Parasitology International 66 (2017) 643–651

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Evaluation of novel oocyst wall protein candidates of *Toxoplasma gondii*

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ARTICLE INFO

Keywords:
Toxoplasma gondii
T. gondii oocyst wall protein

ABSTRACT

Oocyst stage of *Toxoplasma gondii* is characterized by a durable wall that confers a strong protection to this protozoan parasite in face of harsh environmental conditions. Thus, it is considered the key for transmission of *T. gondii.* Analysis of oocyst wall composition is mandatory therefore; the aim of this study was to identify novel *T. gondii* oocyst wall proteins and test their use in detection of these oocysts in environmental samples. Five candidates of novel *T. gondii* oocyst wall proteins (TgOWPs) were identified and named TgOWP8 through TgOWP12. Recombinant protein of TgOWP8 was expressed in *E. coli* using glutathione S-transferase as fusion protein. Polyclonal antibody was produced and validated by indirect immunofluorescence antibody assay (IFA). For detection by IFA, we used different methods for fixation and permeabilization of oocysts to improve the antigen-antibody detection. Specificity to wall of *T. gondii* oocyst was confirmed and revealed absence of cross reactivity with bradyzoite cyst wall and tachyzoites. Although some TgOWPs were identified previously, our study represents a continuation of molecular investigations of oocyst wall proteins as an essential structure for the longevity and infectivity of this stage and also provided new trial to improve *T. gondii* oocysts detection.

1. Introduction

Toxoplasma gondii (*T. gondii*), is an intracellular apicomplexan protozoan parasite with worldwide distribution. This parasite is able to infect most of animals, birds and human [1]. In human, *T. gondii* infection can be life threatening for congenitally infected infants and immunocompromised patients, as a result of either acute or reactivated infection [2]. Felids, particularly cats, are the only definitive hosts known to shed *T. gondii* oocysts in their feces which can be deposited in soil, grass, water, or elsewhere. These oocysts are resistant to the harsh environmental conditions being an excellent reservoir for the parasite survival. Therefore, the environmental contamination with *T. gondii* oocysts is considered a potential risk for both humans and animals [1,3].

Humans or animals can acquire toxoplasmosis either congenitally or orally. Oral transmission is mediated by either bradyzoites contained in cysts or sporozoites enclosed in the environmentally resistant oocysts [2]. Although the tissue cysts in animal meat are considered an important source of *T. gondii* infection, the significant risk of contaminated environment by oocysts may equalize the hazards of cysts. This observation was based on the detection of sporozoite-specific protein antibodies in sera of 43% of recently infected pregnant women in Chile [4]. Also, some evidence suggested that oocyst-induced infections in humans may have more clinical aspect than those acquired *via* tissue cyst-infections [5]. Recent studies reported that the majority of the congenital infections and postnatal acute infections in the United States were resulted from oocysts [6,7]. These studies increased concern about the severity and outcomes of infections primarily induced by oocyst. Moreover, toxoplasmosis outbreaks were greatly owed to the contaminated water or soil with *T. gondii* oocysts [8]. In addition, consumption of contaminated municipal drinking water resulted in high incidence of water-borne toxoplasmosis in both animals and humans [5,9–12]. These outbreaks magnified the risk of *T. gondii* oocysts as hazardous infectious stage and therefore, efforts had been raised toward development of sensitive methods for detection of *T. gondii* oocysts in natural samples [13–16].

T. gondii stages are three walled forms: oocysts, sporocysts, and tissue cysts [17]. Oocyst stage is characterized by a bi-layered extremely robust wall that acts as a main barrier either to physical or chemical attacks. Thus, the common disinfectants and extreme

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http://dx.doi.org/10.1016/j.parint.2017.05.009
Received 12 April 2017; Received in revised form 17 May 2017; Accepted 23 May 2017
Available online 30 May 2017

environmental conditions cannot kill the parasite inside the oocyst [18,19].

Characterization of *T. gondii* oocyst wall structure was previously investigated. A gene encoding one COWP homolog named TgOWP1 was identified by Templeton et al. [20]. Moreover, a family of cysteine-rich proteins which is homologous to the most abundant *Cryptosporidium* oocyst wall proteins (COWPs) were previously characterized [21]. Furthermore molecular researches of *T. gondii* oocyst and sporozoites proteome expressed strong clues of host independency and long-standing characters of oocyst structure toward the external stressful conditions [22,23]. In the present study we are focusing on the protein composition of *T. gondii* oocyst wall, as primary guard of the worldwide parasite. We performed Expression Sequence Tagged (EST) database screening of *T. gondii* oocyst wall proteins in order to identify novel candidates. Antibody against one of these candidates was produced in mice. Immunofluorescence analysis revealed the reactivity of the antibody toward *T. gondii* oocyst wall.

2. Materials and methods

2.1. Enzymes and chemicals

Restriction enzymes were purchased from Toyobo Co., Ltd. (Osaka, Japan) New England Biolabs Japan (Tokyo, Japan) and Promega (USA). All reagents used were commercially available and of analytical grade.

2.2. Animals

Cats: two specific pathogen-free (SPF) domestic short hair male kittens, 2 months old were obtained from Liberty research, USA. Cats were kept separate in isolated cages and fed only dry cat food. These cats were used for experimental infection with *T. gondii* to get the oocysts from their feces.

Mice: six to eight weeks old female BALB/c and ICR mice purchased from CLEA, Japan were used in this study. BALB/c mice were used for antibody production of *T. gondii* oocyst wall protein. ICR mice were used for maintaining *T. gondii* cysts through monthly passage. The experiments were conducted according to the guidelines for the Care and Use of Laboratory Animals issued by Obihiro University of Agriculture and Veterinary Medicine.

