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SEROLOGY USING rROP2 ANTIGEN IN THE DIAGNOSTIC OF TOXOPLASMOSIS IN PREGNANT WOMEN

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SUMMARY

Toxoplasma gondii causes severe fetal disease during acute infection in pregnant women, thus demanding early diagnosis for effective treatment and fetus preservation. Fetal tests are inefficient and risky, and diagnosis is based on maternal IgM serology, which had weak screening ability due to increased sensitivity, with alternative IgG avidity tests. Here, we performed ELISA and avidity assays using a recombinant *T. gondii* antigen, rROP2, in samples from 160 pregnant women screened from a large public hospital who were referred due to positive IgM assays. IgG serology and avidity assays were compared using whole *T. gondii* extract or rROP2. ELISA IgG detection with rROP2 showed good agreement with assays performed with *T. gondii* extract, but rROP2 IgG avidity assays were unrelated to whole extract antigen IgG avidity, regardless of the chaotrope used. These data show that avidity maturation is specific to individual antigen prevalence and immune response during infection. ELISA rROP2 IgG assays may be an alternative serological test for the diagnosis of toxoplasmosis during pregnancy, although our data do not support their use in avidity assays.

KEYWORDS: Toxoplasmosis; Pregnancy; Serology; Recombinant proteins; Avidity.

INTRODUCTION

Toxoplasmosis, which is usually asymptomatic, affects approximately 60% of the Brazilian population, resulting in an increased risk of congenital infection in hundreds of pregnancies each year in the Metropolitan region of São Paulo, where 200,000 children are born each year (GUIMARÃES *et al.*, 1993). Diagnosis of acute or chronic toxoplasmosis is generally performed by serological assays, followed by confirmatory parasitological tests of fetal *T. gondii* infection (CARVALHEIRO *et al.,* 2005). Some serological tests use live agents (Sabin Feldman assay), intact parasites, as indirect immunofluorescence assays or whole extracts in enzymatic immunosorbent assays to detect the presence of specific IgM for definition of acute (less than one year) infections during screening for treatment (REMINGTON *et al.,* 2006). The development of commercial assays and the use of ultra-sensitive techniques such as IgM capture tests increased the sensitivity of these assays, but resulted in extended periods of IgM presence after acute infections, with consequent loss of screening efficiency (SUZUKI *et al.,* 2001). IgG avidity was introduced for temporal definition of viral infection in rubella (LEHTONEN & MEURMAN, 1982), and was adapted to toxoplasmosis to distinguish recent and old infections (HEDMAN *et al.,* 1989). Avidity is based on the binding strength of specific IgG to a multivalent antigen of the parasite, which is produced at low concentrations after the primary infection, but is selectively increased over time. IgG avidity is the summation of specific affinities of antibodies present in polyclonal sera against an extract or antigen (PELLOUX *et al.*, 1998). High avidity or chaotrope-resistant antibodies are characteristic of memory immune response and chronic infection, and the prevalence of low avidity or chaotrope-sensitive antibodies could indicate acute or recent infection. Urea is the common chaotrope used (CAMARGO *et al.,* 1991), an alternative to ammonium thiocyanate (FERREIRA & KATZIN, 1995). Several studies have reported that the avidity of antibody IgG is a good index of the time of the infection, defining if the acute infection has occurred in the past few months despite conflicting results between tests (BARBERI *et al.,* 2001). Recombinant antigens were frequently tested in serology for toxoplasmosis (SUZUKI *et al*, 2001), usually with adequate but weak screening results compared to whole extracts in ELISA (VAN GELDER *et al.,* 1993). The association of recombinant proteins (AUBERT *et al.,* 2000, NIGRO *et al.,* 2003, PIETKIEWICZ *et al.,* 2007) or chimeric constructions (BEGHETTO *et al.,* 2006) has improved these tests, with sporadic use in IgG avidity assays (BEGHETTO *et al.,* 2003). Most of these studies were performed on selected sera panels defined by other tests, which could interfere in efficacy determination. Here, we standardized and tested "in house" ELISA and avidity assays using a recombinant rROP2 protein, and compared the results to standard assays using whole parasite extract in the serology and avidity determination of a population of IgM-positive pregnant women from São Paulo, Brazil who were referred to our specific fetal infections research group.

