Identification, production and assessment of two *Toxoplasma gondii* recombinant proteins for use in a *Toxoplasma* IgG avidity assay

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The IgG avidity assay is an important tool in the management of suspected toxoplasmosis in pregnant women. This study aimed to produce new *Toxoplasma gondii* recombinant proteins and to assess their usefulness in an IgG avidity assay. *Toxoplasma* positive and negative serum samples were used, the former were categorized into low (LGA) and high (HGA) IgG avidity samples. Immunoblots were performed on 30 *T. gondii* cDNA clones to determine the reactivity and IgG avidity to the expressed proteins. Two of the clones were found to have diagnostic potential and were analyzed further; AG12b encoded *T. gondii* apical complex lysine methyltransferase (AKMT) protein and AG18 encoded *T. gondii* forkhead-associated (FHA) domain-containing protein. The His-tagged recombinant proteins, rAG12b and rAG18, were expressed and tested with LGA and HGA samples using an IgG avidity western blot and ELISA. With the IgG avidity western blot, rAG12b identified 86.4% of LGA and 90.9% of HGA samples, whereas rAG18 identified 81.8% of both LGA and HGA samples. With the IgG avidity ELISA, rAG12b identified 86.4% of both LGA and HGA samples, whereas rAG18 identified 77.3% of LGA and 86.4% of HGA serum samples. This study showed that the recombinant antigens were able to differentiate low avidity and high avidity serum samples, suggesting that they are potential candidates for use in the *Toxoplasma* IgG avidity assay.

Keywords: *Toxoplasma gondii*, Toxoplasmosis, cDNA clones, Immunoblot, Recombinant proteins, IgG avidity, Western blot, ELISA

Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that causes toxoplasmosis, a human infection with worldwide prevalence. The rate of prevalence of *T. gondii* infection varies among countries ranging from 10 to 80% of the population.¹ As an example, the age-adjusted *T. gondii* seroprevalence rate in the United States was reported to be 12.4% among persons of more than 6 years old using samples from the National Health and Nutrition Examination Survey (NHANES) collected in 2009–2010.² In Poland, the mean prevalence of *T. gondii* among 8281 pregnant women aged 18–47 years old between the years 2004 and 2012 was reported to be 40.6%.³ Whereas in Brazil, the reported *T. gondii* seroprevalence rate was 19.5–84.5% among children of 6–15 years old and 36–92% among pregnant women.⁴ In Malaysia, the seroprevalence of *T. gondii* has been steadily increasing among healthy individuals and pregnant women. Among healthy individuals, the rate was reported to be 13.9–20% in 1971–1980, and increased to 25–30% in 1981–1990,

whereas the prevalence among pregnant women was reported to be 23–27.4% in 1971–1980, 27.9–31.6% in 1991–2000, and 49% in 2003.^{5,6} A recent study reported a 42.3% *T. gondii* seroprevalence rate among a population of pregnant women.⁷

Immunocompetent individuals with acute toxoplasmosis are commonly asymptomatic or show mild symptoms. However, pregnant women who acquire acute (primary) infection during pregnancy can develop congenital toxoplasmosis with fetal and neonatal complications including severe neurologic and ocular diseases that may last for life.⁸ Therefore, assessment of the stage (acute or chronic) of *T. gondii* infection in pregnant women is crucial for appropriate patient management. Serodiagnosis remains the most common approach for laboratory diagnosis of *Toxoplasma* infection, as well as assessment of the stage of infection. This is usually performed by detection of *Toxoplasma*-specific IgG and IgM antibodies, however, the presence of these antibodies does not provide ideal information on the stage of *Toxoplasma* infection due to the fact that the IgG antibody usually lasts for life while the IgM antibody may persist for years at high levels.^{9,10}

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Measurement of IgG avidity has been shown to be effective in differentiating acute from chronic toxoplasmosis and is widely used in combination with IgG and IgM detection assays.^{11,12} The avidity status of the specific IgG is low at the initial stage of the infection and gradually increases over time. Low IgG avidity indicates a probable recent *Toxoplasma* infection, whereas high IgG avidity confirms a chronic *Toxoplasma* infection.¹³ A number of *Toxoplasma* IgG avidity assays are commercially available. Most of these assays use *T. gondii* tachyzoite whole lysate antigen, however, this has several limitations such as variability in the quality of the antigen and the risk of contamination with host cells due to the lack of method standardization.^{14–17} The use of recombinant proteins can overcome these limitations and produce more reliable test results. Over the past decade, a number of recombinant antigens have been tested in IgG avidity assays.^{16–21} However, there is still a need to identify more antigens to improve the performance of such avidity assays.^{18,21} In this study, two *T. gondii* cDNA phage clones that expressed proteins with the potential to be used in IgG avidity assay were identified. The corresponding purified recombinant antigens were produced and their effectiveness in IgG avidity assays was assessed.

Materials and methods

Toxoplasma gondii cDNA phage clones

A *T. gondii* cDNA phage library was previously constructed via incorporation of *T. gondii* RH strain cDNA in Lambda ZAP II system (Stratagene, CA). The system combined high efficiency of lambda library construction and allowed *in vivo* excision of pBluescript phagemid.²² This library was previously screened with *in vivo* induced antigen technology (IVIAT) using pooled serum from individuals with suspected acute *Toxoplasma* infection and probed with anti-human IgG (HRP-conjugated).²³ Out of the many positive clones obtained, a total of 30 cDNA clones were randomly selected for use in the present study.

Serum samples

Human serum samples were tested with commercial kits to determine their *Toxoplasma* serological status. They were first tested with Platelia TOXO IgG and TOXO IgM ELISAs; those with positive IgG and IgM were then tested with TOXO IgG avidity ELISA (Bio-Rad, Marnes-la-Coquette, France) according to the manufacturer's instructions. The serum samples were categorized into three groups: Group I serum samples were collected from healthy individuals. Meanwhile, serum samples from patients clinically suspected of toxoplasmosis were categorized into either Groups II or III based on their serological profile. Group I ($n = 10$) samples from healthy individuals were negative for both *Toxoplasma*-specific IgG and IgM antibodies; Group II ($n = 32$) samples were positive for both *Toxoplasma*-specific IgG and IgM antibodies, and had low IgG avidity (LGA); while Group III ($n = 32$) samples

were positive for both *Toxoplasma*-specific IgG and IgM antibodies, and had high IgG avidity (HGA). Two types of pooled serum were created: pooled negative serum from all samples of Group I, and pooled positive serum from 10 random LGA samples from Group II.