2.3. Parasites

The high virulent Type I strain of RH and low virulent, cyst-forming Type II strains of Beverley, PLK and ME49 of *T. gondii* were used. RH strain was used for preparation of tachyzoites lysates for western blotting as described below. Beverley strain was used for establishing *T. gondii* chronic infection in mice. ME49 strains were used for *in vitro* differentiation of bradyzoites. PLK strain was used as a template to carry out PCR. Tachyzoites of all *T. gondii* strains, RH, Beverley, PLK and ME49 were maintained in our laboratory through serial passage in Vero cells or human foreskin fibroblasts (HFF) cultured in modified Eagle's medium (Sigma-Aldrich, UK) supplemented with 5% heat-inactivated fetal bovine serum (FBS).

Purification of *T. gondii* tachyzoites and preparation of lysate were performed as following; infected HFF cells were washed with cold phosphate-buffered saline (PBS). Cells pellets were suspended in medium, lysed 6–10 times through 27-gauge needle and filtered by a 5.0 μm-pore filter (Millipore, Bedford, MA). After centrifugation at 2000 x*g* for 5 min at 4 °C, pellet was resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.25 mM sodium deoxycholate, 0.1% Triton X-100 and 1% Nonidet P-40). Tachyzoites lysate was recovered after centrifugation at 2000 × *g* for 5 min. Protein concentration was measured by Coomassie Protein Assay reagent kit using bovine serum albumin as a standard (Pierce, USA). Prepared lysates were kept at − 30 °C until use.

T. gondii cysts of Beverley strain were maintained through successive passages in ICR mice. The *in vivo* bradyzoite cysts were purified from mice brain tissues as previously described [24] with minor modifications. In brief, brains were removed from *T. gondii*-infected mice one month after infection and homogenized in PBS. Brain homogenates were resuspended in 50 ml of PBS and finally cysts were purified from brain homogenate using Gum Arabic (Sigma–Aldrich, UK). Briefly, 2 ml of 1.07 *sg* (specific gravity) Gum Arabic solution were added to each tube and 2 ml of 1.05 *sg* Gum Arabic were overlaid slowly to each tube, followed by addition of 5 ml of brain homogenate. After 10 min of centrifugation at 2000 × *g* at 15 °C, the pellets were collected and washed 3 times with PBS. Purified cysts were counted and used for experimental infection of cats and production of oocysts.

2.4. Purification of T. gondii oocysts

T. gondii oocysts of Beverley strain (genotype II) were extracted from feces of experimentally-infected cat with approximately 400 *T. gondii* bradyzoite cysts according to Dubey and Beattie [25]. Oocyst sporulation was induced by incubation in 2% sulfuric acid at room temperature for 1 week and at 4 °C for prolonged storage. Oocysts were purified by sucrose flotation as described previously [26]. Purified oocysts were used as antigen to test specificity of polyclonal antibodies by immunofluorescence assay.

2.5. In vitro differentiation of T. gondii

Confluent HFF cells grown in 10 cm plates were infected with tachyzoites of ME49 strains and differentiation was induced by culturing in sodium bicarbonate free RPMI 1640 containing 1% FCS, 50 mM HEPES (pH 8.1) at 37 °C without CO_2 [27].

2.6. Expression sequence tagged (EST) database analysis

EST database (GenBank) was used to select *T. gondii* oocyst wall candidates from *T. gondii* cDNA sequence of type III VEG strain; cDNA library from fully and partially sporulated oocysts was used. The putative *T. gondii* oocyst wall proteins are annotated as TGVEG_271580-TGVEG_271590, TGVEG_313000, TGVEG_294600, TGVEG_205090 and TGVEG _272240 in toxoDB (www.toxodb.org). Candidate gene fragment encoding entire TgOWP8 proteins were isolated by PCR using primers 5′-TTGGATCCATGAAGGTTGCCAGCGGTTCC-3′ and 5′-TTGCGGCCGCGAGCTTCTTTGCAGGAGGCGG-3′. Partial DNA fragment for production of recombinant protein was isolated using primers 5′-TTGGATCCTGCCAGAAGCACGAGTTCGAA-3′ and 5′-TTGCGGCCGCGAGCTTCTTTGCAGGAGGCGG-3′. Nucleotide sequences around TGVEG_271580-271590 loci and corresponding regions of RH and ME49 strains were determined after PCR amplifications of two overlapping genome by using primers 5′-CGACCTTACGACTCGAGGACCTCTC-3′ and 5′-ATCGAACTCAAATTGAAAGTGACTT-3′, and 5′-CCAGGTCGATCTGGTGGTACTAGCG-3′ and 5′-TAGGCACTCCTGTGTCGACGCTGCC-3′. Structural and transcriptional data of putative *T. gondii* oocyst wall proteins are shown in Table 1. Amino acid sequence alignment was performed by using ClustalW (http://www.ebi.ac.uk/clustalw).

2.7. Cloning and expression of T. gondii oocyst wall recombinant proteins in E. coli

Genomic DNA was extracted from *T. gondii* PLK strain tachyzoites by phenol/chloroform extraction followed by ethanol precipitation. After centrifugation, pellet was dissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and used as a template to isolate recombinant oocyst wall DNA fragment by polymerase chain reaction (PCR) using the specific primers described above. PCR reaction was performed in 50 μl volume containing 1 × buffer, 0.2 mM dNTPs, 2.5 U Taq

Table 1
Summary of structural and transcriptional data on putative *T. gondii* oocyst wall proteins and genes.

