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Characterization of Soluble and Membrane-Bound Proteins of *Toxoplasma gondii* as Diagnostic Markers of Infection

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Abstract

In the present study, we applied the combination of one-dimensional gel electrophoresis, immunoblot and nano-liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) to identify potential immunogenic proteins of *Toxoplasma gondii* tachyzoites that can be used for the development of reliable assays in the serodiagnosis of acquired toxoplasmosis in immunocompetent subjects. For this purpose, we developed an immunoblot using soluble and membrane extracts of GT1 *Toxoplasma gondii* tachyzoites and tested 194 positive and 100 negative sera obtained from pregnant women.

Five bands of soluble antigens (98 kDa, 36 kDa, 33 kDa, 32 kDa and 21 kDa) and 4 bands of membrane antigens (41 kDa, 35 kDa, 32 kDa and 30 kDa) were selected as the most valuable in terms of sensitivity and specificity. Among these bands, only 2 bands of soluble antigen (33 kDa and 32 kDa) and 2 bands of membrane antigen (32 kDa and 30 kDa) showed a specificity ≥ 90%.

After mass spectrometry and bioinformatics analysis, 7 proteins were identified as potential markers for serodiagnosis of toxoplasmosis. These proteins are: SRS34A, GRA7, GRA1, DG32, MIC5, ROP5 and Toxofilin. These proteins showed a 86% to 100% homology with proteins of both VEG and ME49 strains of *T. gondii* and a 58% to 87% homology with *Hammondia hammondi*; and can be considered as attractive candidates for the development of an immunochromatography test that can be used for the rapid diagnosis of toxoplasmosis and as a confirmatory test when routine techniques give equivocal results.

Keywords: Toxoplasmosis; Serodiagnosis; Tachyzoites; Immunoblot; NanoLC-MS/MS

Introduction

Toxoplasmosis is a parasitic disease caused by an obligate *Apicomplexa* intracellular protozoan parasite, *Toxoplasma gondii* that is widely distributed and can infect humans and warm blooded animals [1-3]. In immunocompetent individuals, toxoplasmosis is usually asymptomatic, whereas in immunocompromised patients (human immunodeficiency virus infected patients, organ and bone marrow transplanted patients), it can cause life-threatening infections. Moreover, in non-immune (without any anti-*Toxoplasma* antibodies) pregnant women, it may cause severe lesions in the fetus, because of the risk of transplacental transmission [4-6]. For this reason, the determination and the correct interpretation of the immune status of the mother towards toxoplasmosis are crucial for appropriate follow-up, prophylactic measures and treatment [5,7].

The diagnosis of toxoplasmosis in immunocompetent individuals is based on serology by the search of IgG and IgM anti-*Toxoplasma* antibodies. For this purpose, a variety of commercial serological tests has been developed; the most widely used being the automated enzyme immunoassays [5,6,8]. Even though most of these assays are overall satisfactory with respect to sensitivity and specificity, equivocal results and discordances between kits are not uncommon especially when the titer of specific IgG is low or close to the cut-off value of the test, despite many efforts for international standardization [9,10].

Tachyzoites of *T. gondii* are responsible of the acute phase of the infection, which they activate a potent host immune response that eliminates most of the parasites [2,11]. The identification and characterization of immunogenic proteins, whether from the soluble tachyzoite antigens ([ST-Ag], cytoplasmic and secreted/excreted antigens) or the membrane tachyzoite antigens ([MT-Ag], constituents of the cytoplasmic membrane), have been the focus of an extensive research. Both antigenic fractions may contain proteins of relevant antigenicity that needs to be identified.

Immunoproteomics combining one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE) followed by immunoblot and nano-liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) analysis is a powerful tool for the identification of proteins with diagnostic value [6,12-14].

In the present study, we applied this approach to identify potential immunogenic proteins of *T. gondii* tachyzoites that can be used for the

development of reliable assays in the serodiagnosis of acquired toxoplasmosis in immunocompetent subjects.

Materials and Methods

Parasites

Seventy female Swiss albino mice weighing 30-35 g were used for the preparation of antigens. Mice were kept at 22-24°C, under humidity level of 45-75% and provided with food and water ad libitum in animal facilities. Then, they were intraperitoneally injected with 50 µL of a suspension of the virulent GT1 *T. gondii* strain tachyzoites at 10^3-10^4 tachyzoites/ml. Three days after infection, mice were euthanized by carbon dioxide inhalation and the peritoneal exudates were collected and pooled. The monitoring of mice was performed according to the criteria set by Montgomery [15]. All experimentations were performed according to the directive 2010/63/UE of the European Parliament and the Council.

