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Efficient production of the *Toxoplasma gondii* GRA6, p35 and SAG2 recombinant antigens and their applications in the serodiagnosis of toxoplasmosis

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Abstract

The description of very efficient system for production and purification of *Toxoplasma gondii* recombinant antigens, GRA6, p35 and SAG2 is given in this study. The usefulness of these antigens for diagnostic of human infections was tested in an ELISA using 99 sera obtained during routine diagnostics. The sera for testing were selected from either acute or chronically infected patients. Both r-GRA6 and r-p35 antigens detected antibodies more frequently ($p<0.01$) from acute (93.9 and 87.9%) rather than chronic (60.6 and 53.0%) infections. The r-SAG2 gave a similar sensitivity in both groups of patients (93.9 and 95.5%).

Key words

ELISA, fusion protein, serological detection, *Toxoplasma gondii* recombinant antigens, GRA6, p35, SAG2

Introduction

The enzyme-linked immunosorbent assay (ELISA), based on native antigen(s) obtained from tachyzoites, is, at present, most commonly used as a commercial kit for diagnosis of toxoplasmosis.

However, it is well known that the whole tachyzoite native antigen test is difficult to standarize and often produces false positive reactions (Hassl *et al*. 1991). Additional limitations are based on the well known fact that the stimulation of antibody production is often not limited to the tachyzoites alone. Moreover, the immune response may vary significantly depending on the nature and structure of the antigen as well as the ability of an individual human being not only to recognize the antigen but also to respond.

It is logical then, that careful and extensive selection of antigens should be done before a commercial test is recommended for routine diagnostics. Our laboratory (Hiszczyńska-Sawicka *et al*. 2003, Pietkiewicz *et al*. 2004) as well as others (Martin *et al*. 1998, Jacobs *et al*. 1999, Aubert *et al*. 2000, Lecordier *et al*. 2000, Li *et al*. 2000, Huang *et al*. 2002) have produced a number of recombinant antigens, with the object of finding the combination of antigens for ELISA that would detect all serologically positive individuals as well as differentiate between acute and chronic infections. This last information is vitally important for pregnant women as the foetus can become infected.

Although, at present, the costs for testing in developed countries are not prohibitively high, however, the undeveloped world would certainly benefit from having accesses to cheaper tests. The costs for the development of a kit will depend, significantly, on the efficient production of recombinant antigens. We (Hiszczyńska-Sawicka *et al*. 2003) have developed a highly efficient system for the production and purification of SAG1, GRA1 and GRA7.

It is well known that an efficient expression system for some proteins may also not be an efficient system for other proteins. In this paper we tested our system with new recombinant antigens such as GRA6, p35, SAG2 and also assessed their diagnostic value.

Materials and methods

Construction of the expression plasmids

The *E. coli* TOP10F' strain (Invitrogen, Carlsbad, CA) was used for the preparation of plasmids and for cloning, and *E. coli* Rosetta (DE3)pLysS (Novagen, UK) was used for the expression of the recombinant antigens. The pUET1 (Dąbrowski *et al*. 1999) plasmid was used for the construction of

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the expression system (Hiszczyńska-Sawicka *et al*. 2003). *E. coli* cells containing the plasmids were cultured aerobically at 37°C in LB medium supplemented with 12.5 μg/ml tetracycline or 100 μg/ml ampicillin for the TOP10F' strain, and with 34 μg/ml chloramphenicol and 100 μg/ml ampicillin for the Rosetta (DE3)pLysS strain. Restriction enzymes were purchased from NewEngland BioLabs. The reagents for PCR were obtained from DNA-Gdańsk II s.c. (Poland). Ni^{2+}-IDA-Sepharose was obtained from Novagen. IPTG, and agarose, and all reagents for the protein purification were purchased from Sigma.