MATERIAL AND METHODS

Population: Pregnant women were referred to the Fetal Infection group of the Hospital das Clínicas da Faculdade de Medicina da

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Universidade de São Paulo (HC-FMUSP) with positive external serology for toxoplasmosis IgM by various commercially available tests for routine prenatal screening. One hundred sixty patients were enrolled in this study after informed consent. All patients were receiving regular spiramycin treatment, instituted at the time of positive IgM at a public health clinic, usually at least one week before entering the study. Blood was collected upon admission for confirmatory serology tests and avidity assays. Patients were then monitored clinically and analyzed by morphological ultrasound and amniotic fluid sampling when indicated, with subsequent parasitological assays or B1 PCR for introducing aggressive therapy. This program was approved and controlled by our Ethical Committee, CAPPesq under no 365/00.

Antigens: Tachyzoites of *T. gondii* RH strain were maintained routinely in the Laboratory of Protozoology of the Institute of Tropical Medicine of São Paulo. Tachyzoites were passaged in serologically negative Swiss mice (20-22g, aged 30-60 days) supplied by our central colony. The animals were injected with the tachyzoite culture intraperitoneally after washing the peritoneal cavity with sterile saline solution containing gentamycin (40 µg/mL). All procedures involving animals followed the rules described either in "Principles of Laboratory Animal Care" (NHI Publication in the 86-23, revised 1985) or "Principles of Ethics in Animal Experimentation" (COBEA-Brazilian School of Animal Experimentation).

Toxoplasma gondii whole saline antigen was prepared as previously described (CAMARGO *et al.,* 1978). Briefly, tachyzoites from PBS washings of the peritoneal cavity of Swiss mice two days after infection were flowed over Sephadex G-25 columns for host cell removal and recovered by centrifugation. The sediment containing $5x10^8$ tachyzoites was suspended in 5.0 mL of water and subjected to sonic rupture, five to 10 periods of 30 seconds of 40 Hz, in an ice bath, until complete parasite disruption as assessed by phase contrast observation. The solution was added to 5 mL of 0.3 M NaCl, which was then homogenized and centrifuged at 10,000g for 30 minutes at 4 ºC. Protein concentration of the supernatant was calculated, and the supernatant was distributed in aliquots of 0.5 mL, frozen at -70 ºC, and used in all experiments as whole extract.

Recombinant purified rROP2 (MARTIN *et al.,*1998, NIGRO *et al.,* 2001) was produced in *Escherichia coli* cells by transfection with the pQE-30 vector containing a fragment of rROP2 mRNA containing 196-561 base pairs and an adjacent His-Tag and stop codon. The inclusion bodies recovered after the cell membranes were disrupted and solubilized with 8 M Urea, and the recombinant protein was affinity-purified on nickel columns. Protein content was determined and samples were distributed and stored at -70 ºC until use.

Antigen identification by SDS-PAGE and immunoblotting: The antigens were characterized by SDS-PAGE and immunoblotting with hyper-immune rabbit serum or anti-*T. gondii* IgG positive and negative human sera. Briefly, antigen was suspended in sample buffer containing 4 M Urea and applied to 10% SDS-PAGE. After running, the proteins were transferred to a nitrocellulose membrane (TransBlot Biorad, USA) by semidry transfer, and individual strips were reacted with three antiserums: anti-*T. gondii* hyper-immune rabbit serum, anti-*T. gondii* IgG-positive human serum and anti-*T. gondii* IgG-negative human serum. After incubation, each strip was treated with adequate concentrations of specific anti-IgG peroxidase conjugates, and bands were visualized with diaminobenzidine and H_2O_2. Strips were mounted according to origin and documented.

Serological and avidity assays: *Toxoplasma*-specific IgG was detected using both an indirect immunofluorescence Assay (IFA) and ELISA, while IgM was detected by IFA alone. IFA was performed on slides recovered with formalin-fixed tachyzoites. Briefly, tachyzoites were obtained from mice and purified as described above, suspended in phosphate buffered saline (PBS) with 0.1% of bovine serum albumin (BSA) and fixed by the slow addition of one volume of buffered saline containing 2% formaldehyde, with constant agitation for 45 minutes at 37 ºC. Fixed parasites were recovered and washed with PBS by centrifugation, adjusted and distributed to multiwell immunofluorescence slides, and then drained and stored for use at 4 ºC. IFA was performed with a 30 minute incubation in a humidified chamber with serial dilutions of serum (1/16, 1/32, 1/64, 1/128, 1/256, 1/512, 1/1024), visualized with fluorescein-conjugated anti-immunoglobulin IgG or IgM, and counterstained with Evans Blue. Positive and negative controls were included in all experiments, and the slides were observed by two trained observers. A reaction was considered positive when adequate membrane fluorescence was clearly seen, although polar or nuclear fluorescence were not considered. Before performing IFA assay, all examined sera were absorbed with rheumatoid factor reagent (*RF-absorbens,* Behring).