Preparation of *T. gondii* tachyzoites antigens

Preparation of ST-Ag: Preparation of ST-Ag was carried out according to Ma et al. with minor modifications [2] (Annex 1).

Preparation of MT-Ag: Preparation of MT-Ag was carried out according to Robinson et al. [16] (Annex 2).

One dimensional SDS-PAGE

1D-SDS-PAGE was performed under denaturing and reducing conditions with a 12% acrylamide-5% bisacrylamide gel as described by Laemmli [17]. The Mini-Protean Tetra Cel (Bio-Rad, Marnes-La-Coquette, France) was used as a vertical electrophoresis system. The molecular weight marker See Blue® Pre-Stained Standard (Invitrogen) was used.

After migration, the gel was stained with Coomassie blue R-250. The images of the gel were obtained by the Chemi Doc TM^{MP} imaging system and then analysed using the Quantity One® Software package (Bio-Rad).

Immunoblot

Once the 1D-SDS-PAGE was finalized, proteins were transferred from the gel to a nitrocellulose membrane for 90 min at 50 V and +4°C. The membrane was cut into strips and blocked in 5% dried skimmed milk in PBS-T (PBS 10 mM containing 0.3% Tween 20) for 2 h at room temperature, then incubated overnight at 4°C with the diluted patient's serum (1/100 in PBS-T) under agitation. After five washes for 5 min in PBS-T and incubation with alkaline phosphatase-goat anti-human IgG antibody (1/6000) (Invitrogen) for 90 min, proteins were revealed using the NitroBlue Tetrazolium–5-Bromo-4-Chloro-3-Indolyl Phosphate (NBT/BCIP) substrate (Invitrogen) according to the manufacturer's instructions and the reaction was blocked with two washes of double-distilled H_2O (ddH_2O). The molecular weight of the revealed bands was determined using the Quantity One® software package.

Sera

All sera used in this study were analysed in the laboratory of Parasitology of Farhat Hached teaching hospital, Sousse, Tunisia. All of them were anonymized before use.

Inclusion criteria: We included sera collected from pregnant women, without any history of immunodeficiency, for routine toxoplasmosis screening. We randomly selected 294 sera we divided into 2 groups:

Group I: Consisted of 194 sera positive by Enzyme-Linked ImmunoSorbent Assay [ELISA-IgG (Platelia Toxo IgG, Bio-Rad, Marnes-La-Coquette, France)] and Indirect Fluorescent Antibody Test [IFAT-IgG (Toxo-Spot IF, Bio-Merieux, Marcy l'Etoile, France)]. Among them, 37 were positive in ELISA-IgM (Platelia Toxo IgM, Bio-Rad, Marnes-La-Coquette, France). All sera were tested in immunoblot with ST-Ag and 126 of them were tested in immunoblot with MT-Ag.

Group II: Consisted of 100 sera negative in ELISA-IgG, ELISA-IgM and IFAT-IgG; all were tested in immunoblot with ST-Ag and 96 of them were tested in immunoblot with MT-Ag.

Exclusion criteria: We excluded sera from patients with symptomatic toxoplasmosis, sera from immunodeficient patients and from neonates.

Statistical analysis

The chi-square test was used to compare the frequencies of each band in both groups of positive and negative sera. The data were coded and entered into Epi Info™ 6.04 software (Centers for Disease Control and Prevention, Atlanta, Georgia, USA). The statistical significance was accepted at the 5% level of significance and a 95% confidence level.

Protein Identification by nanoLC-MS/MS

Sample preparation

Coomassie blue stained protein bands, corresponding to the proteins which were the more reactive with the positive sera, were manually excised from the gel and washed with ddH_2O.

In gel tryptic digestion

In-gel digestion was performed with an automated protein digestion system, MassPREP Station (Waters, Manchester, UK). The gel bands were washed twice with 50 µL of mixture containing 25 mM ammonium bicarbonate/acetonitrile (1:1, v/v) for 10 min in order to remove the Coomassie blue excess. After dehydratation for 5 min with acetonitrile (ACN), cysteine residues were reduced by 50 µL of 10 mM dithiothreitol prepared in 25 mM ammonium bicarbonate at 60°C for 1 h, and alkylated with 50 µL of 55 mM iodoacetamide prepared in 25 mM ammonium bicarbonate at room temperature for 20 min. Gel bands were washed with 100 µL of 25 mM ammonium bicarbonate solution and dehydrated with ACN. Proteins were digested in gel with 20 µL of a 4.7 ng/µL modified porcine trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate at 37°C overnight. Trypsin cleaves proteins on the C-terminal side of lysine and arginine amino acid residues, except when either is followed by proline. Peptides were extracted with 35 µL of an aqueous solution containing 60% ACN (v/v) and 1.0% formic acid (v/v), under agitation for 2 h at room temperature. Excess of ACN was removed by Speed Vac treatment and peptides were resuspended in 30 µL of an aqueous solution containing 0.1% formic acid prior to nanoLC-MS/MS analysis.

