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Technical note

An improved approach to estimate the avidity index of immunoglobulins: Evaluation of the method using IgG anti-*Toxoplasma gondii*

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1. Introduction

The daily clinical practice frequently force physicians to make a decision of whether a patient is coursing an infectious disease at the acute or the chronic stage, since treatment may be different depending on it. This is the case of toxoplasmosis, a worldwide disease caused by the intracellular parasite *Toxoplasma gondii*. When a pregnant woman is infected for the first time by *T. gondii* and is not treated, the parasite can cross the placenta and severely affect the fetus. In case of primary infection, the treatment protocol includes using antiparasitic drugs to prevent serious damages, including death of the unborn baby. However, antiparasitic chemicals have side effects that should be avoided, unless treatment is mandatory. That is why discrimination between long-term and primary *T. gondii* infection turns out to be critical in infected pregnant women (Montoya and Remington, 2008).

The identification of the acute-infection state by only finding specific IgM in serum, a common procedure used for other infection diagnosis, is not reliable because residual IgM may occur for even more than one year after the first exposure to the parasite, turning its diagnosis quite difficult (Montoya and Remington, 2008). IgG-avidity assays can help to differentiate between recently acquired and distant infection, by using the avidity index (AI) (Barros et al., 2017; de et al., 2017). When diagnosing acute toxoplasmosis, although IgG maturation rate varies with each individual, a high AI confirms the chronic phase, thus ruling out a recent infection (Robert-Gangneux and Darde, 2012). On the other hand, a low AI suggests that the sample belongs to a recently infected individual, though exceptions to this outline exist (Drapala et al., 2014; Remington et al., 2004).

As firstly proposed by Hedman et al., anti-*T. gondii* IgG-AI is estimated using two different titration curves obtained by treating each sample dilution with two different solutions, one with and the other without 6 M urea (Hedman et al., 1989). The corresponding end-points (E-P) are determined for a particular cutoff-value, and results are expressed as the percentage calculated on the basis of the E-P ratio. Nevertheless, difficulties may arise in using avidity curves when the number of dilutions is insufficient, *i.e.* when the highest routine dilution renders an optical density (OD) higher than the cutoff-value. An easier alternative to the E-P titration method is the OD one, where triplicate measurements of the IgG amount are performed at a single dilution level, and AI is calculated as the ratio between the mean OD obtained having washed with and without urea (Holliman et al., 1994). The main difficulty of this method is that AI can noticeably vary with the total amount of anti-*T. gondii* IgG in the specimen (Jenum et al., 1997). Prince and Wilson combined the accuracy of assays based on the E-P titers and the simplicity of OD assays, where IgG reactivity was measured quantitatively using a standard curve (Prince and Wilson, 2001).

Here, we have used rP22a, an acute-phase recombinant protein we recently described (Costa et al., 2017), to assess anti-*T. gondii* IgG AI, calculated by two new easier approaches, one based on the area under the avidity curve (AUAC) and the other one based on the E-P titration method with minor variations. We compare our results with those obtained when calculating the AI by the conventional methods above mentioned, and the performance to render a proper sample classification as compared to that obtained having used a commercial kit as standard.

2. Materials and methods

This work was approved by Ethics Committee at the Faculty of Biochemical and Pharmaceutical Sciences, University of Rosario (Res. 1070/2014, Exp. 6060/059).

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2.1. Chemicals, reagents, and recombinant protein preparation

Analytical grade chemicals to prepare saline and buffer solutions were from Sigma (St. Louis, MO), unless otherwise stated. Molecular biology reagents were provided by Promega (Madison, WI). Procedures to construct the plasmid encoding rP22a, those used to express the protein, and those to purify it were described in a previous work where we evaluated the protein antigenicity for diagnosis of recently acquired toxoplasmosis (Costa et al., 2017).