pUETD GRA6

The DNA sequence of the gene encoding a GRA6 antigen from *T. gondii* was obtained from the GenBank database (Accession number L33814). A total tachyzoite DNA from RH *T. gondii* strain was used as the template for amplification of the GRA6 sequence using a standard PCR amplification protocol with Delta2 DNA polymerase [hyperstable Pwo DNA polymerase (DNA-Gdańsk II s.c, Poland)] and primers: 5'-CGC GGA TCC CAT GGG TGT ACT CGT CAA TTC G-3' (forward), 5'-CGC GAA TTC CAT AAT CAA ACA CAT TCA CAC GTT CC-3' (reverse). The primers contained the BamHI and EcoRI sequences (underlined) to facilitate cloning. The PCR product corresponding to nucleotides from 187 to 691 of GRA6 was digested with both BamHI and EcoRI and inserted into the BglII and EcoRI sites of the pUET1 vector. The recombinant plasmid was digested with EcoRI and the cohesive ends were filled by Delta 2 DNA polymerase and religated to introduce, in frame, the DNA sequence encoding for a His-tag domain at the C-terminal of the fusion protein. The resulting plasmid pUETD GRA6 containing a truncated sequence of GRA6 (from 30 to 231 amino acid residues) was embedded, in frame, between the His-tag domains.

pUETD SAG2

The DNA sequence of the gene encoding for the SAG2 (p22) antigen from *T. gondii* was obtained from the GenBank database (Accession number M33572). Total tachyzoite DNA was used as the template for amplification of the SAG2 sequence using a standard PCR amplification protocol and primers: 5'-TCC ACC GGT ACC ACG CCA GCG CCC ATT GAG TG-3' (forward), 5'-AGA ACC GAA TTC GTG AGA GAC ACA GGG TCA AAC CCA G-3' (reverse). Primers were designed to contain the KpnI and EcoRI sequences (underlined), respectively, to facilitate cloning. The PCR product corresponding to nucleotides from 90 to 510 was digested with both KpnI and EcoRI and inserted into the KpnI and EcoRI sites of a pUET1 vector. The resulting plasmid, pUETD SAG2, containing the truncated sequence of SAG2 (from 30 to 170 amino acid residues) was embedded, in frame, between the His-tag domains.

pUETD p35

The DNA sequence of the gene encoding for the p35 antigen from *T. gondii* was obtained from the GenBank database (Accession number AF310261). Total tachyzoite DNA was used as the template for amplifying the p35 sequence using a standard PCR amplification protocol and primers: 5'-AGA AGA TCT CGG TCC TTT GAG TTA TCA TCC-3' (forward), 5'-TAT GAA TTC GCA GCT GTC GTG GTT GTC GTA AC-3' (reverse). Primers were designed to contain the BglII and EcoRI sequences (underlined), respectively. The PCR product corresponding to nucleotides from 74 to 435 was digested with both BglII and EcoRI and inserted into the BglII and EcoRI sites of a pUET1 vector. The resulting plasmid, pUETD p35, containing the truncated sequence of p35 (from 26 to 170 amino acid residues) was embedded, in frame, between the His-tag domains.

The nucleotide sequence of the inserts was verified by sequencing using the dideoxy termination method.