NanoLC-MS/MS analysis

Peptides were analyzed on a nano-ACQUITY Ultra-Performance-LC system (UPLC, Waters, Milford, MA, USA) hyphenated to a MaXis4G (Bruker Daltonics, Bremen, Germany).

Peptides were first trapped on a 0.18 mm × 20 mm, 5 µm Symmetry C18 pre-column (Waters), and then separated on an ACQUITY UPLC BEH130 C18 column (Waters), 75 µm × 250 mm with 1.7 µm particle size. The solvent system consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in ACN (solvent B). Trapping was performed for 3 min at 5 µL/min with 99% solvent A and 1% solvent B. Elution was performed at a flow rate of 450 nL/min, using a 6-35% gradient (solvent B) over 30 min at 60°C.

The mass spectrometer was equipped with a Captive Spray source and a nano-Booster operating in positive mode with the following settings: source temperature was set at 150°C while drying gas flow was at 3 L/min. The nano-electrospray voltage was optimized to -1300 V. External mass calibration of the TOF was achieved before each set of analyses using Tuning Mix (Agilent Technologies, Palo Alto, CA, USA) in the mass range of 322–2722 m/z. Mass correction was achieved by recalibration of acquired spectra to the applied lock masses hexakis (2,2,3,3,-tetrafluoropropoxy) phosphazine ($[M+H]^+$ = 922.0098 m/z]).

For tandem MS experiments (Collision-Induced Dissociation), the acquisition was performed by sequentially selecting the maximum of precursors for a cycle time of 3 s, with a preference for multiply charged ions and strict exclusion of monocharged ions. The fragmentation was performed using argon as collision gas.

Acquisition speed in MS/MS was adjusted according to the intensity of the precursor. Selected ions were excluded for 1 min and optionally reconsidered if the measured intensity was three times higher than the previous measured intensity. The complete system was piloted and controlled by Hystar 3.2 software (Bruker Daltonics, GmbH, Bremen, Germany).

Protein identification

Raw data collected during nanoLC-MS/MS analysis were processed and converted into "mascotgenericfile"(mgf) with DataAnalysis 4.0 (Bruker Daltonics, Bremen, Germany). All mgf files were interpreted using MASCOT 2.4.3 search engine algorithm (Matrix Science, London, UK) running on a local server. Searches were performed without any molecular weight or isoelectric point restrictions against an in-house generated protein database composed of protein sequences of *T. gondii* (ToxoDB database, 24 April 2014) and known contaminant proteins such as human keratins and trypsin. All proteins were concatenated with reversed copies of all sequences (49.328 entries) with an in-house database generation toolbox [18]. Trypsin was selected as enzyme, carbamidomethylation of cysteine (+57 Da) was set as fixed modification, oxidation of methionine (+16 Da) were set as variable modification and both precursor and fragment mass tolerances were adapted according to instrumental mass accuracy. The MASCOT results were loaded into 3.6.5 Scaffold software (Proteome Software, Portland, OR) to validate peptide identifications. The target-decoy search strategy allowed us to control the protein false discovery rate which was set to a maximum of 1% [19]. .

Bioinformatics analysis

The proteins listed in the Raw data with coverage ≥ 40% were extracted and blasted against ToxoDB.org (14 April 2015) and NCBI (National Center for Biotechnology Information) non-redundant protein sequence database, respectively [20]. Both blasts were carried out to check the similarity of these proteins against cogent proteins of their closest relatives (*Hammondia*, *Neospora*, and *Eimeria*), any related other organisms, and of humans.

Results

ST-Ag-immunoblot

A total of 28 bands with an apparent molecular weight ranging from 6 to 250 kDa were detected by positive and negative sera. Out of the 28 bands, 5 bands (ST-98, ST-36, ST-33, ST-32 and ST-21 kDa) showed the highest specificity and/or sensitivity and were selected as the most relevant markers of infection (Table 1 and Figure 1).

