RESEARCH REPORT
PORK SAFETY

Title: Validation of a serological assay using an 18kd oocyst-specific protein from *Toxoplasma gondii* for differentiation of oocyst versus tissue cyst induced human infection – **NPB #04-136**

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Date Received: October 18, 2005

II: Abstract:

Reduction of risk of human and food animal infection with the zoonotic parasite, *Toxoplasma gondii*, is hampered by the lack of data documenting the predominant routes of infection (oocyst vs tissue cyst exposure). Existing serological assays can determine previous exposure to the parasite, but not the infection route. We have identified an oocyst specific 18.3kDa protein that can differentiate between oocyst vs tissue cyst induced *T. gondii* infection in pigs and humans. In the present study, we selected the cDNA clone of the protein from a library constructed from *T. gondii* sporulated oocysts. Following successful subcloning into the pMal 2 plasmid expression vector, the recombinant protein was expressed and purified. Human serum of known infection route was obtained from a variety of sources, including the CDC, the University of Chicago, and Case Western Reserve University, and were used to validate an ELISA serological assay using the expressed recombinant protein.

III: Introduction:

Toxoplasmosis, caused by the protozoan parasite *Toxoplasma gondii*, is one of the most common parasitic infections of man and other warm-blooded animals. It has been found worldwide from Alaska to Australia. Nearly one-third of humanity has been exposed to this parasite. In most adults it does not cause serious illness, but it can cause blindness and mental retardation in congenitally infected children and devastating disease in immunocompromised individuals. Humans become infected by congenital transmission from mother to fetus, through ingestion of tissue cysts in under cooked or uncooked meat, or by ingesting food or water contaminated with sporulated oocysts from infected cat feces. Food animals, such as pigs, become infected by the same routes, resulting in meat products containing tissue cysts which could infect consumers. Currently, there are no tests which can differentiate between oocyst ingestion versus tissue cyst ingestion as the infection route. Development of such a test would make epidemiological studies possible to determine predominant infection routes, could lead to the development of fact-based strategies to reduce transmission, and could increase public perception of pork as a safe food product. Increased consumer awareness of the potential risks of acquiring *T. gondii* from fresh pork products represents a potentially serious problem for the U.S. pork industry. Food safety is a critical issue for the swine industry. Foodborne diseases are increasing in industrialized countries and consequently, are more of a concern to consumers. Large outbreaks of foodborne diseases are being reported and covered extensively in the media, and the severe impact on children, the aged, and immunocompromised

individuals has resulted in a heightened awareness of the consumer to the issue of contaminated food. Demands of consumers for pathogen free meat products have focused attention of government regulators and the meat industry on food safety, and the necessity to produce meat that is wholesome, safe and of high quality. Delivery of a safe product is essential for pork to remain a competitive commodity, both in the U.S. and globally.

IV: Objectives:

The objectives of the present study were to use the cDNA clone of an oocyst specific protein (dgp5p) from a library constructed from sporulated *T. gondii* oocysts to express the recombinant dgp5p protein, and validate a recombinant-based ELISA serological assay for differentiation of oocyst vs tissue cyst induced toxoplasmosis in humans and food animals.

V. Materials and Methods:

Toxoplasma gondii RNA, DNA, and protein were extracted from unsporulated and sporulated oocysts, excysted sporozoites, tachyzoites, and bradyzoites stages using Triazol reagent (Gibco/BRL Life Technologies, Gaithersburg, MD) per the manufacturer's instructions. Using the extracted RNA, a cDNA library was constructed for each stage in the λTriplEx vector (Clontech, Mountain View, CA). A total of seven *T. gondii* cDNA libraries covering all the stages were constructed. Extracted protein from each of the stages was used to identify an oocyst-specific protein from *T. gondii* using 2-dimensional Western blots screened with human sera from known oocyst induced infection. Matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) and collision induced dissociation (CID) fragmentation analysis was performed on the identified protein. The protein was identified as a sporozoite dense granule protein (dgp5p).

Polymerase chain reaction (PCR) primers were constructed from the amino acid sequence derived from the mass spectrometry data, and PCR was performed using these primers and DNA extracted from the sporozoite cDNA library. The amplicons produced were cloned into the pTriplEx2 plasmid vector and sequenced on an ABI Sequencer model 3100.

The sequenced PCR amplicons for dgp5p, identified above as a potential gene candidate for further study for developing an assay to identify route of infection with *T. gondii*, were labeled using a non-radioactive method (DIG DNA labeling) for use in library screening to isolate the full length gene for protein expression. Using this method, the sporozoite cDNA library was screened and approximately 20 positive clones were identified. Secondary screening resulted in selection of over 50 positive plaques, and 12 were sequenced and further characterized to confirm the gene.