Production and purification of His-tag recombinant proteins

The *E. coli* strain Rosetta (DE3)pLysS, transformed with pUETD GRA6, pUETD SAG2, pUETD p35 or nonrecombinant pUET1 plasmid, was grown in LB media supplemented with 50 μg/ml of ampicillin and 34 μg/ml chloramphenicol at 30 or 37°C overnight. Next, 1000 ml of LB medium, supplemented with the same antibiotics, was inoculated with 40 ml of the overnight culture. The cultures were grown with vigorous shaking at 30°C (pUETD GRA6, pUETD p35) or 37°C (pUETD SAG2) to an optical density at 600 nm of 0.4. Protein production was then induced with isopropyl-b-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM, and the cells were incubated with vigorous shaking for an additional 3 h (pUETD SAG2) or 8 h (pUETD GRA6, pUETD p35). The cells were harvested by centrifugation at 14000 × *g* and the pellets were resuspended in 20 ml of buffer A1 (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100) for the r-SAG2 and r-p35 proteins purification or in A2 (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 5 mM imidazole, 0.1% Triton X-100, 5 M urea) for the r-GRA6 protein purification. The cells were disrupted by sonication and then the insoluble debris was removed by centrifugation. The clarified supernatant (about 20 ml) was applied directly onto a Ni^{2+}-IDA-Sepharose column (20 ml of bed volume, Novagen) preequilibrated with four volumes of buffer A1 for r-SAG2 and r-p35 and A2 for r-GRA6. The column was sequentially washed four times with buffers A1 and A2 containing 50 mM imidazole and then the His-tagged proteins were eluted with buffer E (20 mM Tris-HCl pH 7.9, 500 mM NaCl, 500 mM imidazole). The recombinant proteins were analysed by SDS-PAGE and Western blotting. The eluted fractions were dialysed against a phosphate-buffered saline (PBS) buffer (1% w/v NaCl, 0.075% w/v HCl, 0.14% Na_2HPO_4 and 0.0125% w/v KH_2PO_4).

Gel electrophoresis and Western blotting

The recombinant antigens (r-GRA6, r-SAG2 and r-p35) were identified by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. To estimate the amount of indi-

vidual proteins the Coomassie brilliant blue stained polyacrylamide gels were photographed. The photographs were scanned and analysed using the BioDoc 1.0 program (Biometra). BSA solution (1 µg/µl) was applied as the reference.

Western blotting was performed as described earlier (Hiszczyńska-Sawicka *et al*. 2003).

Serological examinations

The GRA6, p35 and SAG2 *T. gondii* recombinant antigens were evaluated in immunoglobulin G (IgG) ELISAs. Each antigen was used at a concentration of 10 µg/ml. An in-house IgG ELISA was carried out according to the protocol described previously (Hiszczyńska-Sawicka *et al*. 2003, Pietkiewicz *et al*. 2004). Each serum was examined twice. Results were determined for each serum by calculating the mean value of the optical density (OD) reading for duplicate wells. A positive result was estimated as any value higher than the average OD reading plus three standard deviations (cut-off) and was obtained with nine sera from the negative control serum group. The cut-off values were as follows: 0.412 for r-SAG2, 1.115 for r-GRA6, 0.739 for r-p35 and 1.095 for the native antigen. An ELISA performed with the control *E. coli* Rosetta (DE3)pLysS pUET1 antigen, produced, purified and diluted in the same way as the recombinant antigens, showed an OD consistently below 0.32 (data not shown), which demonstrates that possible contaminations of the recombinant antigens with *E. coli* antigens do not influence the rec-ELISA results. The results of the IgG rec-ELISA were compared with those obtained from the same group of sera examined by commercial VIDAS Toxo-IgG avidity and Toxo-ISAGA plus IgM/IgA tests (all from bioMérieux, Marcy l'Etoile, France) performed according to the manufacturer's instructions.

For each examination, three sample pooled sera: high positive (400 IU IgG), low positive (12.5 IU) and negative (< 6 IU) were used. The inter-assay reproducibility is shown in Table II.

Sera

Serum samples were obtained during routine diagnostic procedures. Sera were from pregnant women, adults and children. Some of the sera were from persons with adenopathy or from seroconversion. All sera from *T. gondii* infected persons were positive using the standard IFA technique with different titres (IU), generally above 200 IU, with low or high IgG-avidity and were also positive or negative in the ISAGA IgM/IgA tests. These sera were reexamined with an in-house ELISA performed with the native *T. gondii* antigen, and only sera positive in this test were used for further examination with recombinant antigens. Written informed consent was obtained from each study participant.