Bands kDa[a]	Positive sera [n=194]	Negative sera [n=100]	Sensitivity% [CL[b] 95%]	Specificity% [CL 95%]	PPV[c]% [CL 95%]	NPV[d]% [CL 95%]	P value (<0.05)
ST[e]-98	165	13	85.1 [79.1-89.6]	87.0 [78.4-92.6]	92.7 [87.6-95.9]	75.0 [65.9-82.4]	$<10^{-4}$
ST-36	182	11	93.8 [89.2-96.6]	89.0 [80.8-94.1]	94.3 [89.8-97.0]	88.1 [79.8-93.4]	$<10^{-4}$
ST-33	139	7	71.6 [64.7-77.8]	93.0 [85.6-96.9]	95.2 [90.0-97.9]	62.8 [54.5-70.5]	$<10^{-4}$
ST-32	181	8	93.3 [88.6-96.2]	92.0 [84.4-96.2]	95.8 [91.5-98.0]	87.6 [79.4-93.0]	$<10^{-4}$
ST-21	156	12	80.4 [74.0-85.6]	88.0 [79.6-93.4]	92.9 [87.6-96.1]	69.8 [60.9-77.5]	$<10^{-4}$

[a]Kilo Dalton, [b]Confidence Level, [c]Positive Predictive Value, [d]Negative Predictive Value, [e]Soluble Tachyzoite antigens.

Table 1: Intrinsic characteristics of the 5 relevant bands (98, 36, 33, 32, and 21 kDa) selected in immunoblot with soluble tachyzoite antigens (ST-Ag).

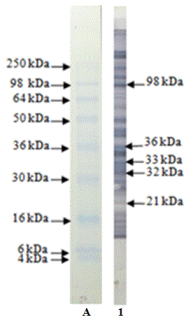

Figure 1: Example of pattern revealed in immunoblot with soluble tachyzoite antigens (ST-Ag) by one positive serum in ELISA and indirect fluorescent antibody test. A: Molecular weight marker. Lane 1: Pattern with the 98, 36, 33, 32 and 21 kDa bands.

Out of the 5 bands, the ST-32 kDa and ST-33 kDa bands showed the highest specificity (92.0% and 93.0%, respectively).

MT-Ag-immunoblot

A total of 26 bands with a apparent molecular weight ranging from 6 to 250 kDa were detected by positive and negative sera. Out of the 26 bands, 4 bands (MT-41, MT-35, MT-32 and MT-30 kDa) showed the highest specificity and/or sensitivity and were selected as the most relevant markers of infection (Table 2 and Figure 2). Out of the 4 bands, the MT-30 kDa and MT-32 kDa bands showed the highest specificity (99.0% and 97.9%, respectively).

All 9 bands were analysed by nanoLC-MS/MS and were subjected to bioinformatics analysis. In addition, the ST-32 kDa, ST-33 kDa, MT-32 kDa and MT-30 kDa that showed a specificity ≥ 90% were subjected to further analysis in order to identify and characterize the proteins they contain.

Bands kDa[a]	Positive sera [n=126]	Negative sera [n=96]	Sensitivity% [CLb 95%]	Specificity% [CL 95%]	PPV[c]% [CL 95%]	NPV[d]% [CL 95%]	P value (<0.05)
MT[e]-41	96	10	76.6 [67.6-83.1]	89.6 [81.3-94.6]	90.6 [82.9-95.1]	74.1 [65.0-81.6]	$<10^{-4}$
MT-35	116	11	92.1 [85.5-95.9]	88.5 [80.0-93.9]	91.3 [84.7-95.4]	89.5 [81.1-94.6]	$<10^{-4}$
MT-32	79	2	62.7 [53.6-71.0]	97.9 [92.0-99.6]	97.5 [90.5-99.6]	66.7 [58.2-74.2]	$<10^{-4}$
MT-30	96	1	76.2 [67.6- 83.1]	99.0 [93.5-99.9]	99.0 [93.6-99.9]	76.0 [67.4-83.0]	$<10^{-4}$

[a]KiloDalton, [b]Confidence Level, [c]Positive Predictive Value, [d]Negative Predictive Value, [e]Membrane Tachyzoite antigens

Table 2: Intrinsic characteristics of the 4 relevant bands (41, 35, 32 and 30 kDa) selected in immunoblot with membrane tachyzoite antigens (MT-Ag).

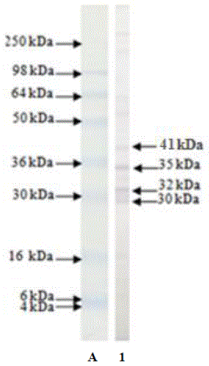

Figure 2: Example of pattern revealed in immunoblot with membrane tachyzoite antigens (MT-Ag) by one positive serum in ELISA and in Indirect Fluorescent Antibody Test. A: molecular weight marker. Lane 1: Pattern with the 41, 35, 32 and 30 kDa bands.

