IDENTIFICATION OF A SPOROZOITE-SPECIFIC ANTIGEN FROM TOXOPLASMA GONDII

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ABSTRACT: Reduction of risk for human and food animal infection with Toxoplasma gondii is hampered by the lack of epidemiological data documenting the predominant routes of infection (oocyst vs. 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 spectrometry and Western blot, to identify a sporozoite-specific protein (T. 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; 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–T. gondii 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–T. gondii 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 T. gondii 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 transmitting the pathogen to the fetus. Congenital infections acquired during the first trimester are more severe than those subsequently acquired (Desmonts and Couvreur, 1974; Remington et al., 2005). Congenital infection can cause a spectrum of disease syndromes, ranging from chronic infection with unapparent clinical symptoms, to blindness and mental retardation in children, to stillbirth. Devastating disease can also result in immunosuppressed patients, such as those given large doses of immunosuppressive agents in preparation for organ transplants or those with acquired immunodeficiency syndrome. In any case, 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 can infect consumers (Smith, 1993; Dubey et al., 1995, 2005). There are no tests that 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 that could lead to the development of strategies to reduce infection in humans and food animals difficult. In the present study, we describe the first identification of a sporozoite-specific protein, T. gondii embryogenesis-related protein (TgERP), that elicits antibody in T. gondii–infected pigs, mice, and humans. The 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. All animal experiments were conducted using approved protocols under the auspices of the Institutional Animal Care and Use Committee, Beltsville Agricultural Research Center, Beltsville, Maryland.

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 microblender (2.5-ml salinebrain). Isotonic Percoll (9:1 Percoll/saline) was added to the homogenate (3:2), mixed well, and centrifuged at 2,600 rpm (2,000 g) for 30 min. The top layer of brain tissue and the supernatant were removed, the pellet was resuspended in saline and mixed well by vortexing, and the suspension was filtered through the edge of a 25-µm 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 (1,179 g) for 10 min. 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 min 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, Manassas, Virginia). T-75 flasks with a 75% confluent cell layer were seeded with 1 × 10⁶ *T. gondii* tachyzoites and maintained at 37 C and 10% CO₂ in high-glucose DMEM containing 3% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mM glutamine, 100 mM HEPES, 1× MEM non-essential amino acids, and 1 mM sodium pyruvate. After 7–10 days of culture, as tachyzoites began to emerge from cells, the medium was switched to HBSS for 2–3 h each day, and emerged tachyzoites were collected from the supernatant by centrifugation at 1,200 g for 7 min 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 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 (Safetypath 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 min 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 500-µm glass beads for 5 min. 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 previously (Hill et al., 2001; Hummon et al., 2007) and per the manufacturer’s instructions (www.invitrogen.com/content/sfs/manuals/trizol_reagent.pdf). Pro- teins were dissolved in 0.1% SDS, and concentrations were determined using a modified Bradford assay (BioRad protein assay; BioRad, Hercules, California). Proteins were then stored in 1% SDS and 8 M urea at −20 C until used.

cDNA libraries were constructed from excysted sporozoites of *T. gondii* using the Smart cDNA synthesis by long-distance PCR protocol in the T7 or T3 vector (Clontech, Mountain View, California). First-strand cDNA synthesis was accomplished using 1 µg total RNA, 1 µM SMART IV oligonucleotide (5′-AAACGACTGATACACGAGATTGCTCAGGCCAT ACGGCCGCGG-3′), 1 µl CDS III′ PCR primer (5′-ATTCTA-GAGGCGCAAGCGCCGACGATG-3′), and the suspension was filtered through the edge of a 25-µm 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 (1,179 g) for 10 min. 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 min 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.