In these examinations, positive sera, selected according to the immunologic profile, were used. The sera were divided

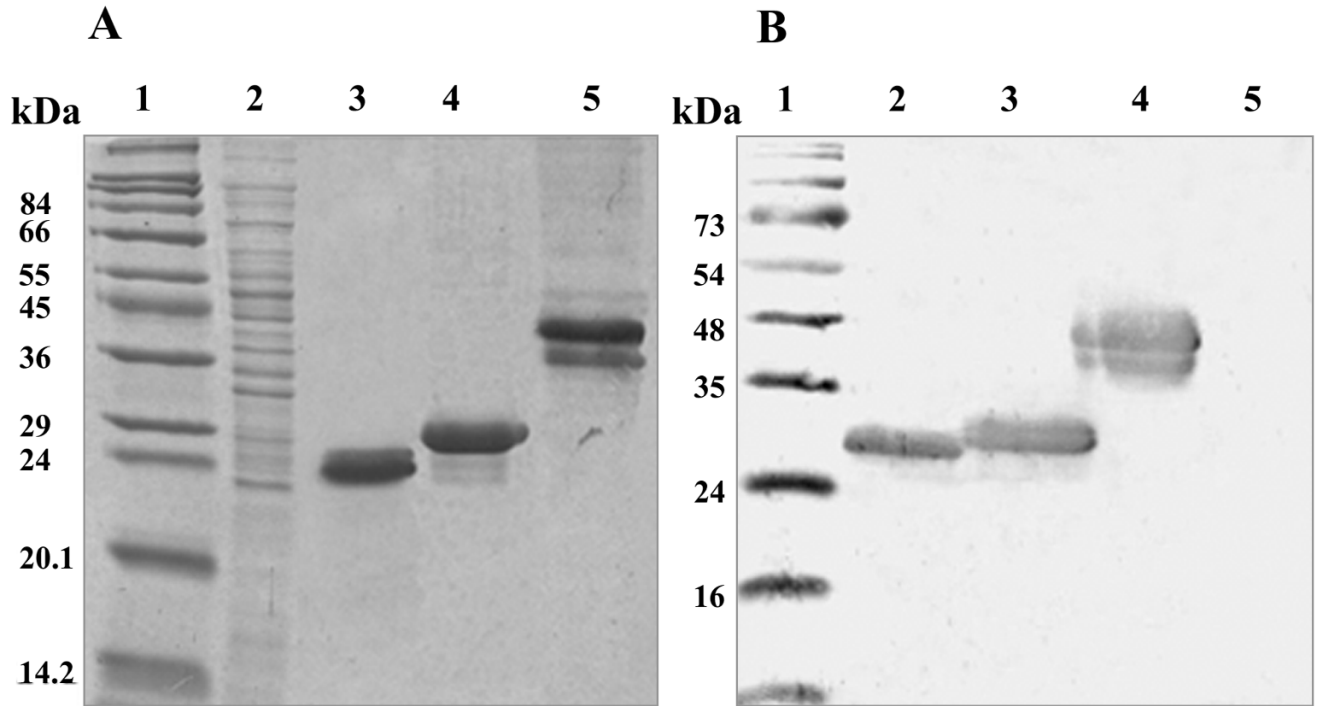

Fig. 1. A – Coomassie blue staining of recombinant fusion proteins after affinity chromatography: lane 1 – molecular mass marker (SIGMA), 2 – soluble protein extracts from *E. coli* strain Rosetta pLysS + pUET1, 3–5 – purified r-SAG2, r-p35 and r-GRA6; **B** – Western blotting of r-GRA6, r-SAG2 and r-p35 recombinant proteins: lane 1 – molecular mass marker (Fermentas); lanes 2–4, purified r-SAG2, r-p35 and r-GRA6, 5 – soluble protein extracts from *E. coli* strain Rosetta pLysS + pUET1, immunoblotted using highly positive (800 IU) anti-*T. gondii* human serum