Characterization of immunogenic proteins using the nanoLC-MS/MS and bioinformatics tools

The Coomassie blue stained protein bands which were the more reactive with the positive sera (ST-98 kDa, ST-36 kDa, ST-33 kDa, ST-32 kDa, ST-21 kDa, MT-41 kDa, MT-35 kDa, MT-32 kDa and MT-30 kDa) were manually excised from the gel, digested with trypsin and analyzed by nanoLC-MS/MS. Thereafter, all 9 bands were subjected to bioinformatics analysis. Proteins that are in the 4 bands (ST-32 kDa, ST-33 kDa, MT-32 kDa and MT-30 kDa) that showed a specificity ≥ 90% were selected for bioinformatics analysis with respect to the similarity between GT1 strain and other *T. gondii* strains, on one hand, and between GT1 strain and non-*Toxoplasma* organisms, on the other hand.

A total of 732 proteins were generated from the 9 soluble and membrane proteins bands of *T. gondii* (Table 3).

Proteins with a sequence coverage ≥ 40% were selected for further investigation in order to better characterize the associated proteins. A total of 57 sets of proteins were generated from the 9 soluble and membrane proteins bands. Thirteen of them present sequence coverage ≥ 40% and a closest ortholog ≤ 90% (Table 3).

Four bands restricted to an antigenic specificity ≥ 90% (ST-33 kDa, ST-32 kDa, MT-32 kDa and MT-30 kDa) showed 7 different proteins (Table 3).

Filtering criteria	Included protein bands	Bands specificity in immunoblot (%)	Number of identified peptides	Number of different proteins	Percentage sequence coverage (%)	Number of discriminant peptide(s)
Any protein sequence coverage	ST[a]-98 kDa, ST-36 kDa, ST-33 kDa, ST-32 kDa, ST-21 kDa, MT[b]-41 kDa, MT-35 kDa, MT-32 kDa, MT-30 kDa.	any	1933	732	0.25 to 80	1 to 39
Protein sequence coverage ≥ 40%	ST-98 kDa, ST-36 kDa, ST-33 kDa, ST-32 kDa, ST-21 kDa.	any	61	46	40 to 80	1 to 39
Protein sequence coverage ≥ 40%	MT-41 kDa, MT-35 kDa, MT-32 kDa, MT-30 kDa.	any	33	27	40 to 70	4 to 20
Protein sequence coverage ≥ 40%	ST-98 kDa, ST-36 kDa, ST-33 kDa, ST-32 kDa, ST-21 kDa, MT-41 kDa, MT-35 kDa, MT-32 kDa, MT-30 kDa.	any	94	57	40 to 80	1 to 39
Protein sequence coverage ≥ 40% and a closest ortholog ≤ 90% (NCBI non redundant protein sequence database).	ST-98 kDa, ST-36 kDa, ST-33 kDa, ST-32 kDa, ST-21 kDa, MT-41 kDa, MT-35 kDa, MT-32 kDa, MT-30 kDa.	any	34	13	40 to 80	4 to 21
Protein sequence coverage ≥ 40% and a closest ortholog ≤90% (NCBI[c] non redundant protein sequence database and ToxoDB[d]).	ST-33 kDa, ST-32 kDa, MT-32 kDa, MT-30 kDa.	≥ 90	19	7	41 to 80	4 to 13

[a]Soluble Tachyzoite antigens, [b]Membrane Tachyzoite antigens, [c]National Center for Biotechnology Information, [d]*Toxoplasma gondii* genome Database.

Table 3: Filtering criteria applied to nanoLC-MS/MS to identify specific immunogenic proteins of *T. gondii*.

These proteins are: SAG-related sequence SRS34A, dense granule protein GRA7, dense granule protein GRA1, dense granule protein DG32, microneme protein MIC5, rhoptry protein ROP5 and Toxofilin.

The Blastp of the 7 proteins with ToxoDB database to check the homology of these proteins against their closest relatives (other *Toxoplasma* strains and *Hammondia*, *Neospora*, and *Eimeria*) showed a 86% to 100% homology with proteins of both VEG and ME49 strains of *T. gondii* and a 58% to 87% homology with *Hammondia hammondi* (Table 4). The blastp against NCBI non-redundant database confirmed the homology with H. hammondi proteins and eliminated any similarity with proteins from human (the host) or from other related species.