Gel electrophoresis

Sporozoite proteins were compared to tachyzoite and bradyzoite proteins using 2-dimensional (2D) difference in gel electrophoresis (DIGE). Equal amounts of protein extracted from sporozoites, tachyzoites, and bradyzoites as described above were labeled by CyDye DIGE fluor (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 second dimension (Applied Biomics, 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 (GE Healthcare, Piscataway, New Jersey) software was used to generate the CyDye image, followed by DeCyder software analysis (GE Healthcare LifeSciences, Piscataway, New Jersey) 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*. Electroblotting was carried out on unfixed gels by transfer of proteins onto Immobilon (PVD/F) nylon blotting membranes (Millipore) using a Novex gel transfer apparatus (Novex, San Diego, California) set at 40 V for 80 min in 25 mM Bis-Tris, 25 mM Bis-Tris, 1 mM EDTA, 20% methanol, and pH 7.2 blotting buffer. For Western blotting, the membranes were rinsed in 50 mM 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 ≥1:1400) pool prepared from sera taken (week 4 through week 12 post-infection [PI]). Horseradish peroxidase-conjugated goat anti-pig IgG (Sigma Chemical, St. Louis, Missouri) was used as the second-step antibody at a dilution of 1:800. Sporozoite, bradyzoite, and tachyzoite proteins recognized by porcine anti-*T. gondii* antibodies were visualized using the 4 CN membrane developer kit (Kirkegaard and Perry). Western blot images were captured using the ProExpress proteomics image acquisition system (PerkinElmer, Boston, Massachusetts); spot matching and image analysis of the 2D Western blot images were accomplished using the PDQuest software system (BioRad). Differentially expressed protein spots in the sporozoite protein sample that were also immunoreactive in Western blots as described above were picked from the 2D gel and identified by peptide fingerprint mass mapping.
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: ACC GTT CTT TGT CTT CGT AT). PCR was performed using the primers listed above and DNA extracted from the sporozoite cDNA library. The amplicons were ligated into the pTrilEx2 plasmid vector using 1.5 μl of CDNA and following the vector manufacturer’s 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, Indianapolis, Indiana) for use in library screening to isolate the full-length gene for protein expression using the kit manufacturer’s protocol. The sporozoite cDNA library was plated onto LB-ampicillin plates overnight at 37°C and transferred to nitrocellulose filters. Filters were placed sequentially on filter paper soaked with 0.5 M NaOH, 1 M Tris-Cl, and 0.5 M Tris Cl/1.25 M NaCl, then dried at 80°C in a vacuum oven. Filters were probed with the DIG-labeled DNA probes described above in SSC hybridization buffer at 45°C, 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 40°C 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 immunoblot 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 10 mM 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 centrifrip spin column to a minimum of 1 mg/ml. The fusion protein was cleaved using Factor Xa and purified using DEAE-Sephacel 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. SDS reducing sample buffer (1 M 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 (Lunde and Jacobs, 1983). Blood was collected from the periorbital plexus and terminally by heart puncture 60 days PI. 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 antibodies to T. gondii were performed as previously described (Remington et al., 2005) on sera from the pregnant Amish women, laboratory workers, mothers, and their children who are part of the National Collaborative Chicago-based Congenital Toxoplasmosis Study (NCCCTS, Chicago, Illinois), and newborn children. Differential agglutination and avidity assays were also performed for pregnant women in the NCCCTS prior to receipt of sera (Remington et al., 2005; McLeod et al., 2006). Serologic testing using sera from persons in the Atlanta epidemic caused by T. gondii were as previously described (Teutsch et al., 1979).

In Group 1, 6 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 (Beltsville, Maryland) 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 the 8 mo 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.

In 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 an indirect fluorescent antibody test (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 of IgG-positive). 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.

In Group 3, sera from 182 T. gondii seropositive Hispanic women of childbearing age (18-43 yr) 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, Oberursel, 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 ELISA, Ani Labsystems Ltd., Vantaa, 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 (unclarified with Factor Xa) was diluted to a concentration of 2 μg/ml in 0.1 M carbonate buffer, pH 9.6. ELISAs were carried out essentially as described by Gamble et al. (2005). Reference positive and negative controls were established using a pool of 10 positive and a pool of 10 negative sera. One replicate of each of the 4 sera with a high avidity index was used as a positive control. Secondary screening resulted in selection of over 50 positive plaques, and 12 were sequenced to confirm the gene.

The MASCOT search engine was used to identify proteins from 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 (unclarified with Factor Xa) was diluted to a concentration of 2 μg/ml in 0.1 M carbonate buffer, pH 9.6. ELISAs were carried out essentially as described by Gamble et al. (2005). Reference positive and negative controls were established using a pool of 10 positive and a pool of 10 negative sera. One replicate of each of the 4 sera with a high avidity index was used as a positive control. Secondary screening resulted in selection of over 50 positive plaques, and 12 were sequenced to confirm the gene.

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In 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 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 were otherwise available to determine how the family became infected.
Mets et al., 1996; Patel et al., 1996; Brézin et al., 2003; Boyer et al., 2005; Remington et al., 2005; McLeod et al., 2006; Roizen et al., 2006; Arun et al., 2007; Benevento et al., 2008; Jamieson et al., 2008; Phan et al., 2008a, 2008b). 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).

In Group 6, sera from 114 pregnant women from the Lancaster County Amish community, many with similar risk factors as the Amish family described previously, were screened for IgG and IgM antibodies (Remington et al., 2005) to T. gondii 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 2D electrophoresis protein maps were produced using proteins isolated from T. gondii sporozoites, tachyzoites, and bradyzoites. DIGE analysis revealed >20 protein spots that were unique to the sporozoite protein sample as compared with the bradyzoite or tachyzoite protein samples (Fig. 1). Western blot analysis of identical 2D gels probed with sera from the 10

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**FIGURE 1.** Protein extracted from (A) bradyzoite (bz), (B, 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 first dimension, and 4–12% SDS gradient gel in the second 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 is indicated by an arrow. Molecular weight bars on left: (A, D) top, 150 kD; center, 30 kDa; and bottom, 14 kDa.
pigs infected p.o. with ~1,000 oocysts revealed a protein spot in the 10–12 kDa 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). The spot was not seen on Western blots from the sporozoite preparation probed with sera from pigs with tissue cyst–induced infection, and it 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 11 kDa sporozoite protein related to embryogenesis (TgERP, Fig. 3A). TgERP corresponds to gene model “TGVEG_027520” in Toxodb and XP_002370433 in NCBI (INV 20-MAY-2009) and is annotated as “a putative late embryogenesis abundant domain-containing protein.” Expression data in sporozoites and functional analysis are not currently available.