	Gene/protein ID (ToxoDB)	Protein name (this study)	Chromosomal location	Protein length	Leader peptide	ESTs		
						Oocyst	Tachyzoite	Bradyzoite
1	TGVEG_271580 TGVEG_271,590	TgOWP8	VIII	573	Yes	273	2	0
2	TGVEG_313000	TgOWP9	XI	604	Yes	20	0	0
3	TGVEG_294600	TgOWP10	Ia	565	Yes	58	0	0
4	TGVEG_205090	TgOWP11	VIIa	575	Yes	24	0	0
5	TGME49_272240	TgOWP12	VIII	634	Yes	21	1	0

polymerase, specific primers and template. Reaction condition was 1 cycle at 94 °C for 2 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; and a final 72 °C for 10 min. PCR product was analyzed by 1% agarose gel electrophoresis stained with ethidium bromide and visualized under ultraviolet light. The amplified product was purified by phenol/chloroform extraction followed by ethanol precipitation. After dissolving in TE buffer, DNAs were double digested with restriction enzymes *Bam* HI and *Not* I. The digested product was electrophoresed on 1% agarose gel, purified by a gel extraction kit (Qiagen, USA) according to the manufacturer's instructions and subcloned into the pGEX6P-2 (GE Healthcare Bio-Sciences). Recombinant protein was expressed as a GST fusion in *E. coli* BL21. The transformed bacteria were centrifuged and lysed by a combination of detergent Triton X-100, lysozyme (20 μg/ml) and sonicated for 10 min. The suspension was purified in Glutathione sepharose (GE Healthcare) for overnight and after centrifugation, the supernatant containing purified recombinant proteins were kept at − 20 °C until further use. The amount of recombinant protein was evaluated using both SDS–PAGE and the Coomassie protein assay reagent kit using BSA as a calibration standard according to the manufacturer's protocol (Pierce Biotechnology, Inc., USA).

2.8. Generation of polyclonal anti-TgOWP8 antiserum

The polyclonal anti-TgOWP8 antiserum was produced in BALB/c mice. Mice immunization was performed as described previously [28]. In brief, mice were immunized subcutaneously at three different inoculation sites with 10 μg of recombinant *Tg*OWP8 protein emulsified with an equal volume of Freund's complete adjuvant. Two weeks later, mice were immunized with the same dose of antigen emulsified with Freund's incomplete adjuvant. Another dose of antigen was emulsified with incomplete adjuvant and given on day 28. Seven days later, mice were euthanized by isoflurane anesthesia and blood was collected for serum extraction. The reactivity of collected serum was tested using Western blotting.

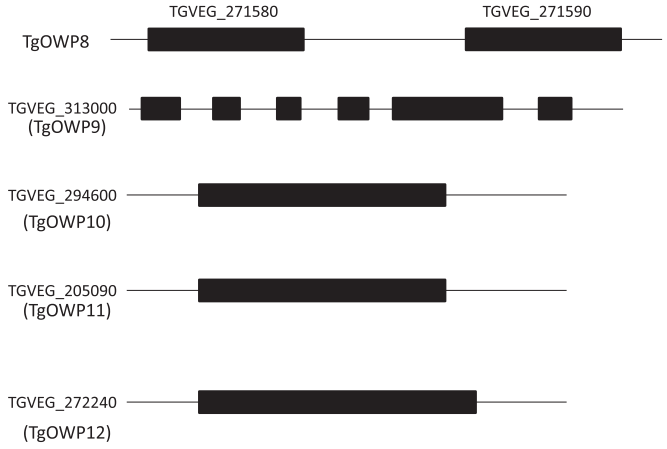

Fig. 1. Schematic diagram showing the loci of the oocyst wall candidates.

2.9. Expression of TgOWP8 in 293T mammalian cells

The human embryonic kidney-derived 293T cells were transfected with *Tg*OWP8 entire coding region cloned into pcDNA6/V5-HisC or empty vector as a control using the calcium precipitation method as previously described [29]. Cells were harvested 48 h after transfection and lysed with RIPA buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 0.25 mM sodium deoxycholate, 0.1% (w/v) Triton X-100, 1% (w/v) Nonidet P-40). After centrifugation, the extracted proteins of cell lysates were used as an antigen to carry out Western blot experiments.

2.10. Western blot analysis

Samples were dissolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 140 mM 2-mercaptoethanol, 10% glycerol, 0.02% bromophenol blue) heated at 95 °C for 5 min and separated on a 12% polyacrylamide gel. All separated proteins were electrically transferred onto polyvinylidene fluoride (PVDF) membranes (Immobilon-P Transfer Membrane, Millipore, Billerica, MA, USA) using a Western blot apparatus (HorizeBlot Type AE-6677, ATTO Bioscience- & Biotechnology, Tokyo, Japan). After blocking for 1 h in phosphate-buffered saline containing 1% skimmed milk (PBS-SM), membrane was probed with mouse serum immunized by TgOWP8, diluted at 1:500 with PBS-SM for one hour. The membrane was washed 3 times for 10 min with phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and then probed with horseradish peroxidase (HRP)-conjugated anti-mouse IgG diluted at 1:2000 in PBS-SM for one hour at RT. After washing with PBS-T, membranes were immersed in the detection solution (0.1 M Tris, pH 8.5, 1.25 mM luminol acid, 0.2 mM coumaric acid, 0.075% H_2O_2) and exposed to X ray film. Prestained molecular mass standards (GeneDireX, Biospeed, USA) were used.

2.11. Indirect immunofluorescence antibody assay (IFA)

Oocysts recovered from cat feces were purified using Diethyl Ether followed by Histopaque 1077 density gradient centrifugation (Sigma) in order to remove debris. The purification was performed as previously described [30,31] and according to manufacturer's instructions with minor modifications. Oocysts were washed three times with distilled water in order to remove 2% H_2SO_4 and resuspended in 15 ml PBS. To cleanse the oocysts and get rid of lipids in fecal samples, five milliliters of Diethyl Ether were added followed by gentle mixing and centrifugation at 1500 × *g* for 10 min at 4 °C. Pellets were resuspended in 3 ml PBS and transferred into 15 ml tube containing 3 ml of Histopaque 1077, then centrifuged at 1500 × *g* for 20 min at 4 °C. The lower layer was harvested, washed once with 10 ml PBS and resuspended in 0.5 ml PBS. For preparation of antigen, the purified oocysts were air-dried on glass slides and treated by different fixation and permeabilization protocols (Table 3). Slides were then washed with PBS (2 times with 5 min intervals), after drying, slides were probed with anti-TgOWP8 antibodies, diluted at 1:100 in 3% BSA/PBS and incubated for one hour in room temperature. Slides were washed three times in PBS with 5 min

Table 2
Amino acid sequence identities (%).

