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IDENTIFICATION OF A SPOROZOITE-SPECIFIC ANTIGEN FROM *TOXOPLASMA GONDII*

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Abstract

Reduction of risk of human and food animal infection with *Toxoplasma gondii* is hampered by the lack of epidemiological data documenting the predominant routes of infection (oocyst versus tissue cyst consumption) in horizontally transmitted toxoplasmosis. Existing serological assays can determine previous exposure to the parasite, but not the route of infection. We have used difference gel electrophoresis in combination with tandem mass spectroscopy and Western blot to identify a sporozoite-specific protein (*Toxoplasma gondii* embryogenesis-related protein, TgERP) which elicited antibody and differentiated oocyst- versus tissue cyst-induced infection in pigs and mice. The recombinant protein was selected from a cDNA library constructed from *T. gondii* sporozoites, and this protein was used in Western blots and probed with sera from *T. gondii* infected humans. Serum antibody to TgERP was detected in humans within 6–8 mo of initial oocyst-acquired infection. Of 163 individuals in the acute stage of infection (anti-*Toxoplasma* IgM detected in sera, or <30 in the IgG avidity test), 103 (63.2%) had detectable antibodies that reacted with TgERP. Of 176 individuals with unknown infection route and in the chronic stage of infection (no anti-*Toxoplasma* IgM detected in sera, or >30 in the IgG avidity test), antibody to TgERP was detected in 31 (17.6%). None of the 132 uninfected individuals tested had detectable

antibody to TgERP. These data suggest that TgERP may be useful in detecting exposure to sporozoites in early *Toxoplasma* infection and implicates oocysts as the agent of infection.

Toxoplasmosis, caused by *Toxoplasma gondii*, is one of the most common parasitic infections of humans and other warm-blooded animals. It has been found worldwide, and nearly one-third of humanity has been exposed to the parasite (Dubey and Beattie, 1988; Dubey, 2009). In most adults, infection rarely produces severe clinical manifestations, however there have been recent reports of focal ocular toxoplasmosis in otherwise healthy adults (Aramini et al., 1998, 1999; Jones et al., 2006; Phan et al., 2008a; Wallace and Stanford, 2008). Congenital infection usually occurs when a woman becomes infected during pregnancy and transmits the pathogen to the fetus. Congenital infections acquired during the first trimester are more severe than those acquired in the second and third trimesters (Desmonts and Couvreur, 1974; Remington et al., 2005). Congenital infection can cause a spectrum of disease syndromes ranging from chronic infection with inapparent clinical symptoms, to blindness and mental retardation in children, to stillbirth. Devastating disease can result in immunosuppressed patients, such as those given large doses of immunosuppressive agents in preparation for organ transplants and in those with acquired immunodeficiency syndrome (AIDS). The immunosuppressed host may die from toxoplasmosis unless treated (Hill et al., 2005). Besides congenital infection, humans become infected through ingestion of tissue cysts in undercooked or uncooked meat, or by ingesting food or water contaminated with sporulated oocysts from infected cat feces (Dubey and Beattie, 1988; Cook et al., 2000; Lopez et al., 2000; Tenter et al., 2000; Jones et al., 2009). Food animals, such as pigs, become infected by the same routes, resulting in meat products containing tissue cysts which could infect consumers (Smith 1993; Dubey et al., 1995; Dubey et al., 2005). There are no tests which can differentiate between oocyst (the stage excreted in cat feces) ingestion and tissue cyst (the stage found in meat) ingestion as the infection route, making epidemiological studies which could lead to the development of strategies to reduce infection in humans and food animals difficult. In this study, we describe the first identification of a sporozoite-specific protein, TgERP, that elicits antibody in *T. gondii* infected pigs, mice, and humans. Presence of this antibody differentiates infection via sporulated oocysts versus tissue cysts in pigs, and clearly identifies people infected through ingestion of oocysts within 6–8 mo of initial exposure. Human sera from 2 North American *T. gondii* outbreaks considered to have resulted from oocyst exposure, sera from individuals with acquired infections whose exposure history was not well characterized, and sera from uninfected individuals were tested by Western blot or ELISA using the recombinant TgERP to characterize the antibody response to the protein in these groups.

MATERIALS AND METHODS

Oocyst (sporozoite) production and pig infection

Toxoplasma gondii (VEG strain) oocysts were collected by sucrose flotation from feces of cats fed tissues of mice experimentally infected with *T. gondii*; these procedures have been previously described (Dubey et al., 1970; Dubey and Frenkel, 1976). Oocysts were sporulated in 2% H₂SO₄ while shaking for 7 days at room temperature and were stored at 4°C until used. Ten *T. gondii* seronegative pigs (~50 kg each, 5 mo old, Ernst Farms, Clear Spring, Maryland) were infected *per os* (p.o.) with 1,000 sporulated *T. gondii* VEG strain oocysts. Serum was collected on a weekly basis from each pig by venipuncture for 9 mo. Ten uninfected seronegative pigs were maintained as controls.

