RESEARCH REPORT
PORK SAFETY

Title: Use of molecular assays to assess *Toxoplasma gondii* burden in commercial meat samples **NPB #02-101**

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Abstract: To improve food safety, our efforts have been aimed at developing diagnostic tests to confirm that pork products that contain foodborne microbial pathogens are reliably identified. Specifically, this grant was aimed at determining whether our parasite DNA test could detect pig products that contain the foodborne parasite, *Toxoplasma gondii* (Tg). Our fluorogenic real-time Tg DNA test, developed with previous NPB funding, is a quantitative molecular technique to detect Tg DNA and was recently patented. In collaboration with USDA IFAFS project "Retail meats survey for *Toxoplasma gondii,"* coordinated by Drs. J.P. Dubey and D. Hill, APDL, BARC, we used our fluorogenic Tg DNA assay to test commercial pork products for parasite contamination. Over a 3 year period commercial meat samples were collected under the IFAFS funded project from market sampling areas (MSA) nationwide using a statistically validated process. Results from that grant, assessing Tg burden using cat bioassays and meat juice analyses, will be reported separately.

Aliquots of the IFAFS pork samples were provided to this NPB project for testing for Tg contamination using parasite DNA assay. First, pretest controls were performed; these confirmed that the Tg DNA assays were positive when used on pig products collected from known Tg infected pigs. Then the IFAFS-associated commercial pork samples were tested. For each MSA, 75 pork products were obtained and processed; aliquots of 8-13 mixed pools of ~5 ground pork samples were provided for molecular testing. Over the course of this NPB project a total of 236 aliquots of pooled pork samples were tested; this represented 1180 individual meat products. For efficient sampling and molecular testing DNA was prepared from only 25mg of each pooled meat sample. None of the 226 pooled pork samples tested has been confirmed as positive for Tg by the parasite DNA assay whereas all 226 samples were clearly positive for 18S DNA, affirming DNA quantity. Therefore, the commercial pork sample molecular testing indicates the possible absence, or low level, of Tg parasite contamination.

More pork samples, and larger aliquots of these samples, [at much higher costs] would need to be tested to absolutely prove the lack of Tg parasite contamination especially since such a small sample was tested for the molecular assays. A follow-up survey of 108 individual samples from 1 MSA was performed and 1 potential Tg+ product identified. This is being tested further. Indeed, the definitive test results will be those collected, and reported separately, with the more detailed bioassay and meat juice antibody tests performed during Drs. Dubey and Hill's USDA NRI IFAFS project. All molecular test results will be compared to those results, expected later in 2005. Hopefully, all of our results will affirm the very low contamination of the commercial US pork supply with the foodborne parasite, Tg.

Introduction: Infection of swine with the protozoan parasite, *Toxoplasma gondii* (Tg), is a food safety concern (1,2). The parasite can be transmitted to humans by consuming Tg tissue cysts in raw or undercooked pork or lamb meat. Pork is considered the most important meat source of Tg in the US (3,4). Recent work has clearly identified waterborne sources of Tg infection. Pigs and humans can ingest Tg oocysts in water contaminated with infected cat feces or poorly washed food exposed to that water (5). There are now numerous reports of widespread Tg contamination in marine mammals in the US (2).

Infection with the Tg parasite causes potentially serious consequences in humans; for pregnant mothers and their newborns, causing birth defects and perinatal mortality, and for immunosuppressed individuals and AIDS patients Tg reoccurrence in the brain, or toxoplasmic encephalitis (3,4,6-9). Thus, preventing Tg contamination of pork is a food safety priority for pork producers. Under current production conditions toxoplasmosis does not heavily affect profitability in pig farms; Tg infections occur, causing clinical, or more often subclinical, symptoms and some reproductive failures (10). However, pigs raised outdoors are clearly more susceptible to Tg infection (11-13). From a consumer's point of view, Tg contamination in pork products poses a food safety problem and thus can have a negative impact on pork consumption. In addition, Tg-free status for US pork products will be required for full access to expanding international trade markets.