	TgOWP8	TgOWP9	TgOWP10	TgOWP11	TgOWP12
TgOWP8					
TgOWP9	29				
TgOWP10	70	29			
TgOWP11	37	28	37		
TgOWP12	61	31	58	37	

intervals; air-dried and secondary antibody (IgG anti-mouse conjugated with Alexa Fluor 488, Invitrogen) was added at dilution of 1:500 and incubated in same conditions. After washing and drying, slides were mounted with Mowiol (4.8 g Mowiol, 12 g glycerol in 50 mM Tris HCl, PH 8.5) and examined on a Leica TCS NT Confocal Laser Scanning Microscope (Leica Microsystems, Wetzlar, Germany) using the appropriate settings. Green fluorescence and differential interference contrast (DIC) images were recorded using the Leica PowerScan software.

Testing the cross reactivity of anti-TgOWP8 antibodies with *T. gondii* tachyzoites and bradyzoites was performed by indirect immunofluorescence as described previously [32] with some modifications. Glass coverslips with confluent monolayers of HFF cells were infected with *T. gondii* (ME49 strains) tachyzoites and incubated in a CO_2 incubator for 24 h at 37 °C. For preparation of bradyzoites, the differentiation was induced as described in section 2.5. Both tachyzoites and bradyzoite cultures were used as antigens. After washing 3 times with PBS, cells were fixed and permeabilized with 4% formaldehyde and 0.2% Triton X-100 in PBS (pH 7.2) for 15 min. Cells were then washed 3 times with PBS and blocked for 30 min in 3% bovine serum albumin (BSA) in PBS. Staining was performed by incubating coverslips with anti-TgOWP antibodies produced in mice and diluted at 1:500 in 3% BSA/PBS along with rabbit anti-inner membrane complex (IMC1) antibodies to outline the tachyzoites. While, rabbit anti-BAG1 antibodies and *Dolichos biflorus* lectin (DBL) were used to show the bradyzoite stage. After washing 3 times with PBS, coverslips were incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG and Alexa Fluor-488 conjugated goat anti-mouse IgG (Invitrogen) diluted at 1:1000 in 3% BSA/PBS. Coverslips were washed twice in PBS and once in distilled water. Mowiolwas used as a mounting medium. Samples were examined on a Leica TCS NT Confocal Laser Scanning Microscope (Leica Microsystems, Wetzlar, Germany) using the appropriate settings. Green and red fluorescence and differential interference contrast (DIC) images were recorded using the Leica PowerScan software.

3. Results

3.1. Identification of novel candidate genes encoding oocyst wall proteins

T. gondii oocyst wall proteins TgOWPs (from 1 to 7) were previously identified and characterized based on homology search against COWP [21]. In addition, *Cryptosporidium* oocyst wall proteins (COWPs) were known to have type I repeat between periodical cysteine residues