Tissue cyst (bradyzoite) production and pig infection

Swiss-Webster mice were orally inoculated with 50 *T. gondii* oocysts. After 60 days, the mice were bled out, and the brains of mice were removed, washed in saline, and homogenized in saline using a micro blender (2.5ml saline/brain). Isotonic Percoll (9:1 Percoll:saline) was added to the homogenate (3:2), mixed well, and centrifuged at 2,600 RPM (2,000g) for 30 min. The top layer of brain tissue and the supernatant were removed, the pellet was resuspended in saline, mixed well by vortexing, and the suspension was filtered through the edge of a 25µm sieve. Saline was used to recover tissue cysts retained on the sieve. Recovered tissue cysts were washed in saline by centrifugation at 2,000 RPM (1,179g) for 10 minutes. Washed tissue cysts (5,000 to each pig) were inoculated directly to 10 pigs p.o. or were treated with 0.25% trypsin for 10 minutes to release bradyzoites from cysts, which were extracted for collection of protein as described below. Serum was collected from each pig on a weekly basis as described above.

Tachyzoite production *Toxoplasma gondii*

VEG strain tachyzoites were produced in the HCT-8 cell line (ATCC #CCL-244). T-75 flasks with a 75% confluent cell layer were seeded with 1×10^5 *T. gondii* tachyzoites and maintained at 37C and 10% CO₂ in high glucose DMEM containing 3% FCS, 50U/ml penicillin, 50µg/ml streptomycin, 2mM glutamine, 100mM HEPES, 1x MEM non-essential amino acids, and 1mM sodium pyruvate. After 7–10 days of culture, as tachyzoites began to emerge from cells, the media was switched to HBSS for 2–3 hr each day, and emerged tachyzoites were collected from the supernatant by centrifugation at $1200 \times g$ for 7 minutes until the cell monolayer was completely disrupted. Collected parasites were washed 2–3 times in HBSS, and forced through a 26 gauge needle to disrupt any collected cells containing parasites. The parasite preparation was then passed through a 5 µm Millex-SV syringe filter (Millipore, Bedford, Massachusetts) to remove cell debris, washed twice in HBSS, centrifuged as above, and were extracted for collection of protein as described below.

Analysis of pig sera

The presence of antibodies to *T. gondii* in experimentally infected pig sera was determined using the modified agglutination test (MAT) and by ELISA as described previously (Dubey et al., 1996; Gamble et al., 2005). For both tests, sera were tested on the day of infection and weekly throughout the course of the experiment. For the MAT, serum samples were tested at doubling dilutions from 1:25 to 1:3,200. Positive and negative control sera diluted from 1:25 to 1:3,200 were included in each test. Sera with a titer of 1:25 or higher were considered positive. Serum antibodies to *T. gondii* were also determined in the pigs using a validated commercial ELISA kit (Safepath Laboratory, Carlsbad, California). This ELISA kit uses formalin-fixed whole tachyzoites as antigen, and has been validated for use with pork samples (Gamble et al., 2005; Hill et al., 2010). Sera were tested at a 1:50 serum dilution, and positive and negative controls provided by the manufacturer were included on each plate. Plates were read at 405 nm using a Vmax ELISA reader (Molecular Devices, Sunnyvale, California). Samples with optical densities greater than 0.200 were considered positive.

cDNA library construction from *T. gondii* sporozoites

For collection of *T. gondii* RNA, DNA, and protein from sporulated oocysts, oocyst walls were first disrupted by treating intact oocysts with 5.25% sodium hypochlorite in water for 30 minutes at room temperature. Oocysts were washed 3–4 times by centrifugation in water to remove the sodium hypochlorite, and were then disrupted by vortexing with 500µm glass beads for 5 minutes. Collection of parasite nucleic acids (sporozoites) and proteins from

sporozoites (as well as tachyzoites and bradyzoites) was accomplished using the TRIZOL reagent followed by sequential precipitation with isopropyl alcohol (RNA), ethyl alcohol (DNA), and isopropyl alcohol in the organic phase (proteins)(Gibco/BRL Life Technologies, Gaithersburg, Maryland) as described (Hill et al., 2001; Hummon et al., 2007) and per the manufacturers instructions (www.invitrogen.com/content/sfs/manuals/15596026.pdf). Proteins were dissolved in 0.1% SDS and concentrations were determined using a modified Bradford assay (BioRad protein assay, Hercules, California). Proteins were then stored in 1% SDS and 8M urea at -20C until used.

cDNA libraries were constructed from excysted sporozoites of *T. gondii* using the Smart cDNA synthesis by long distance PCR protocol in the λ TriplEx vector (Clontech, Mountain View, California). First strand cDNA synthesis was accomplished using 1 μ g total RNA, 1 μ l SMART IV oligonucleotide (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3'), 1 μ l CDS III/3' PCR primer (5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)₃₀N₋₁N-3'; (N = A, G, C, or T; N₋₁ = A, G, or C)) following the manufacturers instructions. Second strand cDNA synthesis used 2 μ l of the first strand cDNA, 2 μ l of the 5' PCR primer, and 2 μ l of the CDS III/3' PCR primer again following the manufacturers instructions. Amplification was accomplished using a Gene Amp 9600 thermal cycler under the following conditions: 95C, 20 sec, then 95C, 5 sec, 68C, 6 min for 20 cycles. Double stranded DNA amplicons were treated with Proteinase K (20 μ g/ μ l), digested with Sfi I restriction enzyme, and fractionated using a CHROMA-Spin-400 column to select larger (>200 kb) cDNAs following the manufacturers instructions. Digested and fractionated cDNA was directionally ligated into the Sfi I-digested, dephosphorylated λ TriplEx2 vector and packaged using Gigapack III Gold packaging extracts (Stratagene/Agilent, Santa Clara, California); titering revealed at least 1×10^6 independent clones in the unamplified library. Plasmids containing cDNA inserts were recovered from the recombinant λ TriplEx phage using XL1Blue cells, and the library was amplified using XL1Blue cells to titers ranging from 10^9 – 10^{10} pfu/ml, and stored at 4C until used.