ELISA was performed overnight using polystyrene microwell plates coated with antigen (10 µg/mL) in carbonate buffer (0.1 M pH 9.0). Free sites were blocked with 1% non-fat dry milk in PBS containing 0.02% Tween-20 (PBST). All subsequent steps were performed with four intermediate washes with PBST. Appropriate dilutions of serum samples in PBST were then applied, and bound antibody was visualized following incubation with appropriate dilutions of peroxidase-conjugated anti-human IgG (Sigma). After a 30 minute incubation with 0.05M sodium citrate, pH 5.8, containing 0.4 mg/mL o-phenylenediamine (OPD), and 0.03% H_2O_2, the reaction was stopped with the addition of 4 N HCl, and 492 nm absorbance is detected in a microplate reader. Avidity assays were performed similarly, with the addition of a chaotrope washing step between the serum and conjugate incubations. Chaotrope solutions were carefully adjusted to pH 7.0 and contained 8 M Urea and 1 M Ammonium Thiocyanate. Avidity was expressed as percent of specific IgG that remained in the well after being washed with chaotrope compared to control wells that were maintained in PBST, subtracting the optical density of background conjugate wells. As several dilutions of sera were performed in each assay, only the dilution that gives an optical density without chaotrope between 0.5 - 1.5 were used for calculation. Avidity was arbitrarily defined as low when the percent of the remaining absorbance was equal to or lower than 50%, and considered to be high when the percent of the remained absorbance was higher than 70%.

RESULTS

Immunoblotting clearly showed that a specific protein of approximately 40 KDa in size was recognized by both rabbit hyper-immune anti-*T. gondii* antiserum and anti-*T. gondii* IgG human serum without any labeling with negative human sera, as shown in Figure 1. This data allowed us to use an ELISA for IgG using rROP2 antigen, which provided similar results to whole saline extract in the same test, as seen in Table 1. Using whole saline extract ELISA for IgG as the gold standard, the rROP2 assay was in good agreement, with 87% (95%

Confidence Interval or CI 60-98%) sensitivity and a efficient specificity of 88% (95% IC 82-93), with a high predictive positive value of 98% (95% CI 94-100%) despite a low negative predictive value of 43% (95% CI 26-62%).

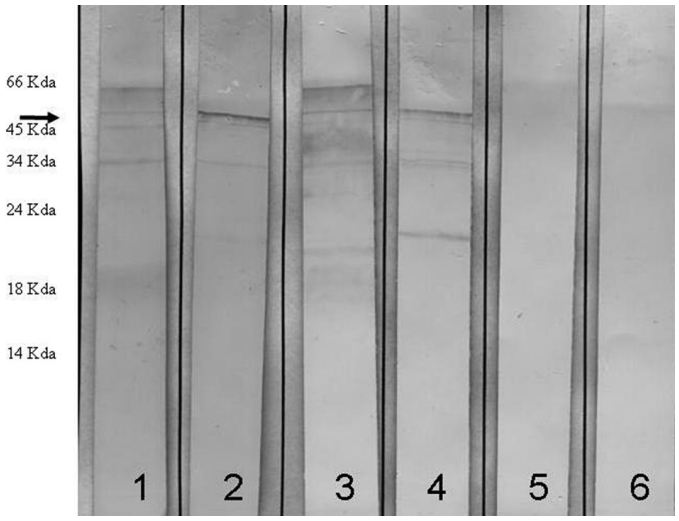

Fig. 1 - Immunochemical characterization of whole extract antigen and rROP2 antigen was revealed by SDS-PAGE with appropriate conjugates. Lanes 1, 3 & 5: Whole *T. gondii* extract. Lanes 2, 4 & 6: purified rROP2. Lanes 1 & 2: Antigen challenged with anti-*T. gondii* rabbit hyper immune serum. Lanes 3 & 4: Antigen challenged with ELISA-positive human sera. Lanes 5 & 6: Immunoblot using negative human sera. The arrow depicts rROP2 mobility.