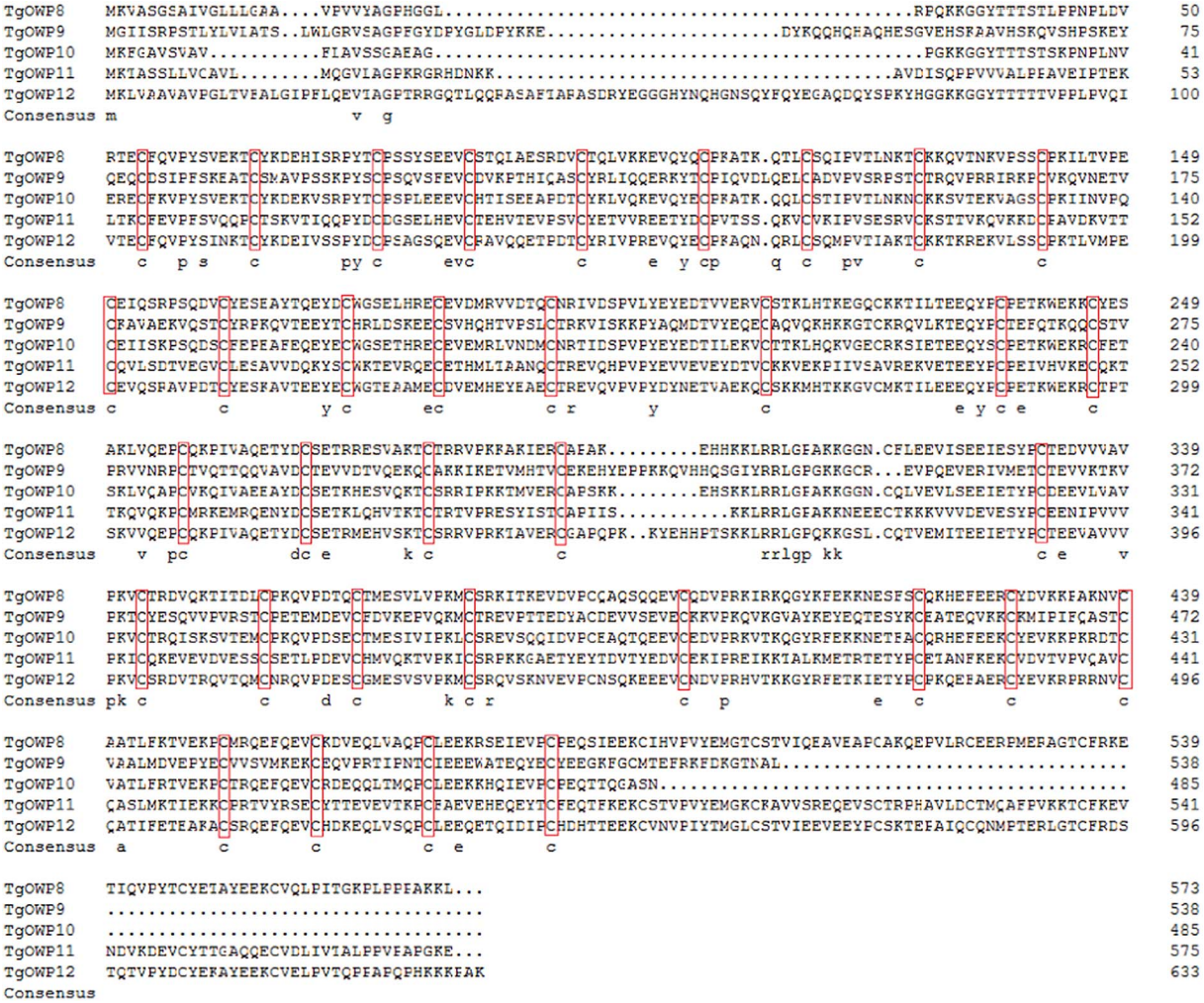

Fig. 2. Alignment of amino acids sequence of *T. gondii* oocysts wall proteins. Conserved amino acids including the cysteine residues shown in figure and cysteine amino acids are marked by red box. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

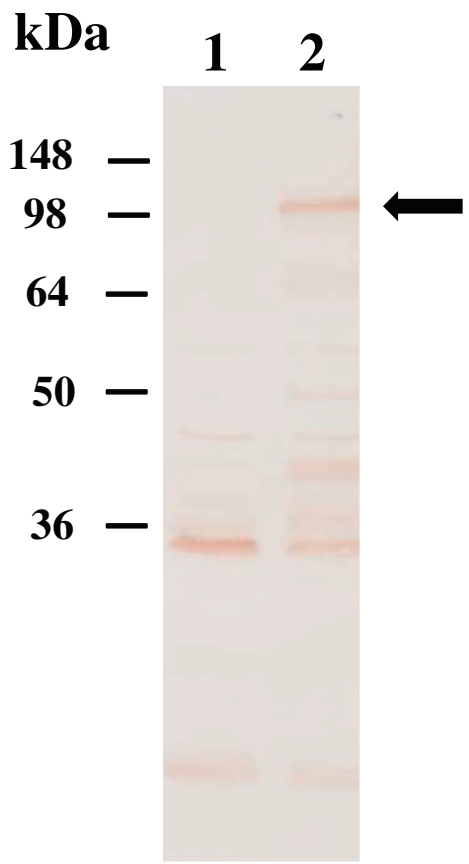

Fig. 3. Western blot analysis. Testing antibodies production of anti-TgOWP8. Lane 1 represent (negative control). Lane 2 showed the specific antibodies reaction observed at 98 kDa (black arrow). The analysis was performed by applying an equal amount of 293T mammalian cells lysates transfected with pcDNA6/V5-HisA vector as a negative control (lane 1) and pcDNA6/V5-HisA encoding TgOWP8 as the tested protein.

appear every 10–12 amino acids were investigated by Templeton et al. [20]. In current study, we searched EST database of *T. gondii* based on two criteria. First one is the existence of periodical cysteine residues and second, is the presence of EST clones specifically derived from sporulated and partially sporulated oocysts of VEG strain cDNA library. In our search, we focused on the identification of five candidate genes corresponding to TGVEG_271580-TGVEG_271590, TGVEG_313000, TGVEG_294600, TGVEG_205090 and TGVEG_272240 (ToxoDB) that herein named as TgOWPs 8, 9, 10, 11, and 12 respectively (Table 1). Regarding TgOWP8, the nucleotide sequence around TGVEG_271580-TGVEG_271590 loci and corresponding genomic regions of other strains seemed to be incorrect in ToxoDB. Therefore we amplified those regions by PCR and sequenced. The result revealed that TGVEG_271580 and TGVEG_271590 are almost identical (1717/1722 nucleotides and 572/573 amino acids identities) and located as a tandem separated with 1.8 kbp intervals (Fig. 1, Supplementary Figs. 1 and 2). In addition, there was no significant sequence variation of this region among RH, ME49 and VEG strains (Supplementary Figs. 1 and 2).

Encoded proteins of novel OWP candidates have high homology to each other as shown in Table 2. However, low homology with COWPs and the previously reported seven TgOWPs [21]. The identified members of TgOWP family displayed an N-terminal leader peptide sequence. Their protein size is ranging from 565 to 634 amino acids. Encoding gene locations are distributed on several chromosomes with the exception of TgOWP8 and 12 that are located on same chromosome number VIII (Fig. 1 and Table 1).

Although TgOWPs 8 to 12 are not analogous to COWPs in their structure, all of them shared cysteine repeats character. In addition, none of these members has histidine-rich amino acid sequence. Transcriptional data integrated in ToxoDB revealed absence of mRNA expression in the tachyzoite stage for TgOWPs with the exception of TgOWPs 8 and 12 that represented by two and one ESTs respectively (Table 1) whereas, all five members were not present at all in the EST dataset from *in vivo* bradyzoites. The whole transcriptome analysis deposited in ToxoDB revealed up-regulated expression of TgOWP 8 to 12 genes during oocyst sporulation. According to deposited genomic sequences in Toxo DB, TgOWPs 8, 10, 11 and 12 have one exon while TgOWP 9 has 6 exons. The predicted amino acid sequences of TgOWP from 8 to 12 are 573, 604, 565, 575 and 634 respectively (Table 1). Amino acid sequence alignment between the five TgOWPs showed an average identity of 50.53% (Fig. 2 and Table 2) accompanied by the presence of the cysteine repeats.

3.2. Testing anti-TgOWPs antibodies production

Because we could not obtain enough amounts of oocysts for western blot analysis, we decided to use mammalian expressed recombinant protein as an antigen for western blot analysis. Here, we cloned C-terminal ORF region encoding TgOWP8 and used for recombinant protein production in *E.coli*. Entire coding region of TgOWP8 was cloned for expression in 293T cells.