Discussion

In our study, we identified 7 potential immunogenic proteins which seem to be very useful in the serodiagnosis of toxoplasmosis in immunocompetent individuals. For this purpose, we used the combination of 1D electrophoresis, immunoblot, nanoLC-MS/MS and bioinformatics analysis. This approach is a high throughput immunoproteomics method to identify immunodominant antigens with diagnostic value [6]. In contrast to two-dimensional (2D) electrophoresis known to have a higher resolution power, 1D electrophoresis is more suitable for the separation of membrane proteins that may precipitate under isoelectric focusing conditions in 2D electrophoresis and of proteins with high pH and molecular weight [21].

In order to select proteins that present strong similarity with *T. gondii* sequences and in the same time the lowest similarity with any non-*T. gondii* sequences, we used a ToxoDB database search with MASCOT followed by a NCBI non-redundant protein database to deduce sequence homology of these 7 proteins against their closest relatives (*Hammondia*, *Neospora*, and *Eimeria*) and other organisms including humans [14,22].

The 7 proteins were selected according to the following highly selective criteria: (i) specificity of the revealed band ≥ 90% in

immunoblot, (ii) proteins sequence coverage ≥ 40% and closest ortholog ≤ 90% in MS/MS. These proteins are: SRS34A, GRA7, GRA1, DG32, MIC5, ROP5 and Toxofilin.

Protein name	Length (amino acids)	Bands kDa[a]	Theoretical molecular weight kD[a]	Protein accession numbers	Homology with ME49 and VEG strains	Homology with *Eimeria*, *Hammondia* and *Neospora* in ToxoDBb	Homology with other related organisms in NCBIc	Percentage sequence Coverage (%)
SAG-related sequence SRS34A (SAG2A)/*T. gondii* GT1	186	MTd-30 kDa MT-32 kDa	18.980	TGGT1_271050	100% TGVEG_271050 99% TGME49_271050	83% HHA_271050 *Hammondia hammondi* (strain_H.H.34)	82% XP_008882475.1 *Hammondia hammondi*	43
dense granule protein GRA7/*T. gondii* GT1	236	STe-33 kDa MT-30 kDa MT-32 kDa	25.857	TGGT1_203310	99% TGME49_203310 97% TGVEG_203310	60% HHA_203310 *Hammondia hammondi* (strain_H.H.34)	60% XP_008884816.1 *Hammondia hammondi*	50
dense granule protein GRA1/*T. gondii* GT1	190	ST-32 kDa ST-33 kDa MT-30 kDa MT-32 kDa	20.149	TGGT1_270250	99% TGVEG_270250 98% TGME49_270250	85% HHA_270250 *Hammondia hammondi* (strain_H.H.34)	84% XP_00888240.1 *Hammondia hammondi*	51
dense granule protein DG32/*T. gondii* GT1	219	MT-30 kDa	24.154	TGGT1_297880	99% TGVEG_297880 99% TGME49_297880	87% HHA_297880 *Hammondia hammondi* (strain_H.H.34)	87% XP_008886071.1 *Hammondia hammondi*	43
microneme protein MIC5/*T. gondii* GT1	181	ST-32 kDa ST-33 kDa MT-30 kDa	19.930	TGGT1_277080	99% TGME49_277080 99% TGVEG_277080	87% HHA_277080 *Hammondia hammondi* (strain_H.H.34)	86% XP_008888560.1 *Hammondia hammondi*	44
rhoptry protein ROP5/*T. gondii* GT1	549	ST-33 kDa	60.777	TGGT1_308090	100% TGVEG_308090 97% TGME49_308090	58% HHA_308096 *Hammondia hammondi* (strain_H.H.34)	75% AIT72053.1 *Hammondia hammondi*	43
Toxofilin /*T. gondii* GT1	245	ST-33 kDa MT-30 kDa	27.132	TGGT1_214080	97% TGME49_214080 86% TGVEG_214080	59% HHA_214080 *Hammondia hammondi* (strain_H.H.34)	58% XP_008885392.1 *Hammondia hammondi*	49

[a]kiloDalton, [b]*Toxoplasma gondii* Genome Database, [c]National Center for Biotechnology Information, [d]Membrane Tachyzoite antigens, [e]Soluble Tachyzoite antigens.

Table 4: List of proteins candidate of *T. gondii* retained after nanoLC-MS/MS analysis for the development of a diagnosis toolkit.