Pork producers established a Toxoplasmosis Working Group to actively assess and address the Tg problem. One task has been to develop proper diagnostic tools. Serology, using MAT, ELISA and other assays, is only one alternative to verify Tg infections. Despite many advantages, serology has some problems: false positive reactions due to cross reactivity, and false negative reactions due to tests of animals early in Tg infection (6,14). Identification of viable Tg by cat and mouse bioassays is considered by some to be the gold standard for Tg detection. However, bioassays are expensive, use live animals, and are only able to give an indirect estimation of how many cysts, not the number of infectious organisms present in tissue samples. With previous NPPC and NPB funding we established a fluorogenic real-time polymerase chain reaction (PCR) assay for Tg DNA that is sensitive and quantitative (15,16). There are many reports of PCR detection assays for Tg (17, 18). There are only two reports of PCR use for Tg diagnosis in ready-to-eat cured meats, but the sensitivity limit/gram meat is low or unknown (19,20). Since most field samples have low levels of Tg a very sensitive PCR protocol is necessary, thus we tested our fluorogenic Tg DNA assay (15,16).

Most reports on Tg prevalence in pigs are based on serology from samples taken from market and breeding age animals. Surveys showed that the Tg infection rate at the individual pig level is in steady decline. Tg incidence can be decreased further when wildlife, cat and rodent access to pig production facilities and their water supplies is

better controlled; this presents clear challenges for outdoor rearing facilities (11-13). However Tg contamination in the final pork product available to the consumer is the true test of whether there will be foodborne transmission of this parasite. Dubey, Hill and their CDC collaborators, received a USDA IFAFS grant # 2001-35212-10863 for "Retail meats survey for *Toxoplasma gondii."* Commercial pork, chicken and beef samples were collected from stores nationwide in a statistically validated sampling procedure. With their grant, samples were screened for Tg using meat juice MAT and ELISA assays and cat bioassays. Results from that grant are still being summarized and will be reported separately. This NPB grant expanded that study to include molecular assays, to determine whether the fluorogenic Tg DNA assay could be used as a reliable indicator of Tg burden in commercial cuts of meat and be a suitable alternative to the bioassay or the meat juice MAT assay.

Stated Objectives from original proposal

1) Assess commercial pork products for *Toxoplasma gondii* (Tg) contamination by comparing results from meat juice anti-Tg antibody and cat bioassay results with the fluorogenic Tg DNA assay
2) Improve technology to process tissue samples to detect Tg DNA
3) Test the performance of the fluorogenic Tg DNA assays for assaying chicken and beef products

Materials & Methods:

A. Tg infected pig samples: As positive sample controls, we collected muscle samples from Tg infected pigs. Test pigs were orally infected with Tg (VEG strain) oocysts. Mesenteric lymph nodes, lungs, liver, intestinal, muscle and spleen samples were collected for testing the Tg TaqMan assay. Tissues were tested directly or were trypsin digested for one hour, centrifuged and DNA was prepared using DNeasy™ Tissue Kits (Qiagen, Valencia, CA). Our previous NPB grant #01-082 had affirmed this procedure as being the best for these samples.
B. Chicken samples: Prior to testing samples from infected chickens muscle and brain from control and Tg infected chickens were processed as noted for pig samples in A.
C. Market samples: Pork, beef and chicken meat products were collected nationwide for the Tg survey coordinated by Drs. Dubey and Hill. Completion of this NPB grant research was delayed because collection of market samples for the meats survey was not completed until August 2004. For that survey, pools of 5-100g samples were fed to cats and bioassayed. For our comparative molecular assay, 1g aliquots of these pools were frozen and stored at -20°C. After thawing, DNA was directly extracted from 25 mg of each 1 g pool sample using DNeasy, without previous trypsin digestion.
D. Tg positive control: DNA preparations were produced from Tg tachyzoites, harvested from RH strain Tg infected mice.
E. Fluorogenic Tg DNA: After DNeasy™ procedure, DNA concentration was estimated by spectrophotometry at 260nm. Real time fluorogenic PCR assay (15)was performed on 100 ng DNA using 1) universal 18S rRNA primers to confirm DNA presence; these primers react with all animal species DNA as well as the parasite DNA; and 2) Tg specific ITS1 region primers to detect Tg. Results were analyzed using ABI 7700 Sequence Detector System (SDS) and SDS software. A positive (+) test with 18S primers affirms DNA presence; a + test with Tg primers affirms Tg presence. All Tg + tissues should be + with both sets of primers; control, non-

infected tissues should be negative for Tg but + for 18S; no DNA controls should be negative for both tests.

Results: Because we needed to develop methods for high throughput analyses of samples to meet the demands of the commercial meats survey we first worked to improve our technologies. Therefore this summary will summarize our results on Objective 2 first.