Gel electrophoresis

Sporozoite proteins were compared to tachyzoite and bradyzoite proteins using 2-dimensional difference in gel electrophoresis (DIGE). Equal amounts of protein extracted from sporozoites, tachyzoites, and bradyzoites as described above were labeled by CyDye DIGE fluors (size and charge matched) and co-separated by isoelectric focusing in the first dimension (pH 4–9), and SDS-PAGE on a single multiplexed gel in the 2nd dimension (Applied Biomix, Hayward, California). After electrophoresis, the gel was scanned using a Typhoon image scanner, revealing the CyDye signals (Cy2 and Cy5) from the individually labeled *T. gondii* stages. ImageQuant software was used to generate the CyDye image, followed by DeCyder software analysis to locate and analyze multiplexed samples in the same gel. Two-dimensional gels resolving tachyzoite, bradyzoite, or sporozoite proteins as described above were subjected to Western blot and screened with swine sera from known oocyst- induced infections to identify sporozoite-specific antigens from *T. gondii*. Electroblothing was carried out on unfixed gels by transfer of proteins onto Immobilon (PVDF) nylon blotting membranes (Millipore, Bedford, Massachusetts) using a Novex gel transfer apparatus (Novex, San Diego, CA) set at 40V for 80 min in 25mM Bicine, 25mM Bis-Tris, 1mM EDTA, 20% methanol, pH 7.2 blotting buffer. For Western blotting, the membranes were rinsed in 50mM Tris buffered, 0.85% saline (TBS) and unbound sites on the membranes were saturated with Detector Block solution (Kirkegaard and Perry, Gaithersburg, Maryland). The membranes were incubated in a pool of porcine sera (diluted 1:500) from 10 pigs with acute oocyst-induced *T. gondii* infection (positive MAT titer of 1:400; pool prepared from sera taken from wk 4 through wk 12 post infection). Horseradish peroxidase conjugated-goat anti-pig IgG (Sigma Chemical. St. Louis, Missouri) was used as

the 2nd step antibody at a dilution of 1:800. Sporozoite, bradyzoite, and tachyzoite proteins recognized by porcine anti-*T. gondii* antibodies were visualized using the 4CN membrane developer kit (Kirkegaard and Perry). Western blot images were captured using the ProExpress proteomics image acquisition system (Perkin Elmer, Boston, Massachusetts); spot matching and image analysis of the 2-D Western blot images was accomplished using the PDQuest software system.

Differentially expressed protein spots in the sporozoite protein sample which were also immunoreactive in Western blots as described above were picked from the 2D gel and identified by peptide fingerprint mass mapping (using MS data) and peptide fragmentation mapping (using MS/MS data). The MASCOT search engine was used to identify proteins from primary sequence databases, and retrieved accession numbers were used to search ToxoDB (<http://www.toxodb.org/restricted/toxoDBblast.shtml>) for the full length sequence (Gajria et al., 2007). Polymerase chain reaction (PCR) primers were constructed from the amino acid sequence derived from the mass spectrometry and database searches (forward: CAA AGG GCT CAT GGA GAG AG; reverse: ACG CGT CTT TTG CTT CGT AT). PCR was performed using the primers listed above and DNA extracted from the sporozoite cDNA library. The amplicons produced were ligated into the pTriplEx2 plasmid vector using 1.5µl of cDNA and following the vector manufacturers instructions, then packaged into the λ-phage vector and titered. Amplicons were sequenced on an ABI Sequencer model 3100. Amplicons were labeled using a non-radioactive digoxigenin (DIG) DNA labeling kit (Roche Applied Sciences) for use in library screening to isolate the full-length gene for protein expression using the kit manufacturers protocol. The sporozoite cDNA library was plated onto LB-ampicillin plates overnight at 37C and transferred to nitrocellulose filters. Filters were placed sequentially on filter paper soaked with 0.5M NaOH, 1M Tris-Cl, and 0.5M Tris Cl/1.25M NaCl, then dried at 80C in a vacuum oven. Filters were probed with the DIG labeled DNA probes described above in SSC hybridization buffer at 45C, and positive clones were detected after fixation and hybridization by an anti-DIG antibody conjugated to alkaline phosphatase. Approximately 20 positive clones were identified and selected. Secondary screening resulted in selection of over 50 positive plaques, and 12 were sequenced to confirm the gene.