Our sample was composed of sera from pregnant women who reported to the public health network of São Paulo and had a positive anti-*Toxoplasma* IgM test, which was performed during routine anti-natal serological screening. We retested the women upon admission to our study, as seen in Table 1. Clearly, there are many potential pitfalls in these screening tests, resulting in large groups of screening errors. The first group consisted of seronegative women, which presented IgG ELISA-negative results in serological retests at least two weeks after screening serology. This group represented 11.3% of the sample (18/160), having no contact with *Toxoplasma gondii*. The second group consisted of another type of false positive. These patients had past infections of chronic toxoplasmosis, a much more frequent condition, occurring in 66.9% (107/160) of the samples. After subtracting these two groups of false positives, only 21.9% (35/160) of the original sample were pregnant women recently infected with *Toxoplasma gondii*.

Quantitative distribution of IgG values in rROP2 ELISA can be seen in Figure 2. The distribution of IgG values against rROP2 was quite diverse in positive samples as compared to negative samples, although there were no differences regarding the presence of specific IgM in the sample. There is no significant relationship between quantitative data from whole extract ELISA as compared to rROP2 ELISA (Fig. 3), both in the chronically infected population (IgG+ IgM-) as well as in the recently infected population (IgG+ IgM+), suggesting that the amount of antibodies against rROP2 do not correlate with whole antigen antibodies.

We evaluated the avidity of IgG antibodies in these two populations using whole extract and rROP2 and two diverse chaotropes in typical

Table 1

Qualitative and quantitative comparison of ELISA assays for detection of specific IgG and its avidity using whole saline extract (WSE) or rROP2 antigen in IgM-screened samples from pregnant women reselected by confirmatory specific anti-IgM and IgG IFA assay. Avidity assays were performed only in positive samples over cut-off of 99% confidence intervals of confirmed negative samples

Chaotrope	Event	IgG and IgM negative	IgG negative and IgM positive	IgG and IgM positive	IgG positive and IgM negative
	Total N	14	4	35	107
	ELISA IgG WSE positive	0	0	34 97.1%	105 98.1%
Urea	WSE Low avidity <50%	NA	NA	2/34 5.9%	0/105 0%
	WSE mean avidity	NA	NA	83% ± 12	88% ± 12
Thiocyanate	WSE Low avidity <50%	NA	NA	3/34 8.8%	3/105 2.9%
	WSE mean avidity	NA	NA	68% ± 15	72% ± 16
	ELISA rROP2 IgG positive	3 21%	1 25%	34 97.1%	105 98.1%
Urea	rROP2 Low avidity <50%	0/3 0%	0/1 0%	1/34 2.9%	0/105 0%
	rROP2 mean avidity	75%	75%	90% ± 12	90% ± 15
Thiocyanate	rROP2 Low avidity <50%	0/3 0%	0/4 0%	8/34 23.6%	19/105 18.1%
	rROP2 mean avidity	79%	85%	61% ± 17	62% ± 18

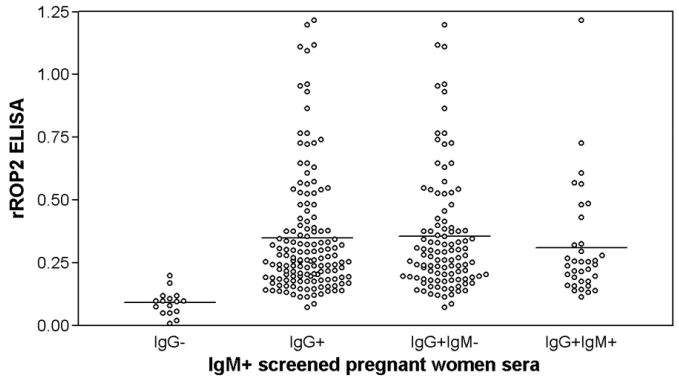

Fig. 2 - Distribution of ELISA using rROP2 antigen according to the *T. gondii* IgG and IgM IFA in at-risk pregnant women. Bars represent the mean value. Cutt-off values based on 99% confidence intervals of standard negative samples was 0.110.