Preliminary expression studies and immunological screening of the recombinant protein using *T. gondii* oocyst-infected human sera confirmed that the positive clones were reactive with the infection sera.

The identified dgp5p gene was subcloned into the Xmn I/Hind III site of pMal-c2 (New England Biolabs, Beverly, MA) 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 purified using amylose resin as described by the manufacturer. The fusion protein was cleaved using Factor Xa, and purified using DEAE-Sepharose ion exchange chromatography. Western blots were carried out to assure continued serological reactivity of the expressed purified protein. The recombinant protein was initially probed with sera from pigs that were experimentally infected with *T. gondii* oocysts (sera collected week 2-6 of infection), followed by probing with human sera from oocyst induced infections.

The recombinant protein was then used to develop an ELISA assay. Human serum from infections resulting from ingestion of *T. gondii* oocysts during an accidental laboratory exposure; sera from a CDC-documented oocyst induced outbreak which occurred near Atlanta, Georgia; serum collected during an outbreak in a religious community in Illinois which was known to have resulted from oocyst exposure; and serum from congenitally infected children were used to validate the specificity and sensitivity of the assay. For the ELISA, optimal dilution of the purified recombinant antigen was initially determined using 2 fold dilutions beginning at 10μg/mL down to 0.1μg/mL and tested in the ELISA format using a positive control human serum, (diluted 1:500), whose anti-*Toxoplasma* IgM EIA value exceeded 6.0 as determined by a commercial laboratory. One hundred microliters (100 μL) of the optimal dilution of the recombinant antigen of 1μg/mL in 0.1M carbonate

buffer, pH 9.6, was used to coat each well of a high binding, flat bottomed microtitre plate (Costar). The plates were incubated overnight at 4 °C, and were then washed with 0.5M phosphate buffered saline containing 0.5% Tween 20 (PBS-T) and incubated with human serum samples diluted 1:500 overnight at room temperature. Anti-human IgM or IgG peroxidase conjugated antibody, diluted 1:1000, was used as the second step antibody and was added to the wells and incubated for 4 hours at room temperature. After washing the plates, ABTS peroxidase microwell substrate (KPL) was added to each well and incubated for 5 minutes. Plates were read at 405 nm using a *V*max ELISA reader (Molecular Devices). Positive and negative control sera were included on each plate. A positive cut-off was established as 5 times the mean + standard deviation of the mean of a set of 14 *Toxoplasma* negative human serum samples. Positive and negative predictive values were calculated (Greiner and Gardner, 2000a,b). All serum samples were also analyzed using anti-*Toxoplasma* MAT as the gold standard for comparative purposes.

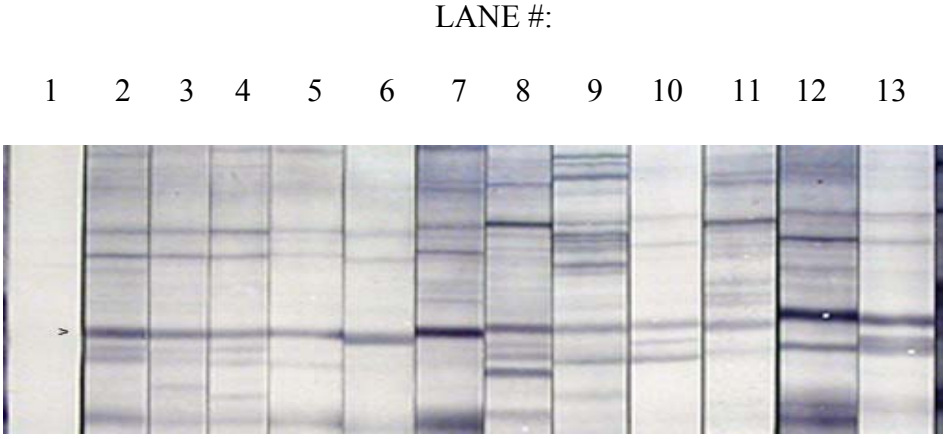
VI. Results:

To test the recombinant antigen, the Factor Xa-cleaved antigen preparation was initially tested in Western blots using sera from pigs infected with *Toxoplasma* oocysts; the results of these tests are shown in Figure 1.

Figure 1: Antibody reactivity to cleaved fusion protein

Lanes 1-13, swine antibody from oocyst induced infection with *T. gondii*.

Lane 1 antigen, uninduced fusion protein.



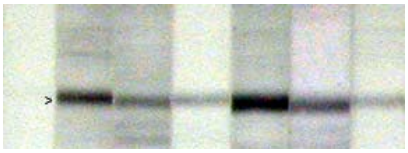
Human sera were also tested in Western blots with the recombinant antigen after passage of the Factor Xa cleaved-preparation over an amylose resin column for purification; these results demonstrated continued serological reactivity of human serum to the recombinant protein after isolation (Figure 2).