The selected clones were diluted in lambda buffer, expanded in XL1 Blue cells, and plated on LB agar at 40C for 5 hr. Expression of the insert was induced in XL1 Blue cells with IPTG-soaked nitrocellulose filters for 4 hr. Filters were removed, blocked with TBS-Tween and 1% gelatin, and immunologically screened using the pig sera from *T. gondii* oocyst-induced infection (described above). Positive results from the immunoscreen confirmed that the clones were reactive with the swine oocyst-induced infection sera. The identified gene was subcloned into the EcoRI/Hind III site of pMal-c2 vector (New England Biolabs, Beverly, Massachusetts) for constitutive protein expression and was expressed as an N-terminal maltose binding fusion protein under the control of the lac repressor. Expression of the fusion protein was induced with IPTG and the protein was purified using an amylose resin column, which binds to the maltose binding protein. The fusion protein was eluted from the column with 10mM maltose, and column fractions were analyzed at 280 nm to determine which fractions contained the fusion protein. The fractions of interest were pooled and concentrated using a centrprep spin column to a minimum of 1mg/ml. The fusion protein was cleaved using Factor Xa, and purified using DEAE-Sepharose ion exchange chromatography. One dimensional gel electrophoresis and Western blotting was carried out on the purified fusion protein using human and pig serum samples essentially as described above for 2nd dimension gels using 4–12% gradient Bis-Tris gels using SDS reducing sample buffer (1M Tris, pH 6.8, 10% SDS, 50% glycerol, 1% 2-mercaptoethanol, and 0.2% bromophenol blue) for solubilization of protein. Goat-anti-pig IgG and Rabbit anti-human

IgG or IgM (all HRP conjugated) were used as the second step antibody in Western blots using pig or human primary sera as probes.

Four month old Swiss-Webster mice (NIH) were separated into 20 groups of 5 each and infected orally with the oocyst (50 mice) or bradyzoite stage (50 mice) of the ME-49 strain of *T. gondii*. Blood was collected from the periorbital plexus and terminally by heart puncture 60 days post infection. Serum was collected by centrifugation and diluted 1:100 before testing individually in Western blots against TgERP (from VEG strain) as described above.

Sources of human serum

Human sera were acquired from sources described below. Prior to receipt, standard serologic testing (the Sabin Feldman dye test, bioMerieux direct agglutination assay, IgM and IgA ELISA, IgM ISAGA) to confirm acute and chronic *T. gondii* infection was conducted as previously described (Remington et al., 2005) on sera from the pregnant Amish women, laboratory workers, NCCCTS mothers and their children, and newborn children. Differential agglutination and avidity assays were also performed for pregnant women in the NCCCTS prior to receipt of sera (McLeod et al., 2006; Remington et al., 2005). Serologic tests using sera from persons in the Atlanta epidemic were as previously described (Teutsch et al., 1979).

Group 1—Six of 23 (26%) laboratory employees were accidentally exposed to oocysts, which resulted in infection with *T. gondii*. These employees had been monitored for >2 yr by the Institutional Occupational Health Management Service, and were known to be seronegative for *T. gondii* before the exposure. The serum was collected from these 6 employees beginning 1 mo after exposure and monthly until 8 mo post infection (PI). Sera from the 17 unexposed workers tested negative for *T. gondii* infection for 3 mo after the exposure event, and subsequently were not tested for antibodies to *T. gondii*. Sera were tested in Western blots as described above.

Group 2—An outbreak of toxoplasmosis occurred in 39 individuals who were frequent visitors to a horse stable in Atlanta, Georgia in 1977 (Teutsch et al., 1979). Thirty-seven of these individuals became ill and tested positive by IFA. Epidemiological investigations revealed that the affected individuals were likely infected by ingestion of aerosolized oocysts from infected barn cats, as no common food sources were identified and meat was ruled out. Eleven of these sera were tested by Western blot for antibodies to TgERP as described above. IgM titers in the 11 sera ranged from 4.2 to >10 (EIA IgM positive = >2.0). Sera were collected between 78 and 149 days after onset of symptoms. Four additional sera of unknown infection date which were unrelated to the stable outbreak were provided with this group of sera. These 4 sera had no detectable IgM titer when tested, but were IgG positive in the dye test and by IFA. These sera were considered to be from chronically infected individuals and were tested by Western blot for reactivity to TgERP in long term infections.

Group 3—Sera from 182 *T. gondii* seropositive Hispanic women of childbearing age (18–43yr) from a highly endemic country were screened for antibodies to TgERP. Initial testing for antibodies to *T. gondii* was performed using the VIR-ELISA, anti-Toxo-IgG (Viro-Immun Labor-Diagnostika GmbH, Germany). Infection dates for these individuals could not be definitively established, however, the avidity index of IgG positive samples was determined using a commercial solid-phase enzyme immunoassay (*T. gondii* IgG Avidity EIA, Ani LabSystems Ltd., Finland). The avidity index was calculated based on titration curves for controls and samples. Results were interpreted as follows: avidity index < 15% is