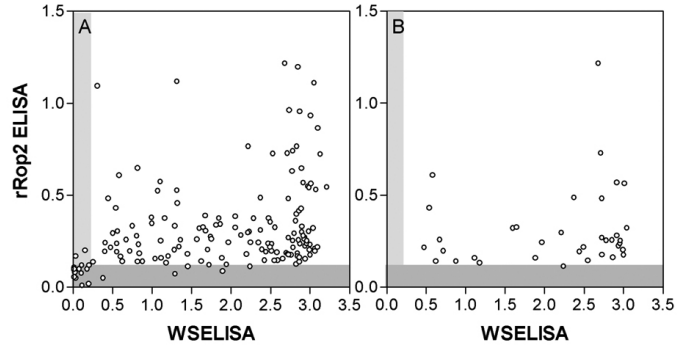

Fig. 3 - Relationship between rROP2 and whole extract quantitative ELISA for detecting anti-*T. gondii* IgG in at -risk pregnant women. A: IgG IFA-positive and B: IgG and IgM IFA-positive. Shaded areas represent negative cut off area of ELISA rROP2(0.110) and WSE(0.199).

elution ELISA avidity assays, as described in the Methods section. As seen in Figure 4 and Table 1, the mean values of IgG avidity were lower in the IgM+ population compared to the IgM- population for the same antigen and chaotrope. There are only a few samples with low avidity indexes, arbitrarily considered less than 50%, precluding frequency comparison between assays and chaotropes. In whole extract avidity, urea was a milder chaotrope, resulting in higher avidity indexes in both antigen preparations, more clearly seen in IgM+ samples. In rROP2 avidity, this chaotrope resulted in no clear definition of recently acquired infections. In both assays, the use of ammonium thiocyanate resulted in lower avidity indexes, most clearly seen in whole extract ELISA, although those indexes were also found in chronic IgM- samples. Whole extract avidity indexes presented no relationship with rROP2 avidity indexes, which only correlated with the same test using another chaotrope, as shown in Figure 5. In fact, the relationship between avidity indexes occurred only when the same antigen was analyzed with different chaotropes.

DISCUSSION

Our patient population was composed of pregnant women screened for IgM. Most mothers presented high avidity antibodies or false positive results, which has been observed in similar studies (SPALDING *et al.,* 2005). Following a positive test, these patients are usually grouped in the

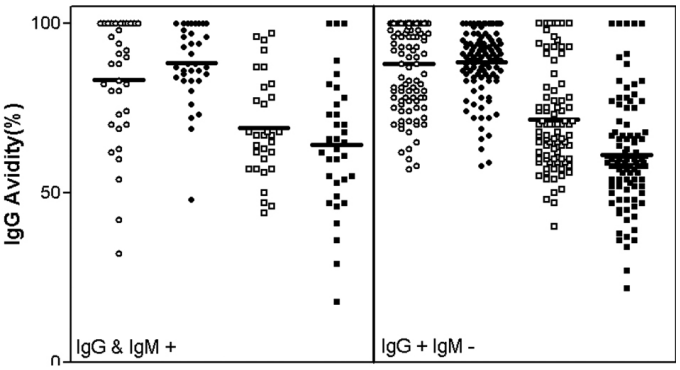

Fig. 4 - Avidity indexes in IgG-positive at-risk pregnant women selected groups using urea or thiocyanate as chaotrope and rROP2 or *T. gondii* whole extract ELISA. Open symbols represent whole extract ELISA; closed symbols represent rROP2 ELISA. Circles represent avidity indexes estimated using 8 M Urea as chaotrope. Squares represent avidity indexes estimated using 1 M sodium thiocyanate as chaotrope. Bars represent mean values.

Fetal infection Group in Brazil (GUIMARÃES *et al.,* 1993), resulting in expensive spiramycin treatment of pregnant women that are not at risk of congenital infection, as well as the psychological burden to the pregnant women following the suspicion of congenital infection. This fact can be attributed to the high proportion of the adult population already infected with *T. gondii* in our area, which results in more IgM false positives compared to countries with lower rates of toxoplasmosis infection, as several reports relate false positive tests to low cut-off values (SUZUKI *et al.,* 2001).