Anti-TgOWP8 antibodies showed a specific signal at approximately 98 kDa (Fig. 3), which did not match the predicted size (65 kDa). This finding may be explained by protein modification that occurred within 293T cells. After confirming specific reaction, IFA was performed to assess the localization. The antibody revealed specific staining signal localized to the oocyst wall (Fig. 4).

3.3. Fixation of T. gondii oocysts

In this study prior to IFA staining, we applied different protocols to fix *T. gondii* oocysts that were harvested and purified from infected cats feces (Table 3). Cats were experimentally infected with bradyzoite cysts taken from brains of mice infected with low virulent type II strain. The idea behind trying different methods and reagents for fixation was to enhance the permeabilization of oocyst walls and consequently improve access of antibody to epitope, antigen-antibody binding and fluorescence signals. We classified these methods to three categories depending on type of used fixative reagent. In the first category (methods I, II and III), we used chemicals only for fixation, either absolute methanol or acetone or combination of both. Second category was depending on detergent (Triton X-100) that was used either alone or combined with acetone (methods IV and V), while, in third category chemicals were used followed by autoclaving (methods VI). In this method, oocysts were fixed on glass slides and combination between absolute methanol and acetone was applied followed by autoclaving at 121 °C in 10 mM citrate buffer, pH (6.0) for 15 min. This protocol provided the most satisfactory results for fixation of oocysts plus the highest antigenicity. Therefore, we applied method (VI) in all experiments to test the antigen-antibody reaction of oocysts wall and anti-TgOWP antibodies (Fig. 4). Other methods, the antigenicity was weak and reflected by poor intensity of fluorescence signal (Fig. 4D and data not shown).

3.4. Testing the specificity of anti-TgOWP8 antibodies to oocyst wall

Cross reactivity of anti-TgOWP8 antibody with either *T. gondii* bradyzoites or tachyzoites was tested. *In vitro* bradyzoite differentiation was induced by growing on HFF under low serum (1%) and high pH (8.0) conditions lacking CO_2. Anti-inner membrane complex antibodies were used to define *T. gondii* tachyzoites (Fig. 5, row A). While, bradyzoite cysts formation was confirmed by IFA using *Dolichos biflorus* lectin (DBL) and anti-BAG1 antibodies as specific bradyzoite markers (Fig. 5, rows B and C). Clearly, anti-TgOWP8 antibodies were not reactive with either ME49 tachyzoites or *in vitro* induced bradyzoites cyst

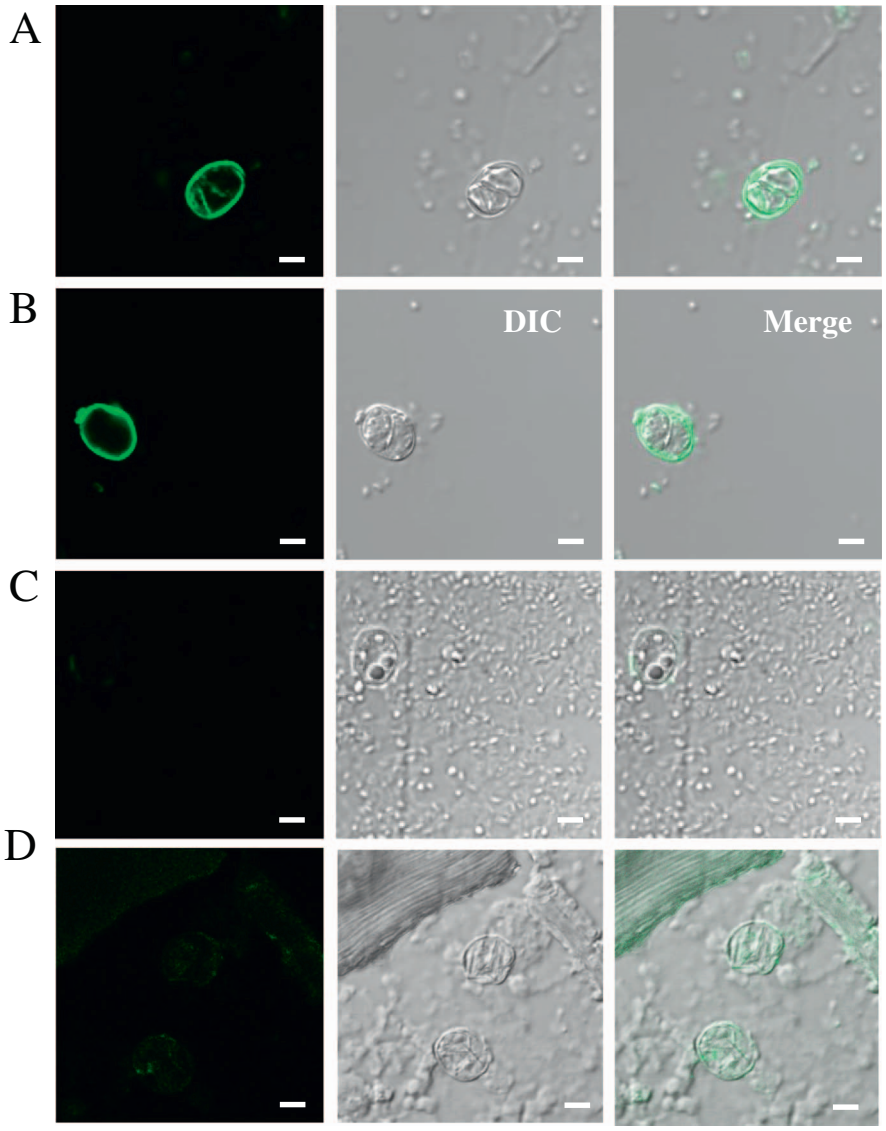

Fig. 4. IFA. Detection of specific reaction and localization of identified TgOWP in *T. gondii* oocyst wall. Fixation protocol VI was used. *T. gondii* oocysts probed with anti-TgOWP8 (A) as tested antibodies. Anti-TgOWP7 antibodies (B) as a positive control, normal mouse serum an a negative control (C). Staining with anti-TgOWP8 antibody using fixation protocol III was also shown as a negative result (D). Stained oocysts wall (left panel), differential interphase contrast, DIC (middle panel) and merge (right panel) (40 ×). Scale bars are 5 μm.

walls (Fig. 5 left panel A, B and C).