Figure 2: Antibody reactivity to purified recombinant

Lane 1, human antibody from congenital infection with *T. gondii*.

Lanes 2-7, human antibody from oocyst induced infection with *T. gondii*.

1 2 3 4 5 6 7



Sera from 40 oocyst infected pigs and 45 tissue cyst infected pigs was tested in ELISA and MAT following confirmation of infection by bioassay. Of the 40 pigs infected with oocysts, 39 of were correctly detected using the recombinant antigen ELISA. Forty-five pigs were infected via consumption of tissue cysts, only 3 of these animals were positive by the recombinant antigen ELISA (Table 1).

ELISA and MAT assays were also completed on 127 human sera known to have resulted from oocyst exposure, 5 sera from infections resulting from congenital infection, and 76 sera from MAT negative individuals. These results demonstrated comparable sensitivity of the 2 assays (Table 2 and 3) and specific detection of antibody in sera from oocyst induced infections using the recombinant antigen. Results from ELISAs using anti-human IgG are shown; recombinant antigen-specific IgM responses were not sufficiently robust and did not persist for more than 4 months in greater that 80% of samples analyzed. Of 76 uninfected sera (as confirmed by MAT) all were negative using the recombinant ELISA. Of 127 oocyst induced infections, the recombinant antigen ELISA detected 119, and did not detect 8, resulting in a PPV of 93.7% as compared to the MAT of 100%. Infections induced by congenital transmission were not detected using the recombinant antigen ELISA.

Table 1: MAT titers and ELISA results for pig sera.

Swine sera sources	**MAT (titer)**					**ELISA (IgG)**	
	0	**1:10**	**1:25**	**1:100**	**1:500**	**Positive**	**Negative**
Swine sera from oocyst induced infection	0	0	0	2	38	39	1
Swine sera from tissue cyst induced infection	0	0	0	6	39	3	42

Table 2.

MAT titers and ELISA results for human sera.

Human sera sources	MAT (titer)					ELISA (IgG)	
	0	1:10	1:25	1:100	1:500	Positive	Negative
Accidental lab exposure to oocysts (n=7)	0	0	0	0	7	7	0
Atlanta outbreak (n=17)	0	0	0	2	15	15	2
Illinois outbreak (n=20)	0	0	0	1	19	19	1
Congenital infection (n=5)	0	0	0	2	3	0	5
Waterborne outbreak (n=83)	0	5	8	27	43	78	5
Negative control serum (n=76)	75	1	0	0	0	0	76

Table 3.

Positive (PPV) and negative predictive values (NPV) for the MAT and ELISA using human sera based on sensitivity.

Samples		MAT	ELISA (IgG)
Oocyst induced infection	PPV	100	93.7
Negative control sera and tissue cyst induced infection	NPV	98.6	100

VII. Discussion:

Analysis of serum from experimentally infected pigs demonstrated that specific IgG was detectable 2 weeks post infection and persisted for 4-6 months in pigs infected with oocysts. Swine IgM generated against the recombinant antigen was weakly detectable 2 weeks post infection, but was not detectable 10 weeks post infection. For many of the recombinant antigen positive human serum samples, the actual infection date is not known, so that no definitive conclusion can be drawn concerning how long the recombinant specific IgG persists in the human host. However, 24 of the recombinant antigen positive human serum samples were drawn from people whose infection date can be closely estimated. In these samples, antigen specific IgG could be detected for at least 6 months post infection. IgG antibody reacting with the recombinant antigen was detectable within 2 weeks of infection in all positive samples tested for which the infection date is known; IgM antibody was also detectable within 2 weeks, however, the specific IgG antibody persisted at higher titer for at least 6 months, while the IgM titer decreased over this time period. The specificity of the antigen can only be estimated, since no human sera were available from patients infected with other protozoan parasites, however, 76 MAT negative sera from human patients were also negative in the recombinant ELISA assay, demonstrating a lack of non-specific binding to the recombinant antigen. These sera were drawn from patients with gastrointestinal nematode infections and patients with inflammatory bowel disease. In addition, *T. gondii* infected human sera which reacted strongly with the isolated recombinant antigen in the Western blots revealed

only limited non-specific binding to large molecular weight proteins (>50kDa) extracted from other protozoan parasites, including the closely related *Neospora caninum*, *Hammondia hammondi*, and *Sarcocystis cruzi.*

VIII: Lay Interpretation:
The ELISA assay developed in this study is both sensitive and specific for the detection of human antibody to *Toxoplasma gondii* infection initiated by consumption of infectious oocysts. The assay will be useful for planned large scale surveys of human serum banks to determine the predominant route of exposure of humans to *T. gondii*, and will be helpful in the development of strategies to reduce and eliminate exposure.

References:
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