There was good agreement between whole extract serology and rROP2 ELISA in our sample of pregnant women screened for Tg IgM serology. There were very few negative samples, resulting in high accuracy as determined by the kappa test. The rROP2 antigen was of good quality as evaluated by immunoblot. rROP2 was further used as an antigen for the diagnosis of toxoplasmosis, although the results were disappointing (VAN GELDER *et al.,* 1993, MARTIN *et al.,* 1998, AUBERT *et al.,* 2000, NIGRO *et al.,* 2003, ALTCHEH *et al.,* 2006, PIETKIEWICZ *et al.,* 2007). Our data was similar to those reported by VAN GELDER *et al.* (1993) and those obtained with the same rROP2 source (MARTIN *et al,*. 1998, NIGRO *et al.,* 2003). It is important to note that the size of the sample used in this study (160 sera) allowed us to confirm with great confidence that the version of rROP2 used was sufficient to diagnose toxoplasmosis by IgG-ELISA, especially when there are several unselected commercial tests in the screening protocol. Our study avoids any selection related to the initial IgM test used, as the study population consisted of suspected pregnant women screened at a reference center under field conditions. Several other recombinant antigens give similar results, but they were not included in the commercial assays. As the results were similar but weaker than for whole extract ELISA, attempts have been made to introduce a mixture of several recombinant antigens or chimeric proteins composed of several epitopes of diverse *T. gondii* proteins (BUFFOLANO *et al.,* 2005; BEGHETTO *et al.,* 2006; PIETKIEWICZ *et al.,* 2007). However, all attempts have given similar results without improving upon the results of the whole extract ELISA. It is likely that the use of rROP2-ELISA could be considered on the basis of its simple and reliable development.

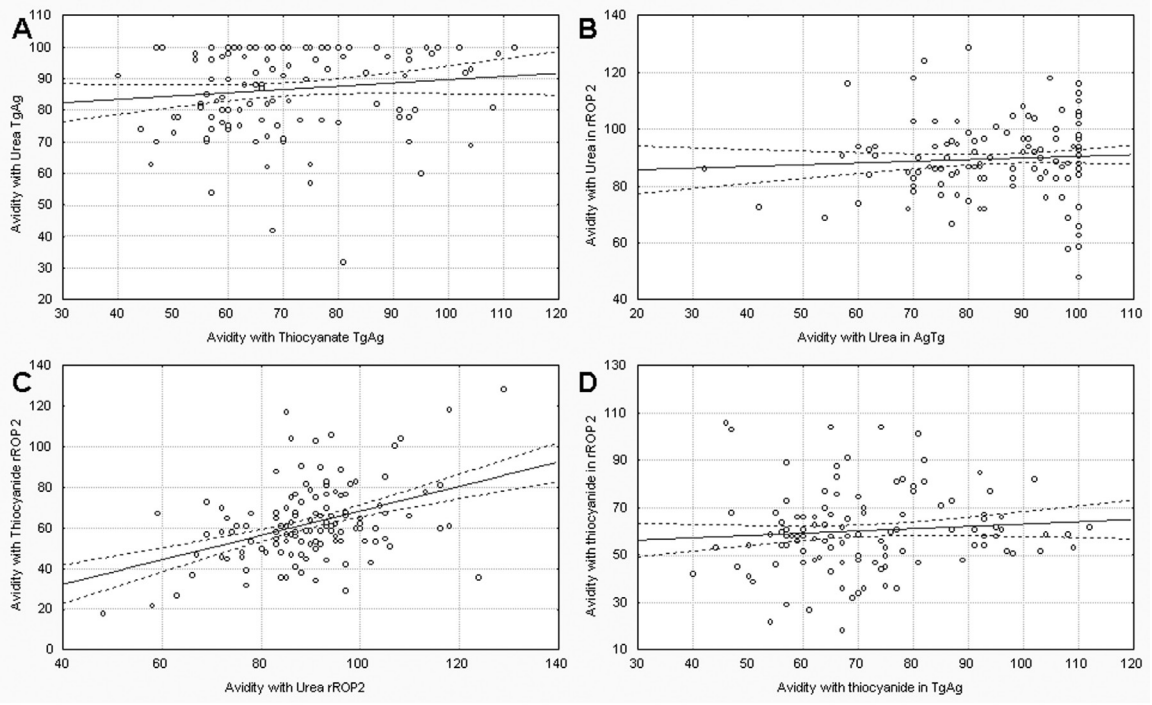

Fig. 5 - Comparison of values of avidity indexes obtained with rROP2 and TgAg using two different chaotropes, Urea or Ammonium Thiocyanate. A: Urea and SCN avidity in WSE assays. B: rROP2 and WSE avidity in urea assays. C: Urea and SCN avidity in rROP2 assays. D: rROP2 and WSE avidity in SCN assays.