IgG immunoblot

The method for immunoblotting of *T. gondii* cDNA clone was based on previous reports.^{23,24} Briefly, *Escherichia coli* host cells XL1-Blue MRF' (Stratagene) were grown overnight in Luria-Bertani (LB) broth. The bacteria suspension was centrifuged and the cell pellet was resuspended in 10 mM MgSO₄ until the OD₆₀₀ reached 0.5. A volume of 10 μ l *T. gondii* cDNA phage clone was mixed with 600 μ l of the *E. coli* cells. The mixture was incubated at 37 °C for 15 min before being added to a melted NZY top agar. The mixture was plated onto a NZY agar plate and further incubated for 5 to 6 h at 37 °C until pinpoint plaques became visible on the agar surface. A nitrocellulose membrane circle (0.45 μ m, Merck, Darmstadt, Germany) pre-treated with 10 mM isopropyl- β -D-thiogalactopyranoside (IPTG, Thermo Scientific, MA) was applied over the agar surface and incubated at 30 °C, overnight. Next day, the membrane was marked to indicate its position on the agar, gently lifted, washed (3 times at 5-min intervals) in Tris-buffered saline with Tween-20 (TBST), and blocked with Superblock solution (Thermo Scientific) with gentle agitation for an hour at room temperature (RT). The nitrocellulose membrane was then washed and cut into six equal triangular pieces. For every assay, the membrane pieces were incubated with individual serum sample (1:100) at 4 °C, overnight. One of the six membrane pieces was incubated with pooled negative or positive serum sample as control. The membranes were washed and incubated with HRP-conjugated mouse anti-human IgG (Invitrogen, CA), diluted 1:2000 in TBST for an hour, RT. After another wash, the membrane pieces were arranged on a film cassette and flooded with enhanced chemiluminescence substrate solution (Thermo Scientific). A CL-XPosure film (Thermo Scientific) was laid on top of the nitrocellulose membrane pieces. The exposed film was developed and fixed with commercial solutions (Sigma, MO).

The film was then superimposed on the agar plate to confirm that the dark spots seen on the film corresponded to actual plaques on the agar. A non-reactive result was shown by generally clear area on the film. A reactive result was shown by dark spots on the film which corresponded to at least 80% of the plaques on the particular section of the agar plate. Each *T. gondii* cDNA phage clone was tested with individual Group I serum samples ($n = 10$) to determine the percent of non-reactive samples. Subsequently, clones with non-reactivity of $\geq 70\%$ were tested with individual serum samples from Groups II and III ($n = 20$) to determine the percent of reactive samples. Clones with reactivity of $\geq 70\%$ were selected for IgG avidity immunoblot.

IgG avidity immunoblot

The method was modified from the aforementioned IgG immunoblot and previously described report.²⁵ A phage clone was plated as described above. The proteins expressed on a plate were blotted on two IPTG-impregnated nitrocellulose membranes by sequential membrane overlay; the first membrane (A) was overlaid on the agar plate for 4 to 5 h and then lifted before a second membrane (B) was applied on the same agar plate for another 4–5 h. Both the membranes were marked, washed, blocked and cut into six pieces as described above. Based on the markings made, membrane pieces from the same area of the agar plate were incubated with the same serum sample and left overnight at 4 °C. The next day, the pieces from membrane A were washed three times with TBST as described above. Meanwhile, washing of the pieces from membrane B was performed using TBST containing 6 M urea. Subsequently, pieces of both membranes (A and B) were incubated with HRP-conjugated mouse anti-human IgG for an hour, washed and developed as described above.

Each of selected *T. gondii* cDNA phage clone was tested with the LGA ($n = 10$) and HGA ($n = 10$) serum samples, respectively. The IgG avidity for each expressed protein was determined by comparing the number of spots observed on the X-ray films. If the number of spots in membrane B (urea-treated membrane pieces) compared to membrane A (non-urea treated membrane pieces) decreased by >50%, the antigen-antibody binding was considered to be of low avidity. Meanwhile, if there was no loss in the number of spots, or if the loss was <50%, the antigen-antibody binding was deemed to be of high avidity.

Clone isolation and identification

In-vivo excision was performed on the selected phage clone according to the manufacturer's instruction for re-circularization of the clone insert contained within the lambda vector to form phagemid. *E. coli* XL1-Blue MRF' and SOLR cells (Stratagene) were grown overnight and resuspended in 10 mM MgSO₄ until the OD₆₀₀ of each cell type reached 1.0. A volume of 250 µl selected phage clone was mixed with 200 µl *E. coli* XL1-Blue MRF' cells and 1 µl of ExAssist helper phage ($>1 \times 10^6$ pfu/µl) (Stratagene), incubated with shaking at 37 °C for 15 min, then added to 3 ml LB broth. Subsequently, the mixture was incubated at 37 °C for 3 h, heated at 65 °C for 20 min, then pelleted. A volume of 200 µl SOLR cells was added separately to 100 and 10 µl of the supernatant, incubated at 37 °C for 15 min before the mixtures being plated on LB-ampicillin agar plates separately. The bacterial colonies grew on the plates were subcultured. Plasmid extraction was performed with QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, then sent for DNA sequencing (1st Base,

Axil Scientific, Selangor, Malaysia) with M13F (-20) and M13R (-24) as forward and reverse primers. The sequence of the insert was analyzed using the ToxoDB database (<http://www.toxodb.org/toxo/>).