Expression and purification followed successful subcloning into the pMal 2-plasmid expression vector (Fig. 3B, C). Western blots were carried out to ensure 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 unaffected control pigs (Fig. 3D). Antibodies were detectable in pigs with oocyst-induced infection for 6–8 mo after initial infection. Sera 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 sera 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 were known to have become infected with T. gondii oocysts on a specific date (Fig. 4A-C). The 6 infected individuals developed IgM and IgG antibodies that 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 I).

Sera collected from 11 visitors to the riding stable in Atlanta (Group 2) were screened for anti-TgERP antibodies using Western blots (Fig. 4D). 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 the 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- to 8-yr-old children have...
Figure 3. (A) MS/MS results from analysis of 11 kDa protein spot excised from DIGE 2D 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 one-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 mo 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; lanes 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) and (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.
antibodies to *T. gondii* putatively 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 United States (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 United States (Smith, 1993), since relatively little lamb is eaten by U.S. consumers (http://www.nass.usda.gov/Publications/Ag_Statistics/2006/CHAP13.pdf). In the United States, pigs are generally thought to be the most common source of tissue cyst–acquired *T. gondii* infection in humans. In 1 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 United States, 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 United States 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 United States. However, in a separate study, associations were found between consumption of raw, or undercooked, meats and *T. gondii* infection (Jones et al., 2009).

In the present study, we have utilized tandem mass spectroscopy in combination with 2D DIGE and Western blot analysis to identify a sporozoite protein (TgERP) that elicits antibody in animals (pigs, mice) and humans that are exposed to sporulated oocysts. No serological assays currently exist that 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). Here significant differences were seen in expressed protein profiles between life cycle stages that 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 is 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 that 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 one-dimensional 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 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). Here significant differences were seen in expressed protein profiles between life cycle stages that 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 is serologically recognized by an infected host, selection of useful biomarkers is simplified significantly.

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Table I. Human study groups, exposures, detectable antibody to TgERP.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
<th>Number</th>
<th>Known exposure</th>
<th>N with detectable antibody/total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Congenital*</td>
<td>Oocyst</td>
</tr>
<tr>
<td>Group 1</td>
<td>Lab outbreak</td>
<td>6</td>
<td>0</td>
<td>6 known</td>
</tr>
<tr>
<td></td>
<td>Uninfected coworkers</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 2</td>
<td>Atlanta outbreak†</td>
<td>11</td>
<td>0</td>
<td>11 suspected</td>
</tr>
<tr>
<td></td>
<td>4 unrelated sera, chronically infected</td>
<td>4</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Group 3</td>
<td>Hispanic women, tested by: Western blot</td>
<td>182</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>ELISA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4</td>
<td>Amish family</td>
<td>8†</td>
<td>1</td>
<td>Unknown</td>
</tr>
<tr>
<td>Group 5</td>
<td>NCCCTS mothers</td>
<td>73</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Victoria epidemic§</td>
<td>3</td>
<td>0</td>
<td>3 suspected</td>
</tr>
<tr>
<td>Group 6</td>
<td>Chronically infected women</td>
<td>59</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td></td>
<td>Seronegative cohorts</td>
<td>55</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
<tr>
<td>Seronegatives</td>
<td>GI nematode/IBD patients</td>
<td>60</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Group 1, laboratory coworkers</td>
<td>17</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Group 6</td>
<td>Unrelated sera, seropositive</td>
<td>55</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Unknown = unknown exposure route.
† Atlanta outbreak was reported previously (Teutsch et al., 1979).
‡ A congenitally infected infant in the NCCCTS study and her mother were index cases for this family cluster. All other family members were seronegative except one 2-yr-old, a sibling of index child. Meat was always well cooked before consumption. Cats frequented their vegetable garden and hen house.
§ Mothers of 3 congenitally infected infants who were part of the Victoria, Canada, epidemic in 1995.

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 in 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 PI) from pigs infected with oocysts. In contrast, all 10 pigs experimentally infected with oocysts had detectable antibody to TgERP during the acute phase of infection (<6 mo PI), as did 18 of 20 (90%) acutely infected people (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 that 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, a putative late embryogenesis abundant domain-containing protein 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 that is contained within the oocyst.

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LITERATURE CITED