suggestive of acute infection, 15–30% is suggestive of primary infection during the last 6 mo, and >30% excludes primary infection within the last 3 mo. Testing for reactivity to TgERP was performed using Western blots as described above, and by ELISA. For the ELISA, TgERP (uncleaved with Factor Xa) was diluted to a concentration of 2 µg/ml in 0.1M carbonate buffer, pH 9.6. ELISAs were carried out essentially as described by Gamble et al. (2000). Reference positive and negative controls were established using a pool of the 6 seropositive samples (P1) and a pool of 6 seronegative samples (P2) from Group 1. ELISA ODs were determined for 20 replicates of P1 and P2, and a mean OD was determined for each (P1r and P2r). P1 and P2 were included on each plate, and a corrected OD value was calculated for each sample using the formula as described: $\text{corrected OD} = (\text{OD sample} - \text{ODP2}) \times \text{ODP1r} / (\text{ODP1} + \text{ODP2r})$. A positive cut-off was established (OD 0.300) as $\text{ODP2r} + 3$ times the standard deviation from ODP2r. Plates were read at 405 nm using a Vmax ELISA reader.

Group 4—Within an Amish family of 8 persons from Lancaster County, Pennsylvania, an infant was born with congenital toxoplasmosis and was enrolled in The National Collaborative Chicago-based Congenital Toxoplasmosis Study (NCCCTS). As a result, the entire family submitted sera for testing to determine if other family members were infected with *T. gondii*. All family members, with the exception of a 2-yr old sibling of the congenitally infected child, were seropositive. While the family's meat was always well cooked, cats were observed in their vegetable garden, hen house, and near a sandbox in which the children played. No data was otherwise available to determine how the family became infected. Sera were obtained within 3 mo of the birth of the congenitally infected child. These sera were stored at –80C and tested in Western blots for the presence of antibodies to the recombinant TgERP.

Group 5—Seventy-six acutely infected mothers in the NCCCTS who transmitted *T. gondii* to their fetuses *in utero* provided their sera for testing near the time their infected child was born between 1981 and 1999. These families, and diagnosis of acutely acquired and congenital infection in their children has been previously described in other publications of the NCCCTS (Swisher et al., 1994; McAuley et al., 1994; Roizen et al., 1995; Mets et al., 1996; Patel et al., 1996; Brézin et al., 2003; Boyer et al., 2005; McLeod et al., 2006; Remington et al., 2005; Roizen et al., 2006; Arun et al., 2007; Benevento et al., 2008; Jamieson et al., 2008; Phan et al., 2008a, b). Three of the mothers described above acquired acute toxoplasmosis during an epidemic of toxoplasmosis in Victoria, British Columbia, Canada that occurred from 1994 to 1995. This epidemic was attributed to oocysts from feral cats contaminating the drinking water of the city of Victoria, however, 1 of the 3 mothers had eaten rare meat during this time (Isaac-Renton et al., 1998).

Group 6—Sera from 114 pregnant women from the Lancaster County Amish community, many with similar risk factors as the Amish family described above, were screened for IgG and IgM antibodies (Remington et al., 2005) to *Toxoplasma* during their pregnancies. Fifty-five (48%) had no antibody (these were considered seronegative controls), 59 (52%) had IgG antibody only (these were chronically infected persons), and 1 of those with IgG antibody also had IgM antibody to *T. gondii*. No specific infection date could be determined for these individuals.

Sera from the 55 seronegative Amish persons (Group 6), 60 seronegative persons involved in studies of gastrointestinal nematode infection or inflammatory bowel disease, and 17 sera from the *T. gondii* negative laboratory personnel (Group 1), were used as negative controls to validate the results of the Western blots. All sera used in the study were coded and tested in a blind manner.

RESULTS

Reproducible 2-dimensional electrophoresis protein maps were produced using proteins isolated from *T. gondii* sporozoites, tachyzoites, and bradyzoites. DIGE analysis revealed >20 protein spots which were unique to the sporozoite protein sample as compared to the bradyzoite or tachyzoite protein samples (Fig. 1). Western blot analysis of identical 2-D gels probed with sera from the 10 pigs infected p.o. with ~1,000 oocysts revealed a protein spot in the 10–12Kda range matching a sporozoite-specific spot appearing in the DIGE gel from the sporozoite preparation; this spot was excised from the DIGE gel for further analysis (Fig. 2). This spot was not seen on Western blots from the sporozoite preparation probed with sera from pigs with tissue cyst induced infection, and was not seen in DIGE resolved protein preparations from tachyzoites or bradyzoites. Analysis of the selected spot by peptide fingerprint mass mapping and peptide fragmentation mapping provided a partial amino acid sequence for this protein. Searching the non-redundant database with Mascot and ToxoDB identified the antigen as an 11Kda sporozoite protein related to embryogenesis (TgERP, Fig. 3A). Expression and purification followed successful subcloning into the pMal 2-plasmid expression vector (Figure 3B, C). Western blots were carried out to assure continued serological reactivity of the expressed protein. The TgERP was initially probed in Western blots with sera from pigs with *T. gondii* oocyst-induced infection (sera collected 4–12 wk PI) to confirm serological reactivity. Results indicated continued reactivity of TgERP with pig sera derived from animals infected with *T. gondii* oocysts, and no reactivity with sera from pigs with tissue cyst induced infections or uninfected control pigs (Fig. 3D). Antibodies were detectable in pigs with oocyst induced infection for 6–8 mo after initial infection. Serum collected 60 days PI from mice infected with bradyzoites of the ME-49 strain of *T. gondii* showed no reactivity to the recombinant protein in Western blots, while serum from mice infected with the oocyst stage of the parasite strongly recognized the protein (Fig. 3F).