Avidity data show much more complex results, as there is no relationship in quantitative analysis between assays with whole extracts or rROP2. The avidity determination was reliable for each isolated antigen regardless of the chaotrope agent, demonstrating that avidity determination is dependent on each antigen and its preparation for determination of recently acquired infections. This data could explain the conflicting results reported when comparing commercial tests available for avidity determination (BARBERI *et al.,* 2001). Avidity is the summation of the specific antibody affinities after an infection. Thus, it is dependent on the strain of the parasite and its virulence as well as on host factors. There are several strains of *T. gondii* with variable virulence and some antigenic differences, despite the fact that all are reactive to whole extract antigens (KONG *et al.,* 2003). These minor antigenic differences could affect ELISA with few epitopes, as observed in most isolated recombinant protein ELISA, which usually present less sensitivity and specificity than whole extract ELISA (BUFFOLANO *et al.,* 2005). Another aspect is the parasite load in human infection, which can be acquired by oocyst ingestion of only eight sporozoites, or by tissue cysts in raw meat, containing several cysts with thousands of bradyzoites, as reported in experimental models comparing these two ways of infection and reporting that cyst induced infection was more acute and severe than oocyst induced infection (DUBEY, 2006). Thus, the immune response could be established more effectively in oocyst infection, controlling the disease before extensive infection and demanding less antibody production and selection. This is the main effect in avidity evolution, as the selection of high avidity B cell clones is dependent on antigen prevalence and the numbers of clones initially produced (TARLINGTON, 2008). Conflicting reports on avidity evolution in human toxoplasmosis has been attributed both to individual variations and to therapy (LEFEVRE-PETTAZZONI *et al.,* 2006).

Diagnosis of recent *T. gondii* infection in pregnant women remains a difficult task despite scientific efforts to solve this problem. Screening tests continue to be based on IgM detection, although our scientific improvement on this detection method has only resulted in more false positive results and unnecessary treatments and psychological burden or, worse, unnecessary pregnancy termination in pregnant women. All efforts should be made to standardize antigen preparation in commercial tests using purified tachyzoites of standard strains, such as the RH strain, with an international consensus on assay procedures, especially for avidity assays.

The use of ELISA for specific anti-rROP2 IgG would be an alternative serological test for the diagnosis of toxoplasmosis during pregnancy, although our data do not support its use in avidity assays for recent infection detection.

RESUMO

Sorologia utilizando o antígeno recombinante ROP2 no diagnóstico da toxoplasmose em mulheres grávidas

A toxoplasmose causa grave doença fetal durante a infecção aguda da gestante, assim demanda diagnóstico precoce para tratamento efetivo e preservação fetal. Os testes fetais são de baixa eficiência e o diagnóstico é baseado na sorologia para IgM materna, que tem fraca capacidade de triagem devido a sensibilidade crescente dos testes, sendo uma alternativa os testes de avidez de IgG. Apresentamos aqui um ELISA e ensaio de avidez de IgG usando antígeno recombinante de *T. gondii*, rROP2, em amostras de 162 gestantes triadas para um grande hospital público por uma sorologia positiva para IgM. O ELISA IgG e o ensaio de avidez de

IgG foram comparados usando o antígeno rROP2 ou extrato total de *T. gondii*. O ELISA usando rROP2 mostrou excelente concordância com os ensaios usando extrato de *T. gondii*, mas nos ensaios de avidez usando rROP2, os valores encontrados não se relacionaram com os encontrados na avidez com extrato de *T. gondii*, independente do tipo de caotrópico utilizado. Estes dados mostram que a maturação da avidez é especifica para a resposta imune ou a prevalência de um antígeno durante a infecção. Os ELISA para IgG anti rROP2 podem ser teste sorológico alternativo para o diagnóstico da toxoplasmose durante a gravidez, embora nossos dados não apóiem seu uso em ensaios de avidez de IgG.

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