RESEARCH REPORT
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PORK SAFETY

Title: Cloning of an 18kd oocyst-specific protein from *Toxoplasma gondii* for serological detection of human infection. **NPB 03-125**

Investigator: Dolores E. Hill, Ph.D.

Institution: United States Department of Agriculture, ARS, Beltsville, MD

Date Received: September 28, 2004

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). Existing serological assays can determine previous exposure to the parasite, but not the infection route. We have identified an oocyst specific 18.3kDa protein fragment 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 sporulated oocysts and validated continued serological responsiveness to the recombinant protein.

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. There are no tests which can differentiate between oocyst ingestion versus tissue cyst 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.

Objectives: Currently, there are no tests which can differentiate between oocyst ingestion versus tissue cyst 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. The objectives of the present study were to select the cDNA clone of the oocyst specific protein from a library constructed from sporulated *T. gondii* oocysts, express the recombinant protein, and test the recombinant in serological assays in order to develop a validated assay for differentiation of oocyst vs tissue cyst induced toxoplasmosis in humans and food animals.

These research results were submitted in fulfillment of checkoff funded research projects. This report is published directly as submitted by the project's principal investigator. This report has not been peer reviewed

For more information contact:
National Pork Board, P.O. Box 9114, Des Moines, Iowa USA
800-456-7675, **Fax:** 515-223-2646, **E-Mail:** porkboard@porkboard.org, **Web:** http://www.porkboard.org/

Procedures: Western blot analysis of protein extracts from unsporulated, 8-hour sporulated, completely sporulated, and sporulated and excysted *Toxoplasma gondii* VEG strain oocysts/sporozoites revealed that the 18.3 kDa protein was expressed in completely sporulated and sporulated and excysted oganisms only, and was recognized by sera from human toxoplasmosis patients infected via oocyst exposure. Concurrent matrix assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF/MS) and collision induced dissociation (CID) fragmentation analysis was accomplished on protein extracts from completely sporulated and sporulated and excysted oocysts, and on the isolated 18kDa protein. For these procedures, proteins were separated in PAGE gels and individual bands were cleaned in alternating rinses with 20 mM ammonium bicarbonate and 100% acetonitrile. Each band digested with 250 ng porcine trypsin (Promega) overnight at 37ºC, purified over a C-18 ZT column. The samples were mixed with dihydroxybenzoic acid (DHB) prepared at 10 mg/ml in 50% acetonitrile:0.3% TFA matrix, and were subjected to MALDI-TOF analysis using a Voyager DE STR (Applied Biosystems) Mass Spectrometer with an ACTH Reflector 500 5000 061103, and analysed using the Voyager Instrument Control Panel (v 5.1) and Data Explorer (v 4.0) software. An external standard peptide mixture was applied for calibration to better than 100 ppm error (Figure 1). The 18.3 kDa protein was identified as a proteolytic fragment of a 36.6 kDa sporozoite microneme protein by searches of the *Toxoplasma* sequencing data base (www.toxodb.org) with amino acid sequence information derived from the MALDI-TOF/MS analysis (Table 1). cDNA libraries were constructed in γTriplEx2 with RNA collected from completely sporulated, and sporulated and excysted *Toxoplasma gondii* VEG strain oocysts/sporozoites. Polymerase chain reaction primers constructed from the MALDI-TOF/MS derived amino acid sequence were used in PCR reactions with cDNA from the constructed libraries to amplify the target sequence, and the amplicons corresponding to a sequence encoding aa 4–349 of the full-length 36.6 kDa protein was ligated into the Eco RI/Sal I restriction sites of pMal-c2 for protein expression. The correct insertion of the PCR product was verified by sequencing automatically on an ABI Sequencer model 3100. The constructs were transformed into competent DH5-α *E. coli* cells and the protein was expressed as an N-terminal maltose binding fusion protein under the control of the lac repressor by induction with IPTG. The expressed maltose fusion protein was purified on an amylose affinity column according to the manufacturer's instructions and cleaved with factor Xa. The purified protein was again passed through the affinity column and purified to homogeneity on a MonoQ (Pharmacia) column eluted at 1 ml/min by loading Buffer A (20 mM bis-TrisHCl-20 mM NaCl, pH 6.2) and washing for 15 min. The protein was then eluted by forming a linear gradient between Buffer A and Buffer B (20 mM bis-TrisHCl-600 mM NaCl). The expression and purification steps were monitored by PAGE. The purified protein had an apparent molecular mass of 36 kDa (Figure 2). Western blot and ELISA techniques were carried out to assure continued serological reactivity of the recombinant protein by probing with sera from humans that were accidentally infected with *T. gondii* oocysts (sera collected week 2-6 of infection) in each assay, as well as human sera from a CDC-documented outbreak which occurred near Atlanta, Georgia, which was known to have resulted from oocyst exposure (sera collected week 8-12 of infection), and serum from a family with oocyst-induced toxoplasmosis. Western blotting was carried out with the recombinant protein electrophoresed on unfixed gels by transfer of proteins onto Immobilon (PVDF) nylon blotting membrane (Millipore, Bedford, MA) 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. The membranes were rinsed in 50 mM Tris buffered, 0.85% saline (TBS) and unbound sites on the membrane were saturated with Detector Block solution (Kirkegaard and Perry, Gaithersburg, MD). The membrane was incubated in human sera (diluted 1:500) from individuals with acute oocyst-induced *T. gondii* infection.