Reactivity of TgERP with human sera was tested by Western blot initially with sera from people in Group 1 described above, since 6 members of this group of serologically monitored laboratory workers was known to have become infected with *T. gondii* oocysts on a specific date. The 6 infected individuals developed IgM and IgG antibodies which recognized TgERP within 1 mo of infection; IgG antibodies were detectable in the individual sera for 5–6 mo, and weakly in 1 individual for 8 mo (Table 1).

Sera collected from 11 visitors to the riding stable in Atlanta (Group 2) were screened for anti-TgERP antibodies using Western blots. Nine (82%) of 11 individuals thought to have been infected with oocysts had detectable antibodies to TgERP. Of the 2 patients that did not have detectable antibody to TgERP, the serum sample had been collected from 1 patient approximately 5 mo after the initial detection of the outbreak. No detectable antibody to TgERP was found in the 4 sera from chronically infected individuals included with this group.

Sera from 182 *T. gondii* seropositive Hispanic women of childbearing age (Group 3) were tested for the presence of antibody to TgERP by Western blot and ELISA. The IgG avidity index suggested that, overall, 63% of the infections (114) were classified as chronic (avidity index >30%), while 37% (68) were classified as recent or acute (avidity index <30%). Overall, 60 of the 182 tested sera had detectable antibody to TgERP in the Western blot (33%), while 122 were seronegative. Twenty-nine of the 60 Western blot positive sera were classified as acute in the IgG avidity test (48%), while 31 were classified as chronic (52%). In the ELISA, 44 of the 182 tested sera had detectable antibody to TgERP (24%). Twenty-three of the 44 ELISA positive sera were classified as acute in the IgG avidity test (52%), while 21 of 44 sera were classified as chronic (48%).

Six (75%) of the 8 Amish family members (Group 4) had detectable antibodies to TgERP in Western blots. The congenitally infected child and a seronegative 2 yr old sibling did not have detectable antibody to the recombinant protein.

Sera from 76 mothers (Group 5) of children who contracted toxoplasmosis congenitally were evaluated by Western blot. Sera were obtained from the women within 2.5 mo of the time their infected children were born. Detectable antibody to TgERP was found in 59 (78%) of these sera; all 3 sera from mothers involved in the Victoria outbreak were positive.

Sera collected from 58 chronically infected persons with IgG but not IgM antibody, and 1 with both IgG and IgM antibody to *T. gondii*, and no determined infection date (Group 6) had no detectable antibody to TgERP.

Fifty-five seronegative persons from Group 6, 60 seronegative GI nematode/IBD patients, and 17 seronegative coworkers from the laboratory outbreak (Group 1) had no detectable antibody to TgERP.

DISCUSSION

Serological methods currently in use for diagnosis or epidemiological surveys of toxoplasmosis in humans and animals utilize whole, fixed tachyzoites, solubilized native tachyzoite proteins, or recombinant tachyzoite proteins (Barberi et al., 2001; Chen et al., 2001; Dando et al., 2001; Roberts et al., 2001; Gamble et al., 2005). These assays, though effective for detection of exposure to *T. gondii*, are not useful for determining infection route.

Available evidence for the oocyst infection route in humans is based entirely upon epidemiological surveys. In certain areas of Brazil, approximately 60% of 6–8 yr old children have antibodies to *T. gondii* linked to the ingestion of oocysts from an environment heavily contaminated with *T. gondii* oocysts (Bahia-Oliveira et al., 2001). One of the largest recorded outbreaks of clinical toxoplasmosis in humans in North America was epidemiologically linked to drinking water from a municipal water reservoir in Victoria, British Columbia, Canada (Isaac-Renton et al., 1998). This water reservoir was thought to be contaminated with *T. gondii* oocysts excreted by cougars (*Felis concolor*) (Aramini et al., 1998, 1999). Three sera from this outbreak were evaluated in this study.

Similarly, human infections resulting from consumption of infected meat products are difficult to enumerate. Previous studies have suggested that consumption of undercooked meat products containing *T. gondii* tissue cysts may account for a significant proportion of *T. gondii* infections in humans in the U.S. (Mead et al., 1999; Roghmann et al., 1999). Dubey et al. (2008) found that 25% of slaughter lambs from the Mid-Atlantic states harbored *T. gondii* tissue cysts; however lamb is considered an insignificant source of *T. gondii* infections in humans in the U.S. (Smith, 1993), since relatively little lamb is eaten by U.S. consumers [[www.nass.usda.QuickStats/Index2.jsp](http://www.nass.usda/QuickStats/Index2.jsp)]. In the U.S., pigs are generally thought to be the most common source of tissue cyst acquired *T. gondii* infection in humans. In one study, viable *T. gondii* was isolated from 17% of 1,000 adult pigs (sows) from a slaughter plant in Iowa (Dubey et al., 1995). In a recent nationwide survey of retail chicken, beef, and pork in the U.S., only pork was found to harbor viable *T. gondii* tissue cysts (Dubey et al., 2005). Viable tissue cysts were isolated from 0.38% of pork samples, and 0.57% of samples had antibodies to *T. gondii*. The northeastern U.S. had a higher number of positive pork samples than other regions of the country, reflecting the higher risk of pig infection due to regional management practices. The low prevalence of *T. gondii* infection in pork reported in Dubey et al. (2005) does not support the contention that pork contributes significantly to human infection in the U.S. However, in a separate study, associations were