Recombinant plasmids and bacteria

The coding region of the selected phage clones was sent to ProteoGenix (Schiltigheim, France) for custom cloning into pET32 prokaryotic expression vector (Novagen, Darmstadt, Germany), using DNA sequences which were codon optimized for expression in *E. coli*. The recombinant plasmid was transformed into *E. coli* strain BL21 (DE3) (New England Biolabs Inc., MA); the DNA inserts were then sequenced for verification (1st Base, Axil Scientific).

Expression and purification of recombinant antigen

A single colony of the recombinant bacteria was grown in Terrific Broth (TB) supplemented with 100 µg/ml ampicillin and incubated in an incubator-shaker at 37 °C, overnight. The next day, a volume of 20 ml culture was inoculated into 500 ml of fresh TB-ampicillin broth. The mixture was incubated for 4–5 h at 37 °C until the OD₆₀₀ reached 0.5. Recombinant protein expression was induced with IPTG (final concentration 1 mM) at 37 °C for 4 h. The bacterial cells were harvested by centrifugation at $10,000 \times g$ at 4 °C for 30 min, and the pellet was used for protein purification.

The cell pellet was resuspended in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, 0.1% Triton-X100, pH 8.0) and sonicated on ice for 2 min (Misonix Sonicator 3000, CT). The suspension was centrifuged at $10,000 \times g$ for 30 min; the resulting supernatant contained the soluble recombinant protein and the pellet contained the insoluble recombinant protein. The Histidine-tagged recombinant protein was purified by affinity chromatography using nickel-nitrilotriacetic acid (Ni-NTA) superflow resin (Qiagen) under native or denaturing approach; depending on the protein solubility. Briefly, in native purification, the supernatant contained the soluble recombinant protein was mixed with Ni-NTA superflow resin (Qiagen) at 4 °C for 60 min. Flow-through was collected, and the resin was washed with 4 different washing buffers (50 mM NaH₂PO₄, 300 mM NaCl, pH 8.0) with an increasing gradient of imidazole concentrations (10, 20, 30 and 40 mM). The purified recombinant antigen was then eluted with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0) at 0.5 ml per fraction for 20–30 fractions. In denaturing purification, the pellet contained the insoluble recombinant protein was resuspended in buffer B (100 mM NaH₂PO₄, 10 mM Tris-Cl, 8 M urea, pH 8.0). The cells were lysed by soft agitation for an hour at RT before another centrifugation to obtain the denatured lysate. The denatured lysate was mixed with Ni-NTA superflow resin (Qiagen) at RT for

60 min. The resin was washed twice with buffer C (which was the same as buffer B, except that its pH was 6.3). The recombinant protein was eluted with 3 ml of buffer D (which was the same as buffer B, except that its pH was 5.9), followed by 5–10 ml of buffer E (which was the same buffer B, except that its pH was 4.5) in 10–20 fractions (0.5 ml/fraction).

Protein analysis

The eluted protein fractions were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue. Western blot protein analysis was performed with the HRP-conjugated anti-His antibody (Novagen). Fractions with high purity were pooled and buffer exchanged into phosphate buffered saline (PBS, pH 7.5). The Coomassie blue stained target recombinant protein band was excised, processed, and analyzed using a MALDI-TOF/TOF 5800 mass spectrometer (AB SCIEX). Mass spectrometry data were searched by ProteinPilot software (AB SCIEX) and MASCOT search engine (Matrix Science, UK) against the SwissProt and ToxoDB databases.

IgG avidity western blot

This method was performed based on previously published reports.^{25,26} The purified recombinant protein, at optimized concentration, was electrophoresed on a 12% SDS-PAGE preparative gel and transferred to a nitrocellulose membrane (0.45 µm, Bio-Rad). The membrane was blocked with protein-free blocking buffer (Pierce, Thermo scientific, MA) at RT for an hour, and then washed with TBST 3 times at 5-min intervals. The membrane was cut into 10 strips, with every 2 strips labeled as a pair. Each strip pair was incubated with the same serum sample diluted at 1:100 overnight. The next morning, one of the strips was washed with TBST, while the other strip was washed with TBST containing 8 M urea, 3 times at 5-min intervals. The strips were then incubated with HRP-conjugated mouse anti-human IgG (1:2000), washed and developed as described above. Each recombinant protein was tested with individual LGA ($n = 22$) and HGA samples ($n = 22$) which were different from those used in the IgG avidity immunoblot. The nitrocellulose strips were imaged and the protein bands were analyzed and quantified using AlphaEase FC imaging software program (Alpha Innotech Corp., CA) with the SpotDenso analysis tool. Band intensities were measured as average density (AVG) by taking AVG of white as 0 and black as 255. The result was determined by the intensity ratio of the strip treated with urea to that without urea treatment. The IgG avidity was considered to be low when the intensity ratio was <0.5 and deemed to be high when the intensity ratio was >0.5 .

IgG avidity ELISA

IgG avidity ELISA was carried out with minor modifications of previously described methods.^{11,19,27} A volume

of 100 µl of each recombinant protein, at optimized concentration, was used to coat wells of a 96-well flat bottom microtiter plate (Nunc Maxisorp, Fisher Scientific, Denmark). The microtiter plate was incubated in a humid chamber at 4 °C overnight and further incubated at 37 °C for an hour the next day. The plate was washed using 200 µl PBS with Tween-20 (PBST) per well, 3 times at 5 min intervals on a plate shaker (700 rpm) at 37 °C (thereafter referred to as 'washing step'). The wells were blocked using protein-free blocking buffer (200 µl per well) at 37 °C, 300 rpm for an hour. A washing step was performed followed by addition of 100 µl individual serum sample (1:100) into each well. Each serum sample was tested in duplicates. The plate was incubated at 37 °C with shaking at 300 rpm for 2 h. Following incubation, one of the two wells incubated with same serum sample was washed with PBST, while another well was washed with PBST-8M urea, 3 times for 5 min each, at 700 rpm in 37 °C. Subsequently, the plate was incubated with 100 µl of HRP-conjugated mouse anti-human IgG (1:2000) and incubated at 37 °C, at 300 rpm, 30 min. A washing step was then performed and the reaction was developed by adding 100 µl ABTS substrate. The plate was incubated at 37 °C, at 300 rpm for 30 min, before the optical density (OD) readings were read by an ELISA spectrophotometer (Multiskan Spectrum, Thermo Fisher Scientific, Finland) at 405 nm with a reference wavelength of 490 nm.