Table I. The results of the IgG ELISAs obtained with *T. gondii* recombinant antigens with sera at low and borderline or high specific IgG avidity

			r-GRA6			r-SAG2 (p22)			r-p35		
		N	A	B	C	A	B	C	A	B	C
I	low and borderline avidity	33	31[1] (93.9)	1.96	0.813–2.372	31 (93.9)	1.29	0.411–2.258	29[4] (87.9)	1.43	0.358–2.223
IIa	high avidity	57	36[2] (63.1)	1.24	0.373–2.245	55 (96.5)	1.15	0.387–2.213	31[5] (54.5)	0.821	0.261–1.784
IIb	low and borderline avidity, but sera were collected more than 4 months after the acquisition of the first serum sample	9	4	1.16	0.489–1.918	8	1.07	0.229–2.083	4	0.480	0.455–1.778
II	together IIa + IIb (chronic toxoplasmosis)	66	40[3] (60.6)	1.23	0.373–2.245	63 (95.5)	1.14	0.229–2.213	35[6] (53.0)	0.840	0.262–1.778
I and II	total	99	71 (70.6)			94 (95.6)			64 (65.2)		
III	control sera – IgG negative	9	0	0.82	0.534–1.09	0	0.25	0.185–0.355	0	0.28	0.14–0.652

N – no. of sera examined; A – no. (%) of positive sera; B – mean absorbance value; C – range absorbance values; [1,2]between group I and IIa, for r-GRA6 statistically significant, $c^2 = 10.408$, $p<0.01$; [1,3]between group I and II, for r-GRA6 statistically significant, $c^2 = 12.052$, $p<0.001$; [4,5]between group I and IIa, for r-p35 statistically significant, $c^2 = 10.550$, $p<0.01$; [4,6]between group I and II, for r-p35 statistically significant, $c^2 = 11.689$, $p<0.001$.

into groups as follows: (1) group I – 33 sera from patients suspected of having recently acquired (acute) toxoplasmosis [30 at low and 3 at borderline avidity, with all but one being positive in ISAGA-IgM (mean index 10.21 ± 3.51) and/or ISAGA-IgA tests (9.48 ± 3.86)]; (2) group IIa – 57 sera from patients with indicative infections acquired in the distant past (chronic toxoplasmosis) [high avidity, ISAGA IgM and/or IgA positive (n = 42) or negative (7.98 ± 4.11 for IgM and 7.90 ± 4.71 for IgA)]; (3) group IIb – 9 sera at low (n = 7) or borderline (n = 2) avidity [all but two being positive in ISAGA-IgM (8.22 ± 4.63) and/or ISAGA-IgA (4.56 ± 4.42)], collected more than four months after the acquisition of the first serum sample; these sera also represented chronic toxoplasmosis; and (4) group III – 9 sera were the control group, and were negative for anti-*T. gondii* antibodies.

Results

Expression of recombinant GRA6, p35 and SAG2 in E. coli

We cloned and expressed three *T. gondii* recombinant proteins (r-GRA6, r-p35 and r-SAG2) as fusion proteins containing six histidyl residues at the N- and C-terminal end.

The previous studies showed that the *gra6* gene encodes a 230-amino-acid-polipeptide containing two hydrophobic regions within membrane domains. The first hydrophobic region is N-terminally located (signal sequence) and the second domain is in the centre of the protein (Lecordier *et al*. 2000). In this study, PCR was used to amplify a 623 bp fragment that encodes amino acids from 30 to the end of the protein (protein without a potential signal sequence). The r-GRA6 fusion protein contains 258 amino acid residues. This protein was expressed in two forms, which was probably a result of post-translational modification. The calculated molecular mass for r-GRA6 with the two His-tagged domains (main band in Fig. 1A) was, approximately, 27.2 kDa.

The p22 *T. gondii* surface protein contains an N-terminal signal sequence and a C-terminal hydrophobic region characteristic of proteins attached to the membrane by a glycophospholipid anchor. In this study PCR was used to produce a 443 bp fragment of the *sag2* gene (without the signal sequence – from 1 to 70 bp and the membrane domain sequence – from 521 to 561 bp), which encodes amino acids from 30 to 170 of the SAG2 protein. The r-SAG2 protein was produced as a soluble His-tagged fusion protein of, approximately, 20.5 kDa (contained 197 amino acid residues).