4. Discussion

Oocyst of *T. gondii*, the worldwide protozoan, plays a central role in the epidemiology of toxoplasmosis. Once oocyst is sporulated, becomes highly infectious and if ingested by a host, new infection will be initiated. Owing to the durability of wall, that confers resistance to both harsh environmental conditions and chemical disinfectants that maintains the integrity of oocyst and consequently, their potential infectivity to both humans and animals [18]. Previous investigations have mentioned that oocyst wall proteins play an important role as a structural component of oocyst [21,23,18]. Therefore, the oocyst wall represents an interesting challenge for molecular analyses of its composition. Progress has been made in this field targeting *Coccidian* oocyst wall formation in order to understand its structure and function [33–38]. Although much emphasis was given to the *Eimeria* species [39,40], attention was also paid to analyze *T. gondii* oocyst wall through proteomic description of oocyst walls, sporocysts and sporozoites [23].

For long time it had been thought that *T. gondii* oocyst wall is similar to that of *Cryptosporidium.* However, it was proved that *Eimeria* is an alternate for *T. gondii* because it has more similarity to *T. gondii* rather than *Cryptosporidium*. Oocysts of *T. gondii* and *Eimeria* are biologically similar, walls of their oocysts and sporocysts are autofluorescent in UV light due to presence of tyrosine rich proteins that form dityrosines [23]. However, *T. gondii* oocyst walls contain much cysteine's that are homologous to the most abundant *Cryptosporidium* oocyst wall proteins, which are called COWPs [21]. On other hand, *Cryptosporidium* oocyst wall owns histidine rich proteins that are lacking in *T. gondii*. *Cryptosporidium* oocyst wall protein-1 (COWP1) is localized to the inner oocyst wall and characterized by a distinctive repetitive architecture named Types I and II repeats based on periodic cysteine-rich motifs that arranged in tandem manner [41]. In addition, another eight members of COWP were also identified through complete genomic sequencing of *C. parvum*, which shares the same characteristics of COWP1 [42]. Collectively COWPs are known to be expressed during oocyst formation and have a contribution to the rigid oocyst wall of coccidian in both *Cryptosporidium* and *T. gondii* [20].

Based on the idea that structurally similar components may contribute to the same functions, *T. gondii* OWPs play a critical role in the formation of oocyst wall as COWPs do. Previously, Possenti et al. [21] identified and analyzed seven members of OWP family; the authors cloned 3 members of TgOWP1, 2 and 3 and confirmed their specificity to oocyst wall. However, much work is needed to extend our knowledge about the structure and function of *T. gondii* OWPs. Therefore, in this study our attention was directed to identify new oocyst wall proteins

Table 3
Methods of fixation and permeabilization of oocysts derived from experimentally infected SPF cats.

Category	Methods	Fixing reagent	Time of fixation	Blocking
1	I	Acetone	5 min at − 20 °C	3% BSA in PBS for 15 min
	II	Methanol	15 min	
	III	Methanol + acetone	Methanol (15 min) followed by acetone (5 min) at − 20 °C	
2	IV	6% Formaldehyde/0.2% Triton X-100	15 min	
	V	6% Formaldehyde/0.2% Triton X-100 + acetone	Formaldehyde/Triton (15 min) followed by acetone (5 min) at − 20 °C	
3	VI	Methanol + acetone + autoclave in 10 mM citrate buffer	Methanol (15 min) followed by acetone (5 min) at − 20 °C then autoclave at 120 °C for 15 min	

that may aid in elucidation of the function of these constituents and their role in the characteristic durable oocyst wall. The other aim of this study was to advance the detection of *T. gondii* oocyst in environmental samples.

At this point, this study describes the identification of five candidates of *T. gondii* oocyst wall proteins (TgOWP8-12). We searched the EST database to identify candidate's that are specific to oocyst wall. TgOWPs are members of family D proteins of *T. gondii*. Here, we are focusing on five new members of this family in VEG strain. These proteins are sharing high homology to each other in the overall structure and characterized by presence of periodically appeared cysteine residues. They also have paralogs in other *T. gondii* strains (GT1 and ME49) and orthologs in *Neospora caninum* (*N. caninum*). Moreover, TgOWPs 8, 10 and 12 share also orthologs with *Eimeria* species. This feature may enrich the evolutionary similarity between *T. gondii* and *Eimeria* and may help in the prediction of function of newly identified genes [23].

In this study, recombinant protein of TgOWP8 was produced. Specificity to *T. gondii* oocyst wall was confirmed by IFA. Thus, in order to analyze the specificity of anti-TgOWP antibodies to *T. gondii* oocyst wall, we orally infected two SPF cats with tissue cysts of low virulent *T. gondii* strain, oocysts shed in their feces. Subsequently, oocysts undergone purification process to get rid of debris in fecal samples and obtain a purified antigen for IFA reaction. Different methods for fixation and permeabilization of *T. gondii* oocysts were performed as described in results and after several trials, method VI (Methanol followed by