Horseradish peroxidase conjugated-goat anti-human IgGAM (Sigma Chemical. St. Louis, MO) was used as the 2nd step antibody at a dilution of 1:1000. The recombinant protein recognized by human anti-*Toxoplasma* antibodies was visualized using the 4CN membrane developer kit (Kirkegaard and Perry, Gaithersburg, MD). Western blot images were captured using the ProExpress proteomics image acquisition system (Perkin Elmer, Boston, MA) (Figure 3). The ELISA was carried out using the recombinant protein on high-binding ELISA plates and human sera (diluted 1:500) from individuals with acute oocyst-induced *T. gondii* infection (Table 2).

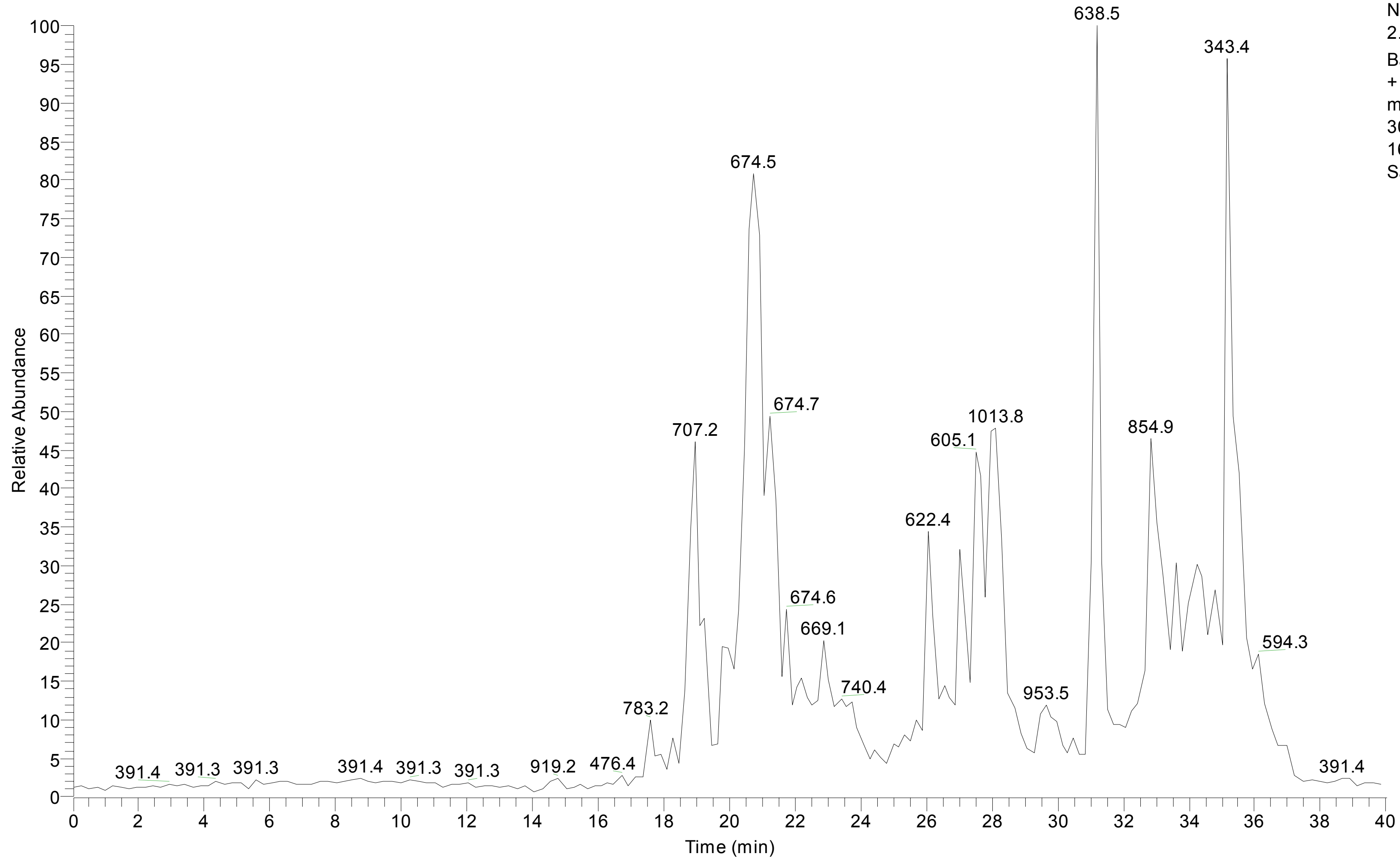
Results: The oocyst-specific protein was identified, cloned, and sequenced. Expression of the recombinant protein was successful, and the expressed recombinant remained detectable by human immune sera in both Western blots and ELISA.

