IgG antibodies against rP35a and rP22a for each serum group, and ROC curves. Avidity percentage was calculated as the ratio between the mean OD of the three replicates washed with urea (ODurea) and the three ones washed with PBS (ODPBS). A, presumably acute (IgG+, IgM+, low-avidity IgG); RC, recently chronic (IgG+, IgM+, high avidity IgG); 18 samples per group. Horizontal lines indicate the mean, and error bars indicates the SEM. Statistical significance was determined by Student´s t test. (1) A *vs.* RC, difference statistically significant (P<0.0001).
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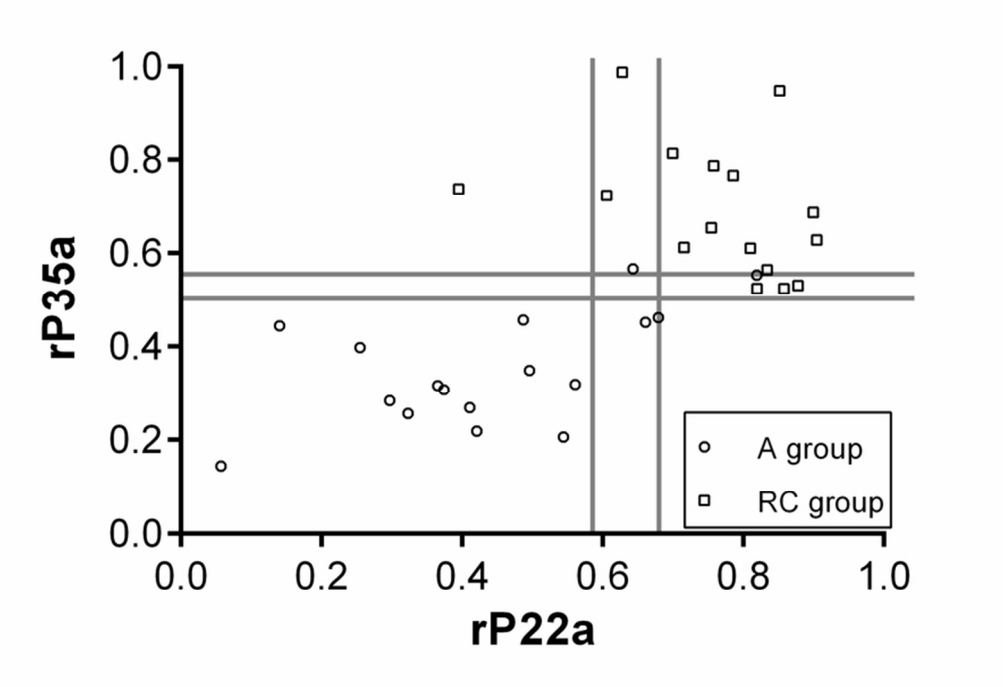

Figure 5: Avidity percentage values of rP35a and rP22a to evaluate complementarity of both antigens. Circles represent sera from A group (IgG+, IgM+, low avidity IgG), and squares represent sera from RC (IgG+, IgM+, high avidity IgG), 18 samples per group. Horizontal and vertical lines indicate upper and lower limit of the undetermined zone, defined as the cut-off value ± 10%, for each antigen.
74x51mm (300 x 300 DPI)