2.2. Serum panel

A typified serum panel containing 48 pregnant-woman samples from Santa Fe Central Laboratory, Argentina, was used. IgG and IgM were detected by indirect immunofluorescence assay and ELISA-IgM-DS (Radim, Pomezia, Italy). Sixteen samples displayed no specific IgG and IgM antibodies (not infected group, NI). Thirty-two samples displayed anti-*T.gondii* IgG and IgM antibodies, and their respective IgG avidity was further tested with the VIDAS-TOXO ELISA kit (bioMérieux, Saint-Vulbas, France). Sixteen of these latter samples displayed low AI, (low-avidity group, LA), whereas the remaining 16 contained specific IgM together with high-AI IgG (high avidity group, HA), these samples presumably belonging to recently chronic infected patients, whose sera still contain IgM produced at early stage but the IgG has already maturated). Differentiation between LA and HA groups is not usually simple. IgG (+) and IgM (−) samples were not included in the serum panel studied because no avidity test has to be performed on them, since these samples belong to chronically infected individuals (Montoya and Remington, 2008).

2.3. IgG-avidity ELISA

IgG-avidity ELISA using rP22a recombinant protein was performed to study the four methods capability to assess IgG-AI. Polystyrene microplates (GBO, Seattle, USA) were sensitized with 500 ng/well of protein (carbonate buffer, pH 9.6), incubated 1 h at 37 °C, and then overnight at 4 °C. Microplates were washed thrice (0.05% Tween/PBS, Croda, Snaith, UK). Unmodified sites were blocked with 5% w/v skimmed milk, SM, (Molico, Argentina) in PBS (30 min, 37 °C). Samples were serially diluted in 1% SM/PBS, from 1:100 v/v dilution in twofold steps, and assayed in duplicate (Hedman et al., 1989). The dilution that allowed the best discrimination (1:100) was tested in 2 series of 3 replicates. Plaques were incubated for 30 min at 37 °C (30′–37 °C), and washed thrice with 0.05% Tween/PBS. Then, doublet wells were treated either with 0.01% Tween/PBS or with 6 M urea in 0.01% Tween/PBS (30′–37 °C), and washed thrice with 0.05% Tween/PBS. Finally, plaques were incubated with peroxidase-conjugated goat anti-human IgG, Fc_γ (Zymed) 1:2000 diluted in 1% SM/PBS (60′–37 °C). The reaction was developed using 100 µL of tetramethyl benzidine (Zymed) in H_2O_2 (10′–25 °C), and stopped with 100 µL of 1000 mM H_2SO_4 (Cicarelli, San Lorenzo, Argentina). ELISA results were registered as OD at 450 nm.

2.4. AI calculation and data analysis

Cutoff-values were calculated as 2-fold the mean OD of samples from the NI group. AI of samples was assessed in four different ways: i) Classical OD method, with AI being expressed as the ratio between the mean OD of the three replicates of 1:100-diluted samples washed with urea/Tween/PBS (OD_{urea}) and those washed with Tween/PBS (OD_{PBS}):

$$AI\% = \frac{MeanOD_{urea}}{MeanOD_{PBS}}$$

ii) Classical cutoff E-P titer, calculated as the ratio between the titers of urea- and PBS- treated specimens. Titers were determined by interpolating their OD *vs.* dilution curves, at the dilution that rendered an OD value equal to 2-fold the ELISA cutoff. iii) 50% E-P titer, calculated as in ii, but interpolating data to the dilution that renders an OD value equal to 50% of the maximum OD, the curve has the highest slope. iv) AUAC, calculated as:

$$AI\% = \frac{AUAC_{urea}}{AUAC_{PBS}}$$

where $AUAC_{urea}$ and $AUAC_{PBS}$ are the areas under the curves obtained when serially diluted samples were treated with urea and PBS, respectively.