found between consumption of raw or undercooked meats and *T. gondii* infection (Jones et al., 2009). In this study, we have utilized tandem mass spectroscopy in combination with 2-D DIGE and Western blot analysis to identify a sporozoite protein (TgERP) which elicits antibody in animals (pigs, mice) and humans that are exposed to sporulated oocysts. No serological assays currently exist which utilize sporozoite proteins as a source of diagnostic antigens, though stage specific antigens have been previously described from tachyzoites, bradyzoites, and sporozoites (Lunde and Jacobs, 1983; Kasper et al., 1984; Kasper, 1989; Omata et al., 1989; Tomavo et al., 1991; Appleford and Smith, 2000; Weiss and Kim, 2000). In the current study, significant differences were seen in expressed protein profiles between life cycle stages which could be exploited to differentiate oocyst induced infection from other infection routes in the Western blot assay, since only a relatively small subset of expressed proteins in each of the life cycle stages are serologically recognized by an infected host; selection of useful biomarkers is simplified significantly.

One problem with selecting useful biomarkers for differentiation of human routes of infection is the lack of availability of human sera from individuals with *T. gondii* infections known to have resulted from consumption of tissue cysts in infected meat. Few outbreaks known to have resulted from this infection route have been investigated (Sacks et al., 1983; Choi et al., 1997; Ross et al., 2001), and sera from these patients were unavailable. Consequently, sera from pigs which were experimentally infected with oocysts or tissue cysts were used to identify the sporozoite protein initially in 2D Western blots, and then from the sporozoite cDNA library for protein expression and purification. Comparisons of protein recognition profiles in 1D Western blots with human and pig serum from known oocyst induced infections using extracted whole sporozoites as the antigen, or serum from chronically infected humans and tissue cyst induced infection in pigs using extracted bradyzoite proteins as the antigen revealed similar antigen recognition patterns (Fig. 3E). These data suggest that antigen recognition by sera from pigs and humans with oocyst or tissue cyst induced infections are also similar, and that the lack of recognition of TgERP by tissue cyst infected pigs may reflect a lack of recognition of this antigen by humans infected by tissue cysts.

Further, all human (mouse and pig) infections result in exposure to tissue cyst antigens during the chronic infection phase. However, of 176 human sera tested from chronically infected individuals, (4 from Group 2, 114 from Group 3, and 58 from Group 6), only 31 (17.6%) had detectable antibody to TgERP. Antibody to TgERP was not detected in sera from tissue cyst infected pigs, or in sera collected during the chronic phase of infection (>8 mo post infection) from pigs infected with oocysts. In contrast, 10 of 10 pigs experimentally infected with oocysts had detectable antibody to TgERP during the acute phase of infection (<6 mo post infection), as did 18 of 20 (90%) acutely infected people known (6 in Group 1) or suspected (11 in Group 2, 3 in Group 5) to have been exposed to oocysts. In addition, of the 163 people with acute infection as defined by the presence of anti- *T. gondii* IgM or <30 in the IgG avidity test, (6 in Group 1, 11 in Group 2, 68 in Group 3, 1 in Group 4, 76 in Group 5, and 1 in Group 6), 103 (63.2%) had detectable antibody to TgERP. The difference seen among these groups in the presence of detectable antibody to TgERP is significant (acute, 63.2% vs chronic 17.6%; G test, $p < 0.001$, 1 degree of freedom). Further, mice infected with a heterologous strain (ME-49), and humans from widely separated geographic areas produced antibody which reacted with TgERP from the VEG strain, indicating that TgERP is not strain-specific, making it useful for detection of oocyst infection from different sources. Antibody to TgERP was detectable for 6 to 8 mo in both pigs and humans, suggesting that TgERP, related to late embryogenesis, does not present a continuing immunological challenge. In contrast, antigens from tachyzoites and bradyzoites present an ongoing challenge to the host, and therefore antibody to these parasite stages persists indefinitely in the host. In totality, these data suggest that TgERP is specifically expressed in

the sporozoite stage, and positive serology is emblematic of early infection and accurately reflects a unique exposure to the stage of the parasite which is contained within the oocyst.

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Figure 1.