SRS34A: formerly SAG2A, SRS34A is one of the major surface antigens of the tachyzoite form [23-25]. It has a theoretical molecular weight of 18.980 kDa and is composed of 186 amino acids. In our study, this protein was only identified in the membrane fraction of tachyzoites (MT-30 kDa and MT-32 kDa bands). It appears to play a role in host cell invasion, immune modulation, virulence attenuation, and it may also promote the parasite survival in the host cell [23,24,26,27].

SRS34A is known as an immunodominant antigen and as a good marker of infection. Its usefulness in the serodiagnosis of toxoplasmosis was evaluated in many studies [24,28-30]. It was shown to be very sensitive in the detection of IgG. Fong et al. using a recombinant form of SRS34A showed that the protein was recognized by all *Toxoplasma* positive sera in immunoblot analysis, while in ELISA, the sensitivity varied from 80% in patients with acute infection to 100% in patients with chronic infection [31]. In contrast, the

majority of investigators underlined the usefulness of SRS34A as a diagnostic tool of the acute phase of toxoplasmosis, with a sensitivity ranging from 90% to 100% against 67% to 70% in the chronic phase [24, 32-36].

In our study, SRS34A was identified within the MT-30 kDa band that showed a sensitivity of 76.2% and a specificity of 99.0%; and in the MT-32 kDa band that showed a sensitivity of 62.7% and a specificity of 97.9%. Our findings argue for the relevance of SRS34A in the serodiagnosis of toxoplasmosis.

GRA7: is a dense granule protein composed of 236 amino acids. It has a theoretical molecular weight of 25.857 kDa. It is expressed by both tachyzoite and bradyzoite stages. It is abundant on the surface and in the cytoplasmic matrix of the host cell, the parasitophore vacuole membrane and within the parasitophore lumen [37-39]. Like all GRA proteins, GRA7 is involved in host cell invasion and associated with the parasite membrane complex, with the tubular elements of the intravacuolar network and with the parasitophorous vacuole membrane through the intravacuolar network during the invasion process [37]. When GRA7 is released from tachyzoites and bradyzoites, it has direct contact with the host immune system, and induces a strong antibody response in both early and late stages of infection [33,40].

GRA7 has been reported as a good marker of infection and showed to be much more sensitive than ROP1, SAG1 and GRA8 that were not included among proteins selected in our study [39]. According to Neudeck et al. GRA7 induces antibodies response earlier than other antigens, such SAG1 and MAG [41].

The overall sensitivity of GRA7 ranged between 81% and 88% and its specificity between 98% and 100% [32,33,35,39,41]. The majority of authors demonstrated the usefulness of GRA7 as a diagnostic marker of the acute phase of toxoplasmosis, with a sensitivity ranging from 94% to 96% against 79% to 89% in the chronic phase of infection [32,39,42-44]. In contrast, Pietkiewicz et al. showed a sensitivity of 68.9% in acute infection and 78% in chronic infection [45]. Altchech et al. underlined the usefulness of GRA7 in the diagnosis of congenital toxoplasmosis [46].

In our study, GRA7 was identified in ST-33 kDa band that showed a sensitivity of 71.6% and a specificity of 93.0% and in both MT-30 kDa and MT-32 kDa bands that showed a sensitivity of 76.2% and 62.7%, respectively; and a specificity of 99.0% and 97.9%, respectively. These findings argue for the potential of GRA7 as a serodiagnosis marker of toxoplasmosis infection.

GRA1: is expressed and secreted by both the tachyzoite and the bradyzoite stages. It has a theoretical molecular weight of 20.149 kDa and is composed of 190 amino acids. The protein is secreted into the parasitophore vacuole and plays an important role in host cell invasion [47-49]. GRA1 is also associated with strong stimulation of the host immune system [47,50,51]. The overall sensitivity of ELISA using the recombinant form of the protein in the detection of anti-*Toxoplasma* IgG varies from 60% to 98% [33,47,48,52]. The protein was described as a good marker of the chronic phase of toxoplasmosis. Ferrandiz et al. reported a sensitivity of the protein of 78.2% in chronic infection against 34% in acute infection [48]. However, recent serological studies showed that GRA1 can be used to detect specific IgG in sera of both acute and chronic phase of disease with a similar sensitivities [33,45,51]. In our study, GRA1 was identified in ST-32 kDa and ST-33 kDa bands that showed a sensitivity of 93.3% and 71.3%, respectively, and a specificity of 92.0% and 93.0%, respectively; and in MT-30 kDa and MT-32 kDa bands that showed a sensitivity of 76.2% and 62.7%, respectively and a specificity of 99.0% and 97.9% respectively. Our findings suggest a potential serodiagnosis role of this protein in the diagnosis of toxoplasmosis.