The mean coefficient of variation ($\overline{C_v}$) was used to estimate the relative dispersion of each method, which is also considered an indicator of precision of a set of data [http://www.ilexmedical.com/files/PDF/ToxoIgGAvidity_ARC.pdf]:

$$\overline{C_v} = \frac{\sum_{1}^{n} C_v}{n}$$

where C_v is the ratio between the standard deviation and the mean AI obtained for each sample tested in duplicate, and *n* the number of samples tested.

Receiver operating characteristic (ROC) curves were obtained. The analysis was performed in order to evaluate the methods capability to discriminate between the sera groups (see Results and discussion in the following section).

3. Results and discussion

Human immunological response generally includes an immunoglobulin selection process that tends to increase the production of that specific IgG antibody displaying more affinity to the particular antigen. Consequently, IgG-antigen association strength mainly depends on the period of time elapsed since the antibody was first produced. An evaluation of the IgG-antigen association strength is therefore a useful approach to assess whether a patient suffers from a distant or a recent infection, with the AI being the proposed parameter to estimate it (Perciani et al., 2007). Taking into account that the objective of this work was to contribute with a confident method to asses AI, we evaluated our new proposal by analyzing the values obtained when performing IgG avidity ELISAs to distinguish between recent and distant *T. gondii* infection in pregnant women, a population for which this distinction is essential. The criterion we applied to determine the patients' infection status was the one already well established. We then assessed the presence of specific IgG and IgM, as well as the IgG-AI value, with the mentioned commercial kits to classify samples of our serum panel, having studied sera from: i) pregnant women who presumably suffered from acute infection, thus displaying specific anti-*T. gondii* IgM, and IgG with low AI values (LA group), and ii) pregnant women who have already maturated their specific anti-*T. gondii* IgG, thus displaying high AI values but still having specific anti-*T. gondii* IgM (HA group). This careful selection of the serum panel was carried out because it is usually a hard task to make a distinction between both sets of samples. However, it should be kept in mind that we used the VIDAS-TOXO ELISA commercial kit from bioMérieux to classify samples as truly belonging to the LA group. This fact does not allow unequivocally exclude the possibility that some of those samples belong to the HA group. Even so, we hold this criterion because we intended to compare the different AI calculation approaches rather than to evaluate the performance of rP22a to detect low AI samples.

In order to compare performances of the methods to calculate AI, we used 3 figures of merit that numerically describe their respective quality, namely the area under the ROC curve, the sensitivity and the specificity. Accuracy of a diagnostic method can be measured by the area under the ROC curve, where an area equal to 1 represents a perfect test (Metz, 1978). In the case we are here studying, the area under the ROC curve measures discrimination, *i.e.* the ability of the test to

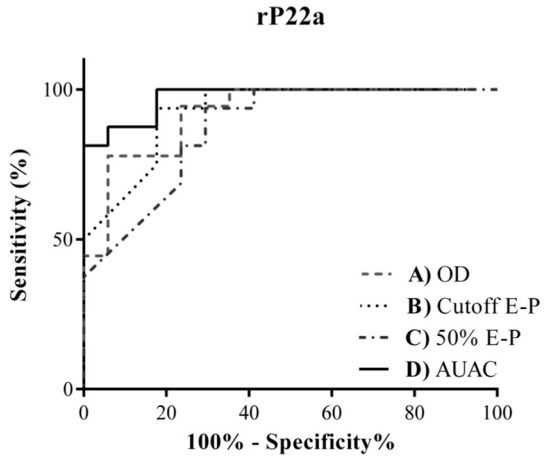

Fig. 1. Comparison between the ROC curves obtained with results of IgG avidity ELISA performed with rP22a, with the AI having been calculated by the following methods: A) OD, B) Cutoff E-P, C) 50% E-P and D) AUAC.

Table 1
Comparison among the four methods used to calculate IgG avidity index. Sensitivity and specificity were determined using the cutoff value obtained by ROC analysis, for the best discrimination among the groups.