A 453 bp fragment of the *p35* gene was achieved by PCR. This DNA fragment encodes amino acids from 26 to 170 of the p35 protein (without the predicted signal sequence – from 1 to 65 bp or the membrane domain sequence at the C-end of the protein). The r-p35 was expressed as a soluble protein with a calculated molecular mass of, approximately, 21.3 kDa (200 amino acid residues).

The highest expression level was obtained 3 h (for r-

SAG2) and 8 h (for r-GRA6 and r-p35) after IPTG induction.

Purification of recombinant proteins

Recombinant GRA6, p35 and SAG2 proteins were purified by a one-step chromatography procedure, using metal affinity chromatography, with Ni^{2+} bound to iminodiacetic acid-agarose (Novagen) and their purity was verified by SDS-PAGE (Fig. 1A). The amount of purified r-GRA6, r-p35 and r-SAG2 from a one litre induced culture was estimated as 60, 80 and 80 mg, respectively. The purified proteins contained some *E. coli* protein contaminants (about 95% purity) (see Fig. 1A), however, this purity is sufficient for serologic tests.

Reactivity of human sera with recombinant GRA6, SAG2 and p35 fusion proteins (*Western blot analysis*)

The immunoreactivity of the purified antigens was confirmed by Western blot analysis with a human serum sample from an individual infected with *T. gondii* (800 IU in IgG-IFA). Immunoblot analysis showed that all recombinant proteins are recognised by this serum (Fig. 1B, lane 2–4). The same human serum did not react with the *E. coli* control antigens (Fig.1B, lane 5).

Reactivity of human sera with recombinant GRA6, SAG2 and p35 fusion proteins (*IgG ELISA*)

Three separate IgG ELISAs were carried out using recombinant GRA6, p35 and SAG2 fusion proteins as coating antigens to evaluate the potential of each individual recombinant antigen for the serodiagnosis of toxoplasmosis.

Table II. Reproducibility of the r-GRA6, r-p35 and r-SAG2 ELISAs (day-to-day test)

	r-GRA6	r-SAG2	r-p35
	mean absorbance value		
Highly positive IgG 400 IU	1.28 ± 0.16	1.85 ± 0.27	1.32 ± 0.26
Low positive IgG 12.5 IU	0.45 ± 0.09	1.04 ± 0.12	0.70 ± 0.22
Negative IgG <6 IU	0.38 ± 0.19	0.51 ± 0.12	0.60 ± 0.13
Diluting buffer	0.16 ± 0.09	0.19 ± 0.03	0.17 ± 0.00

On examination of the positive sera (groups I and II), the sensitivity of the IgG rec-ELISA was high at 95.6% for the r-SAG2 (p22) antigen, and lower for the r-GRA6 and r-p35 antigens, reaching 70.6 and 65.2%, respectively. The results were different depending on the group of sera used. In group I sera (from persons suspected or having acute toxoplasmosis – low or borderline avidity), the sensitivity was high for the r-SAG2 and r-GRA6 antigens at 93.9% and a little lower for the r-p35 antigen, at 86.7%. For group II sera (high avidity – chronic toxoplasmosis), the results for the r-SAG2 antigen were similar to those obtained with group I, reaching 96.5%. In contrast, when r-GRA6 and r-p35 antigens were used, the sensitivities of the IgG rec-ELISAs were significantly lower than those obtained with group I, reaching only 63.1% ($c2$ = 10.408, $p<0.01$) and 54.5% ($c2$ = 10.550, $p<0.01$), respectively (Table I). For group IIb (low or borderline avidity, but with chronic toxoplasmosis), four sera were positive for the r-GRA6 and r-p35 antigens and eight for the r-SAG2 antigen. All the sera from the control group (IgG negative) were also negative with rec-ELISAs (Table I).

Discussion

In this paper we confirm that our (Hiszczyńska-Sawicka *et al*. 2003) expression system and purification method previously developed for SAG1, GRA1 and GRA7 is also very efficient for a new group of *Toxoplasma* recombinant proteins, GRA6, p35 and SAG2 (p22).