DG32: is an antigen of the dense granules [53], composed of 219 amino acids. It has a theoretical molecular weight of 24.154 kDa. DG32 participate in the maintenance of intracellular parasitism in almost all nucleated host cells [54]. To our knowledge, the immunogenicity of this antigen has been poorly evaluated. However, according to our results, this protein can be considered as a serodiagnosis marker of toxoplasmosis because it was identified in the 30 kDa band that showed a sensitivity of 76.2% and a nearly absolute specificity of 99.0%.

MIC5: is a small soluble micronemal protein composed of 181 amino acids. It has a theoretical molecular weight of 19.930 kDa. Like all micronemes proteins, it contains a transmembrane domain [55]. It appears to be one of the most abundant secretory proteins and was shown to be necessary for the correct trimming of invasion-related proteins on the parasite surface [56]. MIC5 seems to be strongly immunogenic [57]. However, the serodiagnosis value of this protein remains poorly documented.

In our work, MIC5 was identified within the ST-32 kDa and ST-33 kDa bands that showed a sensitivity of 93.3% and 71.3%, respectively; and in MT-30 kDa band that showed a sensitivity of 76.2% and a specificity of 99.0%. These findings suggest a potential role of the MIC5 in the diagnosis of toxoplasmosis.

ROP5: it is a pseudokinase composed of 549 amino acids. It has a theoretical molecular weight of 60.777 kDa. ROP5 has been recognized as a key virulence factor of *T. gondii* [58-61].

To date, little is known about the immunogenicity and the mechanisms of immune response induced by ROP5. According to Grybowski et al. ROP5 induces an early IgM response in the acute stage of infection, while the specific IgG response is rather moderate [61].

In our study, ROP5 was only detected in the ST-33 kDa band that showed a sensitivity of 71.6% and a specificity of 93.0% suggesting the usefulness of ROP5 as an additional marker of serodiagnosis.

Toxofilin: is a 27.132 kDa monomeric actin-binding protein composed of a 245 amino acids. It is secreted by the rhoptries and is involved in host cell invasion [62,63]. Toxofilin is known for its ability to bind to parasite and mammalian actin and for its role in the regulation of actin filament disassembly and turnover [64-66]. The immunogenicity of Toxofilin is still unclear and its relevance as a serodiagnosis tool poorly evaluated. However, our findings indicate that Toxofilin could be a valuable marker of toxoplasmosis. Indeed, the protein was detected in ST-33 kDa band that showed a sensitivity of 71.6% and a specificity of 93.0% and in MT-30 kDa band that showed a sensitivity of 76.2% and a specificity of 99.0%.

According to most previous data, it is noted that the specificity of the seven reactive *T. gondii* proteins is mostly >90% but their sensitivity is around 70%. This may be due to the fact that the proteins were present in only low concentrations in the antigen extract.

The analysis of our results according to the IgM response showed that the MT-30 kDa band was much more frequently revealed by IgG positive and IgM negative sera as compared to IgG positive and IgM positive sera ($p<10^{-4}$) (unpublished results). Six of the seven selected

proteins were identified in the MT-30 kDa band. This finding suggest that the reactivity to the six proteins may vary according to IgM response and hence to the stage of infection. The absence of IgM response usually characterizes the chronic phase of infection. However, the differential reactivity of positive sera against the seven proteins needs to be investigated in order to assess the relevance of these proteins in dating toxoplasmosis infection. In this respect, it would be much interesting to test the seven different proteins against antibodies produced over time in an experimental animal model of *T. gondii* infection.

It is worth mentioning that the 7 proteins we described herein and we extracted from the GT1 virulent strain of *T. gondii* showed a 86% to 100% homology with both VEG and ME49 strains. These findings argue for the relevance of these proteins in the serodiagnosis of toxoplasmosis infection independently of the strain causing the disease. On the other hand, our bioinformatic approach revealed a 58% to 87% homology between *T. gondii* and *H. hammondi*. Both species are known to have not only morphological but also antigenic similarities and serological cross-reactions between both organisms have been described [67-70]. It is worthy to note that *H. hammondi* has never been shown to infect humans.

In conclusion, according to our findings, the seven proteins we described in the present study appear to be highly valuable markers of acquired toxoplasmosis in immunocompetent individuals. The use of the recombinant form of these proteins may have many advantages with respect to the standardization of serological techniques. Finally, since there may be differences of reactivity of the seven *T. gondii* proteins identified, it should be considered to produce and optimize mixtures of those proteins in order to achieve comprehensive capture of *T. gondii*-specific serum antibodies. .

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