The cut-off values were determined as the mean plus 2 standard deviations (SD) of the *Toxoplasma*-negative serum samples from group I (IgG-, IgM-), with and without urea treatment. The results were calculated as Avidity Index (AI) which referred to the ratio between the OD with urea treatment to that without urea treatment and expressed as percentage. AI of $<50\%$ was considered as low avidity while AI of $\geq 50\%$ was considered as high avidity.

Ethics statement

The Human Research Ethics Committee at Universiti Sains Malaysia permitted the use of previously banked and anonymized serum samples at our institute for this study.

Results

IgG immunoblot

Nine *T. gondii* cDNA phage clones showed non-reactivity of $\geq 70\%$ when tested against individual serum samples from Group I ($n = 10$). The clones were then tested against individual serum samples from Groups II and III ($n = 20$), and the results revealed seven cDNA clones with reactivity of $\geq 70\%$ (Table 1). Fig. 1 shows representative images of non-reactivity and reactivity of AG12b to serum samples from Group I (Fig. 1(A)), and Groups II and III (Fig. 1(B)).

IgG avidity immunoblot

The seven clones were analyzed against the individual LGA ($n = 10$) and HGA ($n = 10$) serum samples using an IgG avidity immunoblot. The IgG avidity of the seven

Table 1 Reactivity and IgG avidity of *T. gondii* cDNA phage clones in immunoblot

cDNA phage clones	Reactivity (%)		IgG avidity (%)	
	Non-reactive ¹	Reactive ²	Low ³	High ⁴
AG2	70	65	ND	ND
AG5	70	75	40	50
AG9	70	80	50	50
AG10	70	80	60	40
AG12b	90	90	80	80
AG18	70	90	80	70
AG21	80	60	ND	ND
AG28	80	75	40	40
AG30	80	85	70	40

Note: ND – not determined.

¹The percentage of non-reactive serum samples when tested using 10 serum samples from Group I (IgG-, IgM-).

²The percentage of reactive serum samples when tested using 10 serum samples from Group II (IgG+, IgM+, low IgG avidity, LGA) and 10 serum samples from Group III (IgG+, IgM+, high IgG avidity, HGA).

³The percentage of serum samples from Group II (LGA, *n* = 10) that showed the decrease in number of spots was >50% after urea treatment.

⁴The percentage of serum samples from Group III (HGA, *n* = 10) that showed the decrease in number of spots was <50% after urea treatment.

clones ranged from 40 to 80% (Table 1), with two clones showing the best results. Clone AG12b demonstrated low IgG avidity with 80% of the LGA samples and high IgG avidity with 80% of the HGA samples; while clone AG18 demonstrated low IgG avidity with 80% of the LGA samples and high IgG avidity with 70% of the HGA samples. Fig. 2 shows representative images of the IgG avidity immunoblot of clones (A) AG12b and (B) AG18 using LGA and HGA serum samples, when treated with TBST wash or TBST-6M urea wash.

Clone identification

Clone AG12b possessed an insert of 1182 base pairs (bp) and was 100% identical to *T. gondii* ME49 apical complex lysine methyltransferase (AKMT) at nucleotide positions 770 to 1951 (ToxoDB accession number: TGME49_216080; accessed 10 July 2015). The coding

region of AG12b comprised 630 bp (nucleotide positions 1320–1949 of TGME49_216080), which encoded 210 amino acids of AKMT protein.

Clone AG18 possessed an insert of 2481 bp and was 99% identical to *T. gondii* ME49 forkhead-associated (FHA) domain-containing protein at nucleotide positions 2638 to 5127 (ToxoDB accession number TGME49_202840; accessed 10 July 2015). The coding region, which comprised 1584 bp and encoded 528 amino acids, showed 100% identity with *T. gondii* GT1 FHA-domain containing protein, at nucleotide positions 1543–3126 (ToxoDB accession number TGGT1_202840; accessed 10 July 2015). The DNA and amino acid sequences for AG12b and AG18 were deposited in the GenBank database under accession numbers KT380837 and KT380838 respectively.

Recombinant antigens and protein analysis

The nucleotide sequences encoding the coding regions of AG12b (630 bp) and AG18 (1584 bp) were cloned into expression vector pET32. Recombinant protein rAG12b was expressed mainly as soluble protein, whereas rAG18 was expressed mainly as insoluble protein. Native purification was performed for the former and denaturing purification for the latter. Both recombinant proteins were successfully purified and the proteins were verified by SDS-PAGE and western blot analysis. Fig. 3(A) and (B) show the recombinant proteins rAG12b and rAG18 with predicted molecular masses of 38 and 65 kDa, respectively, when analyzed on Coomassie blue-stained SDS-PAGE gels and western blots using anti-His-HRP antibody.

MALDI-TOF/TOF analysis using the ToxoDB *T. gondii* ME49 database confirmed that the correct recombinant proteins had been produced. With rAG12b, a protein score of 234 (cut-off score 52) was obtained, with 6% sequence coverage, along with a peptide score of 113 (cut-off score 26) with five matched sequences. Analysis of rAG18 showed a protein score of 320 (cut-off score 52) with 15% coverage, along with a peptide score of 158 (cut-off score 25) with six matched sequences.

Figure 1 Immunoblot of AG12b with (A) Group I and (B) Groups II and III serum samples. In 1(A), nitrocellulose membrane pieces AS1–AS5 were incubated with individual serum samples from Group I (IgG-, IgM-) showed non-reactivity; PC was incubated with pooled positive serum as positive control. In 1(B), nitrocellulose membrane pieces RL1–RL5 incubated with individual serum samples from Group II [IgG+, IgM+, low IgG avidity (LGA)] and RH1–RH5 incubated with serum samples from Group III [IgG+, IgM+, high IgG avidity (HGA)] showed good reactivity; NC was membrane piece incubated with pooled negative serum as negative control.