Method used to calculate the AI	AU_{ROC}	Sensitivity (%)	Specificity (%)
OD	0.92	77.78	94.12
Cutoff E-P	0.92	83.33	82.35
50% E-P	0.87	81.25	76.47
AUAC	0.96	83.33	94.12

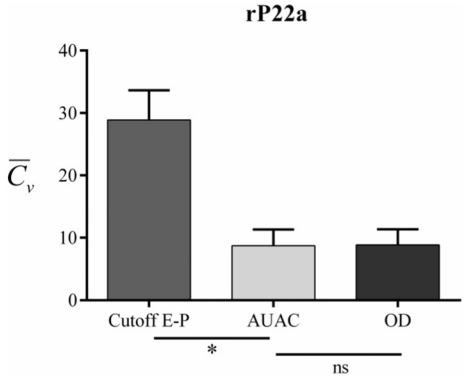

Fig. 2. Comparison of the relative ELISA IgG-avidity results dispersion, estimated by the mean coefficient of variation for the avidity indexes calculated by the cutoff end point method, Cutoff E-P, by the area under the avidity curves new proposal, AUAC, and by the optical density method, OD. *Significantly different from each other ($p < 0.01$), ns not statistically different.

correctly classify samples into low and high avidity group. Fig. 1 displays the ROC curves obtained for each method, when assessing the whole serum panel to discriminate samples from LA and HA groups. Assuming that the more accurate test is the one displaying the curve that follows the left-hand border and the top border of the ROC space (Metz, 1978), from Fig. 1 it can be seen that our proposed AUAC is the one that better fits this premise, thus indicating that this method is the best among the 4 we compared.

A summary of the performances of the four methods studied is depicted in Table 1, where the chosen figures of merit to describe their aptitude to calculate AI are displayed.

When analyzing Table 1, it can be seen that the 50% E-P titer method displayed the smallest area under ROC curve (AU_{ROC}), and also the lowest sensitivity and specificity. This means that this proposal was the worst among the 4 methods used to calculate AI, and we therefore no longer used this method for further analysis. Conversely, the best method to discriminate between LA and HA groups was AUAC, because the $AU_{ROC} > 0.96$, which is very close to the ideal area, 1. Defining the method sensitivity as the rate between the number of samples correctly classified as with LA over the total number of samples displaying LA, the AUAC method together with the cutoff E-P presented the highest sensitivity, which turned out to be > 83%. Meanwhile, specificity, understood as the ratio between the number of samples correctly included in the HA group over the actual number of HA samples in the panel was higher than 94% for both the classical OD method and our AUAC. Even so, remarkably, our new AUAC method substantially enhanced both AU_{ROC} and sensitivity as compared to the classical OD method (0.96 *vs.* 0.92 and 83% *vs.* 78%, respectively).

Another important feature to be evaluated is the capability of our proposal to reproduce results, as compared to the methods nowadays used. We therefore calculated $\overline{C_v}$, as an estimation of the degree of the dispersion of results for each of the methods used to calculate AI (Fig. 2).

As shown in Fig. 1, $\overline{C_v}$ for the AUAC method was significantly lower than that for the cutoff E-P method, whereas $\overline{C_v}$ for the OD method was not significantly different from that for the AUAC method. Therefore, the AUAC and OD methods performed better than the cutoff E-P method, regarding reproducibility.

As a whole, when comparing performances of the approaches displaying the best quality figures of merit, the E-P method can prevent the AI value distortion which depends on the total IgG amount present in the sample. However, if the OD of the most diluted sample is higher than the cutoff-value, it will require further dilutions, as described above. The good AUAC method performance provides a solution to this problem.

4. Conclusion

In this work we used an alternative display and analysis of the avidity data which allowed obtaining reliable IgG-AI values. As a major improvement, our AUAC approach avoids the difficulties to determine the E-P (titration curve method), and IgG quantitation (OD method). This is therefore a simple method for AI calculations when reliable values are needed, such as when diagnosing toxoplasmosis stages in pregnant women.

Disclosure

All authors declare that there is no conflict of interests regarding the publication of this paper.

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