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. Determination of the predominant route of infection of *Toxoplasma gondii* in humans and food animals could help to identify strategies for reducing transmission and could increase public perception of pork as a safe food product.

Figure 1: MALDI-TOF/MS chromatogram of peptide peaks resulting from trypsin digestion of gel purified sample containing 18.3 kDa proteolytic fragment. Sample 16: Base Peak Chromatogram (* see note 1)

C:\Xcalibur\...\Fournet\082703\Sample16 11/17/2003 7:25:54 PM Sample16

RT: 0.00 - 40.01



NL:
2.33E8
Base Peak F:
+ c NSI Full
ms [
300.00-
1600.00] MS
Sample16

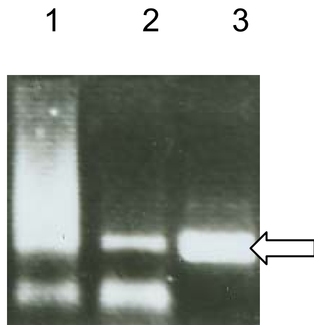
Table 1: TgTwinScan_1521 hits with MALDI-TOF/MS peptide data *

Reference Scan : *T.gondii* microneme protein

Sequence(s) Detected	MH+	Charge / XC		Peptides (Hits)
RTPLEESQEPEGSTPDSQQSR	2358.08	3	3.88	10
CCAAAVVAAESLLWLK	1629.98	3	2.63	9
ISLDGTGNVTCIVR	1429.62	2	3.11	9
QESGCEENGCGPPDAVQSCR	2048.10	2	3.90	7
ENFIATIDASAHITCK	1715.91	3	3.78	6
KPPFEFGK	931.06	2	2.30	3
GGHPVDSEPSK	1091.10	3	3.69	4

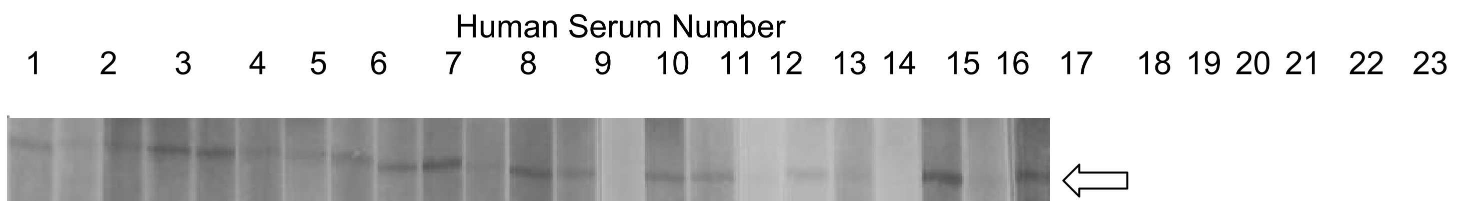
*see note 2

Figure 2: PAGE analysis of recombinant protein purification steps



Lane #
1 : Expressed maltose fusion protein purified on an amylose affinity column
2 : Fusion protein cleaved with factor Xa
3 : MonoQ (Pharmacia) column elute of purified recombinant protein (36 kDa)

Figure 3: Western blot analysis of recombinant oocyst specific protein against human serum from acute oocyst induced toxoplasmosis cases; serum diluted 1:500.



*see note 3

Table 2: ELISA analysis of recombinant oocyst specific protein against human serum from acute oocyst induced toxoplasmosis cases; serum diluted 1:500.

Controls	**ELISA OD**
Negative *	0.016
Positive **	0.808
Positive Cut-Off	0.300
Serum number	**Sera*** (1:500)**
1	1.089
2	.780
3	1.268
4	.976
5	1.447
6	.744
7	.884
8	.767
9	1.276
10	.943
11	.738
12	.826
13	1.082
14	.587
15	.998
16	1.115
17	.715
18	.776
19	.794
20	.561
21	1.159
22	.572
23	1.045

*Toxoplasma negative human serum
** Toxoplasma positive human serum
***see note 3

Notes:

1. MALDI-TOF/MS and CID were conducted at the Proteomics Core Facility, The Johns Hopkins University, 601 Physiology Bldg, 725 N. Wolfe St., Baltimore, MD 21205-2185 USA

2. Preliminary genomic, cDNA, and protein sequence data was accessed via http://ToxoDB.org. and http://prowl.rockefeller.edu.

3. Human serum was the kind gift of Dr. Jeffrey Jones and Marianna Wilson at the CDC Parasitic Diseases Laboratory; and Dr. Rima McLeod at the University of Chicago.