Figure 2 IgG avidity immunoblot of (A) clone AG12b and (B) clone AG18. The decrease in the number of spots on nitrocellulose membrane pieces incubated with HGA serum (RH1, RH2, and RH3) after TBST-6M urea wash was <50% as compared to TBST wash, indicating high IgG avidity reaction. Meanwhile, the number of spots on nitrocellulose membrane pieces incubated with LGA serum samples (RL1, RL2, and RL3) after TBST-6M urea wash was decreased >50% as compared to TBST wash, indicating low IgG avidity reaction.

Figure 3 SDS-PAGE and western blot analysis of purified recombinant proteins (A) rAG12b and (B) rAG18. Lane 1: Coomassie blue-stained SDS-PAGE gel; Lane 2: western blot probed with anti-His-HRP. The molecular mass of rAG12b is ~38 kDa and rAG18 is ~65 kDa. Lane M: molecular weight marker [Precision Plus Protein Unstained Standards (Bio-Rad)].

IgG avidity western blot

The optimal amounts of protein loaded onto gels for SDS-PAGE analysis were determined to be 4 µg for rAG12b and 5 µg for rAG18, with 8 M urea used as the elution agent. Recombinant proteins rAG12b and rAG18 showed low IgG avidity with 86.4 and 81.8% of Group II (LGA) serum samples ($n = 22$), respectively, and high IgG avidity with 90.9 and 81.8% of the Group III (HGA) serum

samples ($n = 22$), respectively (Table 2). Fig. 4 shows representative images of the IgG avidity western blots for both recombinant proteins.

IgG avidity ELISA

The optimal amounts of protein for use in the IgG avidity ELISA were determined to be 2 and 5 µg/ml for rAG12b and rAG18, respectively. The cut-off values obtained from

Table 2 Performance of rAG12b and rAG18 in IgG avidity assays

Type of assay	Recombinant protein	Avidity interpretation	No. of serum samples		
			LGA	HGA	PPV
IgG avidity western blot	rAG12b	Low	19	2	90.5% (19/22)
		High	3	20	87.0% (20/23)
		Results (%)	19/22 (86.4)	20/22 (90.9)	
	rAG18	Low	18	4	81.8% (18/22)
		High	4	18	81.8% (18/22)
IgG avidity ELISA	rAG12b	Results (%)	18/22 (81.8)	18/22 (81.8)	
		Low	19	3	86.4% (19/22)
		High	2	19	90.5% (19/21)
	rAG18	Could not be determined ¹	1	0	
		Results (%)	19/22 (86.4)	19/22 (86.4)	
		Low	17	3	85.0% (17/20)
		High	3	19	86.4% (19/22)
		Could not be determined ²	2	0	
		Results (%)	17/22 (77.3)	19/22 (86.4)	

¹Results could not be determined due to the OD obtained was lower than the cut-off value of rAG12b.²Results could not be determined due to the ODs obtained were lower than cut-off value of rAG18. **Figure 4** IgG avidity western blots of (A) rAG12b and (B) rAG18. Lanes 1 and 2 are paired strips incubated with HGA serum sample while lanes 3 and 4 are paired strips incubated with LGA serum sample. Lanes 1 and 3 were treated (washed) with TBST while lanes 2 and 4 were treated with TBST containing 8M urea. When lane 2 was compared to lane 1, the intensity ratio was >0.5, indicating high avidity binding of anti-*Toxoplasma* IgG to the antigen. When lane 4 was compared to lane 3, the intensity ratio was <0.5, indicating low avidity binding of anti-*Toxoplasma* IgG to the antigen.

the rAG12b-IgG avidity ELISA with and without urea treatment were 0.19 and 0.23, respectively, whereas the cut-off values for the rAG18-IgG avidity ELISA with and without urea treatment were 0.25 and 0.33, respectively. With Group II (LGA) serum ($n = 22$), rAG12b and rAG18 showed low IgG avidity ($AI < 50\%$) with 86.4 and 77.3% of the samples, respectively. By contrast, with Group III (HGA) serum ($n = 22$), both rAG12b and rAG18 showed high IgG avidity ($AI \geq 50\%$) with 86.4% of the samples (Table 2). The AI scores of the serum samples using the two recombinant proteins are tabulated in Fig. 5.

Positive predictive values of the developed assays

The positive predictive value (PPV) of the assay (western blot/ELISA) to detect low IgG avidity samples was determined from the samples from patients with probable acute *Toxoplasma* infection (LGA). Similarly, the PPV of the assay developed to detect high IgG avidity samples was determined from the samples from patients with chronic

Toxoplasma infection (HGA). The results showed that irrespective of the kind of assay or the type of serum samples, rAG12b showed higher PPV values than rAG18 (Table 2).

Discussion

Recent data from the World Health Organization highlights the importance of toxoplasmosis. The disease burden of congenital (excluding stillbirths) and acquired toxoplasmosis was reported to be 1.68 million disability-adjusted life years (DALYs),²⁸ while in another analysis, congenital toxoplasmosis (including stillbirths) was reported to have a global disease burden of 1.2 million DALYs.²⁹ In developed countries with low *T. gondii* seroprevalence, pregnant women without prior exposure to the parasite are susceptible to acquiring acute toxoplasmosis. Although the low levels of parasite circulation in such environments reduce the risk of acute infection, the incidence of congenital toxoplasmosis can be higher than expected.¹ In some countries including France and Austria, a routine maternal toxoplasmosis screening program for pregnant

Figure 5 Avidity index scores of IgG avidity ELISA using (A) rAG12b and (B) rAG18. Avidity index at 50%, indicated with a bold dashed line, was used to differentiate acute and chronic serum samples. Of the 22 LGA serum samples tested, rAG12b demonstrated 19 low IgG avidity and 2 high IgG avidity results. Avidity status of one serum sample could not be determined since the OD was lower than the cut-off value. With the same group of serum samples, rAG18 demonstrated 17 low IgG avidity and 3 high IgG avidity results, while the avidity status of 2 serum samples could not be determined. Both recombinant proteins demonstrated 19 high IgG avidity results and 3 low IgG avidity results with the 22 HGA serum samples.

women has been established to facilitate the early diagnosis of the acute stage of the disease. The French screening program has been reported to be cost-effective compared with the estimated societal damage caused by congenital toxoplasmosis.³⁰

Current commercially available IgG avidity assays are clinically useful in assessing the stage of infection. However, cross-evaluation studies have shown variability in the performances of some well-known commercial assays.^{31–33} Thus, efforts at improving IgG avidity assays are still needed.