Protein extracted from A, bradyzoite (bz), and B and E, sporozoite (sz), and D, tachyzoite (tz) stages of VEG strain *T. gondii* spots resolved on 2D-DIGE gels, pH range 4–9 in the 1st dimension, and 4–12% SDS gradient gel in the 2nd dimension. Protein samples from C, bz and sz or F, tz and sz *T. gondii* stage labeled with Cy2 (green; tz, bz), or Cy5 (red, sz). Equal concentrations of proteins from each stage were mixed loaded onto a 2D-DIGE gel for analysis. Stage-specific proteins from the bz or tz stage resulted in a green spot, proteins specific to the sz stage resulted in a red spot, and shared proteins resulted in a yellow spot. Sporozoite-specific protein selected from gel for assay development indicated by arrow. Molecular weight bars on left, A and D, top, 150kD, center, 30kDa, bottom, 14kDa.

Figure 2.

Western blot of *T. gondii* extracted sporozoite proteins resolved on 2D gel, pH 4–9 in the 1st dimension, and 4–12% SDS gradient gel in the 2nd dimension. Proteins were transferred to PVDF nylon blotting membrane and incubated in a pool of porcine sera (diluted 1:500) from 10 pigs with acute oocyst-induced *T. gondii* infection, followed by horseradish peroxidase conjugated-goat anti-pig IgG. Immunoreactive, sporozoite-specific protein identified in DIGE gel was spot matched and selected from identical 2D gel for assay development; indicated by arrow.

Figure 3.

A, MS/MS results from analysis of 11 kDa protein spot excised from DIGE 2-D gel from which the partial amino acid sequence for the sporozoite protein was derived. **B**, Amino acid sequence of TgERP as translated from DNA sequence of selected immunoreactive clone. **C**, Coding sequence of TgERP. **D**, Individual results of 1-dimensional Western Blots using sera diluted 1:100 from 10 pigs (lanes 1–10) with oocyst- or 10 pigs (lanes 11–20) with tissue cyst-induced *T. gondii* infection, using 11 kDa sporozoite-specific protein as antigen. Antibody persisted ~6–8 months in pigs. **E**, Western blot of *T. gondii* total sporozoite protein extract, Lane 1, human sera from Group 2, *T. gondii* outbreak investigated by the CDC (EIA IgM positive = 4.2), Lane 2, serum from pig experimentally infected with *T. gondii* oocysts; Lane 3 and 4, *T. gondii* tachyzoite total protein extract probed with: Lane 3, human sera from Group 2, *T. gondii* outbreak investigated by the CDC (EIA IgM negative);

Lane 4, serum from pig experimentally infected with *T. gondii* tissue cysts. **F**, Western blot of VEG strain TgERP with individual sera collected 60 days PI from mice infected with A, oocysts, or B, bradyzoites, of the ME-49 strain of *T. gondii* (Fig. 3F).

Figure 4.

Western blots were carried out using human sera and the Factor Xa cleaved-fusion protein purified over a DEAE sepharose column (purified TgERP insert). **A**, Lane 1, Factor Xa-cleaved, DEAE column purified pMal-c2 vector without the TgERP insert. Lanes 2–7, purified insert, probed with individual sera from Group 1, 6 people from laboratory outbreak resulting from oocyst ingestion. Sera collected 6 weeks post-infection. **B**, Left, purified insert, probed with pooled sera from 6 Group 1 individuals, 12 weeks post infection; Right; same, probed with pooled sera from 4 chronically infected persons from Group 2. **C**, purified insert, probed with pooled sera from 6 Group 1 individuals, 5 months post infection. **D**, purified insert, Lanes 1–9, probed with sera of 9 people from stable outbreak (Group 2), Lanes 10–12, probed with sera of 3 IgM positive women from Group 3.

Table 1

Human study groups, exposures, detectable antibody to TgERP

Group #	Description	Number	Known Exposure:			N with detectable antibody/total (%)
			Congenital	Oocyst	Tissue cyst	
Group 1	Lab outbreak	6	0	6 known	0	6/6 (100%)
	Uninfected coworkers	17	0	0	0	0/17 (0%)
Group 2	Atlanta outbreak ^a	11	0	11 suspected	unk	9/11 (82%)
	4 unrelated sera, chronically infected	4	unk	unk	unk	0/4 (0%)
Group 3	Hispanic women, tested by: Western blot	182	unk	unk	unk	60/182 (33%)
	ELISA					44/182 (24%)
Group 4	Amish family	8 ^b	1	unk	unk	6/8 (75%)
Group 5	NCCCTS mothers	73	unk	unk	unk	56/73 (78%)
	Victoria epidemic ^c	3	0	3 suspected	0	3/3 (100%)
Group 6	Chronically infected women	59	unk	unk	unk	0/59 (0%)
	Seronegative cohorts	55	unk	unk	unk	0/55 (0%)
	GI nematode/IBD patients	60	0	0	0	0/60 (0%)
Seronegative Persons	Group 1, laboratory coworkers	17	0	0	0	0/17 (0%)
	Group 6, seronegatives	55	0	0	0	0/55 (0%)

^a Atlanta outbreak was reported previously (Teutsch et al., 1979).

^b A congenitally infected infant in the NCCCTS study and her mother were index cases for this family cluster. All other family members were seropositive except one two-year old, a sibling of index child. Meat was always well cooked before consumption. Cats frequented their vegetable garden and hen house.

^c Mothers of 3 congenitally infected infants who were part of the Victoria, Canada epidemic in 1995. unk=unknown exposure route