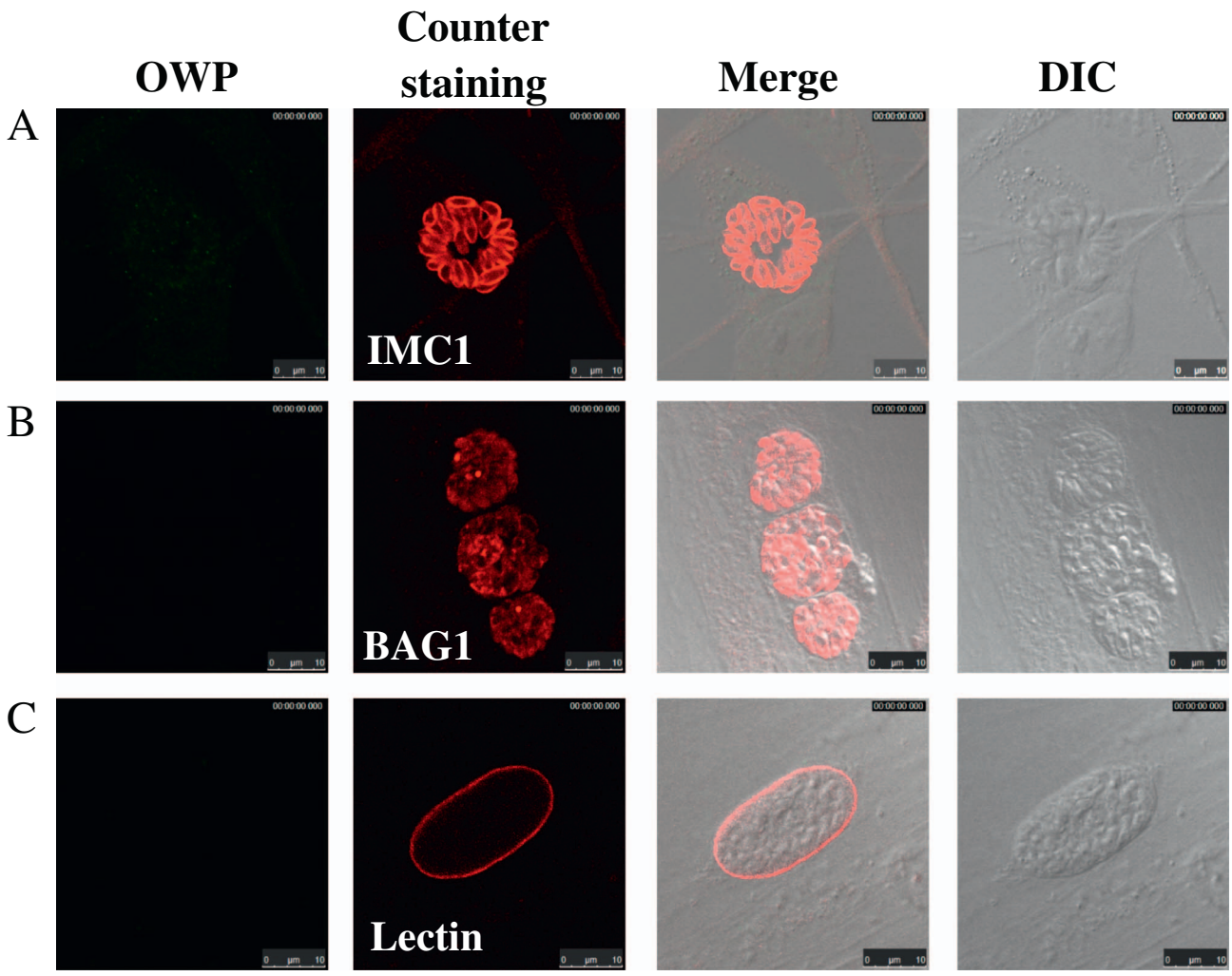

Fig. 5. IFA. Testing the reactivity of anti-TgOWP antibodies produced in mice to either *T. gondii* tachyzoites (5A) or bradyzoite (5B and 5C). Anti-TgOWP antibodies (green) (left Panel). Counter staining performed by rabbit anti-*T. gondii* IMC1 antibodies (5A) (red) for *T. gondii* tachyzoite. For detection of bradyzoites, rabbit anti-*T. gondii* BAG1 antibodies (5B) and lectin staining (5C) (red) were used. Scale bars are 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

acetone and autoclave, Table 3) had achieved the best fixation with minor loss of oocysts and improved antigenicity under our experimental conditions. While, in the other methods, the capacity of antigen to bind antibodies was compromised that reflected in high background around the oocysts in negative controls (data not shown) indicating ineffective permeabilization (Fig. 4).

In reference to the possible expression of TgOWP genes in tachyzoite or bradyzoite stage, some negative results here have important implications for interpreting the specificity of oocyst wall proteins. Reactivity of these proteins toward both tachyzoites and induced bradyzoites in *in vitro* culture was tested (Fig. 5), antibodies raised against TgOWP showed highly specificity to oocysts walls and not to the bradyzoite cyst walls or tachyzoites. These finding can be explained by presence of differences between oocyst and bradyzoite cyst walls in their composition [43].

Cross-reactivity against oocysts of other related parasite should be considered. Several orthologs were found in *Neospora*, *Hammondia*, *Sarcocystis* and *Eimeria* species by ToxoDB database search. Highest homology of amino acid sequence was shown in *N. caninum* ortholog (NCLIV_035420, 92% identity). Therefore, anti-TgOWP8 antibody is quite likely to react with *N. caninum* oocyst. Specificity and cross-reactivity with oocysts against other parasites should be examined for future.

In conclusion, *T. gondii* is a significant water and food-borne pathogen of humans as well as animals. The durability of oocyst wall allows the global protozoan to survive under extreme environmental conditions and resist the use of common disinfectants. Adding, few studies were directed toward studying the oocysts wall proteins due to technical difficulty encountering this work. This study is considered a step in the molecular analysis of the oocyst wall composition through the identification of five new genes specific to the oocyst wall. Hopefully, our work on these candidates will be promising toward improvement of the detection of *T. gondii* oocysts in the environment and in future for the discovery of new strategies to eliminate this parasite from the environment.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.parint.2017.05.009.

Declaration of interest

All authors disclose that there was no conflict of interest of any type from the beginning of the work to the submission of manuscript.

Acknowledgements

This study was supported in part by Grant-in-Aid for Scientific Research, Japan Society for the Promotion of Science (JSPS) (No. 25450419). The first author is supported by foreign researcher fund, Obihiro University, Obihiro, Japan.

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