In the current study, two cDNA clones that showed the highest potential in immunoblot and IgG avidity immunoblot assays were sequenced and investigated further. AKMT, encoded by clone AG12b, is involved in methylation of the apical cytoskeletal structure of *T. gondii*.³⁴ It regulates motility activation in *T. gondii*, thus, deficiency of this protein may affect the parasite's entry into and exit from the host cell, as well as its lytic cycle.³⁵ FHA domain-containing protein, encoded by clone AG18, is a

protein that contributes to phosphothreonine, phosphoserine, and phosphotyrosine epitopes and serves as a site for phosphorylated protein–protein interactions.^{36,37}

Previous studies have identified a number of immunogenic *T. gondii* proteins through the application of western blot techniques in the investigation of the human humoral immune response to the *T. gondii* tachyzoite antigen.¹⁵ These include surface antigens (SAG),^{17–20} dense granule antigens (GRA),^{17,18,20,21} microneme protein (MIC)¹⁸ and rhoptry antigens (ROP).^{20,38} Proteins encoded by AG12b and AG18 belong to none of the above-reported groups since a different approach was used in the present study. The western blot techniques employed in the previous studies may not have discovered all immunoreactive antigens. Hence, the finding of this study provides two new potential proteins for further investigation.

Previously, a commercial *Toxoplasma* IgG western blot assay known as the LDBio-Toxo II IgG test (LDBio Diagnostics, France) was reported to demonstrate a specificity of 100% and a sensitivity of 99.2% compared with the

dye test.³⁹ A major disadvantage of western blot analysis is that interpretation of the results can be complicated by weak band intensity. Therefore, in the present study, the protein band intensity was quantified using an imaging program and software. A related method for serodiagnosis is a line blot assay; this assay has been used to determine *Toxoplasma* seroreactivity to, and the IgG avidity of, five *T. gondii* recombinant antigens.¹⁶ The recombinant antigens ROP1, MAG1, SAG1, GRA7, and GRA8 were applied directly onto a nitrocellulose membrane in different lines. The membrane was blocked, cut into individual strips, and incubated with individual serum samples. The line assay allows differentiation of reactivities and IgG avidities directed against individual recombinant antigens. This test format can thus be used to incorporate new recombinant proteins such as AG12b and AG18 identified in the present study.

Many studies have been performed to investigate the AI ranges of serum samples from acute and chronic toxoplasmosis. A correlation between the phase of *Toxoplasma* infection and the AI has been shown; however, the results were variable.^{11,27,40,41} The AI variations may be caused by several factors such as the estimation of the infection stage at the time of serum collection, antigen preparation, antigen type, the concentration of the elution agent and the type of assay.⁴¹ Indeed, IgG affinity maturation against *T. gondii* recombinant antigens is different from *Toxoplasma* whole lysate antigen and may affect the AI cut-off in an IgG avidity assay.¹⁷ In this study, the intensity ratio and the AI to define acute and chronic toxoplasmosis of the developed IgG avidity assays were determined based on the results of the previous studies^{27,41} and a 50% difference in the IgG titer was found to be appropriate.

The performance of the two recombinant antigens obtained in this study was comparable to, or better than, the results of studies using other recombinant antigens. In a previous study, the recombinant rhostry antigen (rROP1) gave low or borderline avidity with 26 of 34 (76%) suspected acute toxoplasmosis serum samples when compared with the VIDAS IgG Toxo avidity assay (*bioMérieux*, Marcy l'Étoile, France).³⁸ In another study by Bela *et al.*,¹⁹ the recombinant surface antigen (rSAG2A) gave low or intermediate AIs for 60% of acute-phase serum samples and detected 70% of chronic serum samples based on an AI of >60% in an IgG avidity ELISA. Drapala *et al.*²⁰ evaluated two different mixtures of recombinant proteins (rROP1 + rSAG2 and rROP1 + rGRA6) in an IgG avidity assay and compared these with the VIDAS IgG Toxo avidity assay. Using suspected chronic toxoplasmosis samples ($n = 68$), both protein mixtures gave high AI values for all serum samples (100%) and were in good agreement with results obtained using the VIDAS assay. However, both mixtures showed mostly high AI values using serum samples from suspected acute toxoplasmosis (only 6 out of 22 showed low AI, 27.3%); by comparison, the VIDAS assay exhibited low avidity with most (91%) of the samples. The VIDAS Toxo IgG avidity assay utilized whole *T.*

gondii native antigen and is widely used in clinical laboratories in Europe and the United States. However, it must be performed on a VIDAS instrument that is costly and therefore not affordable for most laboratories in developing countries.

The use of more than one recombinant antigen has been reported to enhance the performance of a *Toxoplasma* serological assay compared with using a single antigen. This was demonstrated in previous studies that evaluated recombinant antigens individually, followed by mixtures of these antigens.^{15,42–44} Indeed, a cocktail of recombinant antigens proved more effective since some patient's serum samples may lack specific antibodies toward certain antigens as a result of variable individual immune responses.¹⁵ In the present study, when serum IgG avidity in response to each recombinant protein was considered, the IgG avidity western blot successfully identified the avidity status of 90.9% of LGA and 95.5% HGA samples, whereas the IgG avidity ELISA correctly identified the avidity status of 90.9% of the LGA and HGA serum samples. An IgG avidity assay using a mixture of the recombinant proteins, or inclusion of a third or more recombinant proteins could be investigated as an approach to increase the diagnostic value of the assay. Other further studies that can be performed include testing on serum samples from a patient with known time course of infection, or alternatively testing serum of experimentally infected mice at different time points of infection. In conclusion, based on the results of the present study, rAG12b and rAG18 are useful recombinant proteins for the IgG avidity assay.