The high yields obtained are due to the controlled expression of the cloned genes resulting in a greater fraction of the protein being present in a soluble state in the cytosol. One of the difficulties in using recombinant antigens expressed in *E. coli* for serodiagnosis in humans is the existence of antibodies against the fusion partners or proteins of *E. coli* (Tenter and Johnson 1991, Parmley *et al*. 1992, Redlich and Müller 1998). However, using the His-tag domain avoids this problem. In addition, recombinant antigens could be easily purified using a one-step chromatography procedure.

In this study we also evaluated the reactivity of IgG antibodies with our recombinant antigens in sera from patients with acute and chronic toxoplasmosis. The sensitivity of the IgG rec-ELISA with all toxoplasmosis sera for r-SAG2 (p22) was high (95.6%) and was only a little lower in sensitivity for the r-SAG1 (99.1%) achieved in our previous study (Pietkiewicz *et al*. 2004). The sensitivities of the IgG rec-ELISA for r-GRA6 (70.6%) and r-p35 (65.2%) were similar to those obtained earlier for r-GRA7 (78%). The results of serological tests also indicate that GRA7 (Pietkiewicz *et al*. 2004), GRA6 and p35 recombinant antigens more frequently detected antibodies in a group of sera from patients suspected or having acute toxoplasmosis (according to the immunologic profile), and these results were statistically significant ($p<0.01$). The conclusion reached, therefore, is consistent with results previously published where similar sensitivity was obtained with these antigens in IgG rec-ELISA using sera from acute and chronic patients with toxoplasmosis.

The r-GRA6 protein was used in an ELISA test and it was found to be highly reactive with specific IgG-positive human sera, leading to the development of an IgG ELISA test that reached a 96% sensitivity (Lecordier *et al*. 2000). Using the GRA6 with a GST domain recombinant antigen, Redlich and Müller (1998) obtained positive results with all sera from acute (IgM positive, adenopathy, fever) infections, and about 83% with sera from chronic infections (IgM negative). However, considering various absorbance values, they were able to discriminate between acute and chronic toxoplasmosis, reaching 89% sensitivity and 99.6% specificity.

The r-SAG2 (p22) was also shown to be very sensitive in its ability to detect IgG in sera from both acute and chronic toxoplasmosis – Parmley *et al*. (1992) and present results. Li *et al*. (2000a) examined only small number (10) sera from acute and chronic toxoplasmosis cases with SAG2 recombinant antigen, and obtained eight and no positive results, respectively. However, Aubert *et al*. (2000) did not obtain satisfactory results with this recombinant antigen.

The r-p35 protein has been evaluated in an IgG ELISA test by Aubert *et al*. (2000) and the reactivity reached 98% for acute infections and 37% for chronic infections. These and present results (87.9 and 53%, respectively) are in contrast to the results of Li *et al*. (2000b) who obtained very low sensitivity (8%) with sera from chronic toxoplasmosis. In Li *et al*. (2000a) study, among 10 another sera examined, there were only six which gave positive results from acute infection and one positive result from a chronic infection. However, the reactivity of their r-p35 antigen was very low (OD below 0.14).

The differences between results in different laboratories can depend on many factors, among them the criteria used for discriminating between acute and chronic toxoplasmosis are most important. The immunologic profile is the criterion used for most of these studies.

The final usefulness of *T. gondii* recombinant antigens in diagnosis of chronic or acute toxoplasmosis should be further investigated by combining different recombinant antigens to increase the sensitivity and specificity of ELISA tests.

This study also confirms a significant improvement from the existing methods for obtaining *T. gondii* antigens in large quantities. In addition, our simplified extraction and purification protocols, with a minimum of processing steps, resulted in reduced losses and increased yields of recombinant antigen. The availability of large quantities of *T. gondii* recombinant antigens will facilitate their use in diagnostics.

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