Declaration of interest

The authors report no declarations of interest.

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References

- 1 Robert-Gangneux F, Darde ML. Epidemiology of and diagnostic strategies for toxoplasmosis. *Clin Microbiol Rev*. 2012;25(2):264–296.
- 2 Jones JL, Kruszon-Moran D, Rivera HN, Price C, Wilkins PP. *Toxoplasma gondii* seroprevalence in the United States 2009–2010 and comparison with the past two decades. *Am J Trop Med Hyg*. 2014;90(6):1135–1139.
- 3 Nowakowska D, Wujcicka W, Sobala W, Spiewak E, Gaj Z, Wilczynski J. Age-associated prevalence of *Toxoplasma gondii* in 8281 pregnant women in Poland between 2004 and 2012. *Epidemiol Infect*. 2014;142(3):656–661.
- 4 Dubey JP, Lago EG, Gennari SM, Su C, Jones JL. Toxoplasmosis in humans and animals in Brazil: high prevalence, high burden of disease, and epidemiology. *Parasitology*. 2012;139(11):1375–1424.
- 5 Nissapatorn V, Abdullah KA. Review on human toxoplasmosis in Malaysia: the past, present and prospective future. *Southeast Asian J Trop Med Public Health*. 2004;35(1):24–30.
- 6 Nissapatorn V, Noor Azmi MA, Cho SM, Fong MY, Init I, Rohela M, et al. Toxoplasmosis: prevalence and risk factors. *J Obstet Gynaecol*. 2003;23(6):618–624.
- 7 Andiappan H, Nissapatorn V, Sawangjaroen N, Nyunt MH, Lau YL, Khaing SL, et al. Comparative study on *Toxoplasma* infection between Malaysian and Myanmar pregnant women. *Parasit Vectors*. 2014;7:564.
- 8 Jones JL, Kruszon-Moran D, Sanders-Lewis K, Wilson M. *Toxoplasma gondii* infection in the United States, 1999–2004, decline from the prior decade. *Am J Trop Med Hyg*. 2007;77(3):405–410.
- 9 Hill D, Dubey JP. *Toxoplasma gondii*: transmission, diagnosis and prevention. *Clin Microbiol Infect*. 2002;8(10):634–640.
- 10 Gras L, Gilbert RE, Wallon M, Peyron F, Cortina-Borja M. Duration of the IgM response in women acquiring *Toxoplasma gondii* during pregnancy: implications for clinical practice and cross-sectional incidence studies. *Epidemiol Infect*. 2004;132(3):541–548.
- 11 Hedman K, Lappalainen M, Seppä I, Makela O. Recent primary toxoplasma infection indicated by a low avidity of specific IgG. *J Infect Dis*. 1989;159(4):736–740.
- 12 Liu Q, Wang ZD, Huang SY, Zhu XQ. Diagnosis of toxoplasmosis and typing of *Toxoplasma gondii*. *Parasit Vectors*. 2015;8:292.
- 13 Jenum PA, Stray-Pedersen B, Gundersen AG. Improved diagnosis of primary *Toxoplasma gondii* infection in early pregnancy by determination of anti-*Toxoplasma* immunoglobulin G avidity. *J Clin Microbiol*. 1997;35(8):1972–1977.
- 14 Jacobs D, Vercammen M, Saman E. Evaluation of recombinant dense granule antigen 7 (GRA7) of *Toxoplasma gondii* for detection of immunoglobulin G antibodies and analysis of a major antigenic domain. *Clin Diagn Lab Immunol*. 1999;6(1):24–29.
- 15 Aubert D, Maine GT, Villena I, Hunt JC, Howard L, Sheu M, et al. Recombinant antigens to detect *Toxoplasma gondii*-specific immunoglobulin G and immunoglobulin M in human sera by enzyme immunoassay. *J Clin Microbiol*. 2000;38(3):1144–1150.
- 16 Pfeiffer KI, Enders G, Gohl M, Krczal D, Hlobil H, Wassenberg D, et al. Seroreactivity to and avidity for recombinant antigens in toxoplasmosis. *Clin Diagn Lab Immunol*. 2005;12(8):977–982.
- 17 Pietkiewicz H, Hiszczynska-Sawicka E, Kur J, Petersen E, Nielsen HV, Paul M, et al. Usefulness of *Toxoplasma gondii* recombinant antigens (GRA1, GRA7 and SAG1) in an immunoglobulin G avidity test for the serodiagnosis of toxoplasmosis. *Parasitol Res*. 2007;100(2):333–337.
- 18 Beghetto E, Buffolano W, Spadoni A, Del Pezzo M, Di Cristina M, Minenkova O, et al. Use of an immunoglobulin G avidity assay based on recombinant antigens for diagnosis of primary *Toxoplasma gondii* infection during pregnancy. *J Clin Microbiol*. 2003;41(12):5414–5418.
- 19 Bela SR, Oliveira Silva DA, Cunha-Junior JP, Pirovani CP, Chaves-Borges FA, Reis de Carvalho F, et al. Use of SAG2A recombinant *Toxoplasma gondii* surface antigen as a diagnostic marker for human acute toxoplasmosis: analysis of titers and avidity of IgG and IgG1 antibodies. *Diagn Microbiol Infect Dis*. 2008;62(3):245–254.
- 20 Drapala D, Holec-Gasior L, Kur J, Ferra B, Hiszczynska-Sawicka E, Lautenbach D. A new human IgG avidity test, using mixtures of recombinant antigens (rROP1, rSAG2, rGRA6), for the diagnosis of difficult-to-identify phases of toxoplasmosis. *Diagn Microbiol Infect Dis*. 2014;79(3):342–346.
- 21 Elyasi H, Babaie J, Fricker-Hidalgo H, Brenier-Pinchart MP, Zare M, Sadeghiani G, et al. Use of dense granule antigen GRA6 in an immunoglobulin g avidity test to exclude acute *Toxoplasma gondii* infection during pregnancy. *Clin Vaccine Immunol*. 2010;17(9):1349–1355.
- 22 Short JM, Fernandez JM, Sorge JA, Huse WD. Lambda ZAP: a bacteriophage lambda expression vector with *in vivo* excision properties. *Nucleic Acids Res*. 1988;16(15):7583–7600.
- 23 Amerizadeh A, Khoo BY, Teh AY, Golkar M, Abdul Karim IZ, Osman S, et al. Identification and real-time expression analysis of selected *Toxoplasma gondii* *in vivo* induced antigens recognized by IgG and IgM in sera of acute toxoplasmosis patients. *BMC Infect Dis*. 2013;13:287.
- 24 Amerizadeh A, Idris ZM, Khoo BY, Kotresha D, Yunus MH, Karim IZ, et al. Identification of *Toxoplasma gondii* *in vivo* induced antigens by cDNA library immunoscreening with chronic toxoplasmosis sera. *Microb Pathog*. 2013;54:60–66.
- 25 Marcolino PT, Silva DA, Leser PG, Camargo ME, Mineo JR. Molecular markers in acute and chronic phases of human toxoplasmosis: determination of immunoglobulin G avidity by western blotting. *Clin Diagn Lab Immunol*. 2000;7(3):384–389.
- 26 Ali-Heydari S, Keshavarz H, Shojaei S, Mohebbi M. Diagnosis of antigenic markers of acute toxoplasmosis by IgG avidity immunoblotting. *Parasite*. 2013;20:18.
- 27 Holliman RE, Raymond R, Renton N, Johnson JD. The diagnosis of toxoplasmosis using IgG avidity. *Epidemiol Infect*. 1994;112(2):399–408.
- 28 Torgerson PR, Devleeschauwer B, Praet N, Speybroeck N, Willingham AL, Kasuga F, et al. World health organization estimates of the global and regional disease burden of 11 foodborne parasitic diseases, 2010: a data synthesis. *PLoS Med*. 2015;12(12):e1001920.
- 29 Torgerson PR, Mastroiacovo P. The global burden of congenital toxoplasmosis: a systematic review. *Bull World Health Organ*. 2013;91(7):501–508.
- 30 Stillwaggon E, Carrier CS, Sautter M, McLeod R. Maternal serologic screening to prevent congenital toxoplasmosis: a decision-analytic economic model. *PLoS Negl Trop Dis*. 2011;5(9):e1333.
- 31 Bobic B, Klun I, Vujanic M, Nikolic A, Ivovic V, Zivkovic T, et al. Comparative evaluation of three commercial *Toxoplasma*-specific IgG antibody avidity tests and significance in different clinical settings. *J Med Microbiol*. 2009;58(3):358–364.
- 32 Lachaud L, Calas O, Picot MC, Albaba S, Bourgeois N, Pratlong F. Value of 2 IgG avidity commercial tests used alone or in association to date toxoplasmosis contamination. *Diagn Microbiol Infect Dis*. 2009;64(3):267–274.
- 33 Villard O, Breit L, Cimon B, Franck J, Fricker-Hidalgo H, Godineau N, et al. Comparison of four commercially available avidity tests for *Toxoplasma gondii*-specific IgG antibodies. *Clin Vaccine Immunol*. 2013;20(2):197–204.
- 34 Xiao H, El Bissati K, Verdier-Pinard P, Burd B, Zhang H, Kim K, et al. Post-translational modifications to *Toxoplasma gondii* alpha- and beta-tubulins include novel C-terminal methylation. *J Proteome Res*. 2010;9(1):359–372.
- 35 Heaslip AT, Nishi M, Stein B, Hu K. The motility of a human parasite, *Toxoplasma gondii*, is regulated by a novel lysine methyltransferase. *PLoS Pathog*. 2011;7(9):e1002201.
- 36 Durocher D, Jackson SP. The FHA domain. *FEBS Lett*. 2002;513(1):58–66.
- 37 Li J, Lee GI, Van Doren SR, Walker JC. The FHA domain mediates phosphoprotein interactions. *J Cell Sci*. 2000;113(Pt 23):4143–4149.
- 38 Holec-Gasior L, Drapala D, Lautenbach D, Kuri J. *Toxoplasma gondii*: usefulness of ROP1 recombinant antigen in an immunoglobulin G avidity assay for diagnosis of acute toxoplasmosis in humans. *Pol J Microbiol*. 2010;59(4):307–310.
- 39 Franck J, Garin YJ, Dumon H. LDBio-Toxo II immunoglobulin G western blot confirmatory test for anti-toxoplasma antibody detection. *J Clin Microbiol*. 2008;46(7):2334–2338.
- 40 Joynson DH, Payne RA, Rawal BK. Potential role of IgG avidity for diagnosing toxoplasmosis. *J Clin Pathol*. 1990;43(12):1032–1033.
- 41 Rossi CL. A simple, rapid enzyme-linked immunosorbent assay for evaluating immunoglobulin G antibody avidity in toxoplasmosis. *Diagn Microbiol Infect Dis*. 1998;30(1):25–30.
- 42 Nigro M, Gutierrez A, Hoffer AM, Clemente M, Kaufer F, Carral L, et al. Evaluation of *Toxoplasma gondii* recombinant proteins for the diagnosis of recently acquired toxoplasmosis by an immunoglobulin G analysis. *Diagn Microbiol Infect Dis*. 2003;47(4):609–613.
- 43 Pietkiewicz H, Hiszczynska-Sawicka E, Kur J, Petersen E, Nielsen HV, Stankiewicz M, et al. Usefulness of *Toxoplasma gondii*-specific recombinant antigens in serodiagnosis of human toxoplasmosis. *J Clin Microbiol*. 2004;42(4):1779–1781.
- 44 Li S, Galvan G, Araujo FG, Suzuki Y, Remington JS, Parnley S. Serodiagnosis of recently acquired *Toxoplasma gondii* infection using an enzyme-linked immunosorbent assay with a combination of recombinant antigens. *Clin Diagn Lab Immunol*. 2000;7(5):781–787.