Objective 2) *Improve technology to process tissue samples to detect Tg DNA*

Prior to analyzing commercial market meat samples we confirmed the procedures and fluorogenic Tg DNA assay specificity with small quantities of pig tissues, using samples from known Tg infected pigs. Samples were collected from multiple tissues from pigs at 2, 4, 7 and 14 days after infection (DAI). All tissues were processed with and without trypsin digestion, followed by DNA extraction using DNeasy. Real time PCR detected Tg in 12 of the 13 infected animals, but not in every tissue (Table 1). Uninfected pig tissues were tested and confirmed negative for Tg as controls.

Table 1 – Tg Real-time PCR results expressed as positive/number of pigs.

Day after infection (DAI) / Tissue	2 DAI	4 DAI	7 DAI	14 DAI
Intestine - Ileum	0/3	3/3	NT	NT
Mesenteric Lymph node	2/2	3/3	4/5	1/2
Liver	0/2	2/2	3/5	1/2
Spleen	0/2	3/3	NT	NT
Lung	0/3	2/3	3/5	1/2
Muscle*	NT	NT	0/2	2/2

NT = not tested; * Muscle samples were tested without trypsin digestion.

As expected for those samples collected early in the infection, which starts in the intestine, the mesenteric lymph node (MLN) was the best tissue from day 2 to 7. The muscle tissue was not expected to be positive until later in infection due to Tg spread from the gut.

Real-time PCR results are expressed as C_t numbers (Table 2). Positive sample produces a fluorescent signal that is detected by the machine. Quantitation is possible by analyzing C_t number; low C_t number indicates high amount of Tg DNA. Comparisons between C_t numbers indicate higher number of parasites in mesenteric lymph node than in the other tissues. Amount of parasites is higher during the acute period of the infection, particularly at the 4 and 7 DAI where they were 9-11 Ct values lower (2^9– 2^{11} – fold higher), or 256 to 1024-fold higher parasite burden in the intestine. This decreased at 14 DAI as the parasites spread throughout the pig's body to the muscle.

Table 2 - Average C_t values for Tg fluorogenic PCR assays of DNA from infected pig tissue samples:

Inf. Day / Tissue	2DAI	4 DAI	7 DAI	14 DAI
Ileum	TN	32	N	N
Lymph node	35	24	26	37
Liver	TN	30	32	36
Spleen	TN	34	N	N
Lung	TN	30	28	37
Muscle*	N	N	TN	37

N = not tested; TN = Tg negative

These results showed that Tg molecular assays were positive when used on pig products collected from deliberately Tg infected pigs. As expected the level of Tg DNA was positive but low in muscle tissues.

Objective 1) *Assess commercial pork products for Toxoplasma gondii (Tg) contamination by comparing results from meat juice anti-Tg antibody and cat bioassay results with the fluorogenic Tg DNA assay*

This grant supports the analyses of the fluorogenic Tg DNA assay for presence/contamination of Tg DNA in commercial pork products. Samples hat were collected nationwide, in a statistically validated process, under the USDA NRI IFAFS funded project coordinated by Drs. J.P. Dubey and D. Hill, APDL, BARC over a 3 year period. Aliquots of the 75 pork products collected in each of 28 market sampling area (MSA) were provided for molecular testing. For each MSA 8-13 mixed pools of 5 ground pork samples were prepared. Over the course of this NPB project a total of 236 aliquots of pooled pork product samples have been tested; this represented 1180 individual meat products. DNA was prepared from 25mg of each pooled meat sample and screened using the real-time PCR assays. Overall, 226 of the 236 samples were clearly positive for 18S DNA, affirming DNA quantity (Table 3). None of these 226 pork samples has been confirmed as positive for Tg by the Tg specific PCR assay (15). Therefore, the molecular testing indicates the possible absence, or low level, of Tg parasite contamination in the commercial cuts of pork. This conclusion is limited since a low Tg DNA parasite burden could result in a false negative, particularly because of the small sample size tested. Each sample was provided as a 2 g pool of meat from which 25 mg was selected to prepare DNA from which 100 ng DNA was used for the assay. It is likely that only a high Tg DNA burden would be detected using this method that was designed for high throughput testing. Although the results indicate that, if contaminated with Tg, the commercial pork samples have only small amounts of Tg. This would reduce the risk of serious disease.

Table 3. Results of DNA tests on commercial pork samples.

				Fluorogenic Real-time PCR Results			
MSA #	**Dates of collection**	**Sample Numbers**	**# Pooled Samples Tested**	**18s +**	**Tg –**	**?ID***	**Confirmed Tg +**
MSA1	May/June 2002	1-75	NT				
MSA2	July 2002	76-150	13	13	13	0	0
MSA3	August 2002	151-225	13	13	13	0	0
MSA4	September 2002	226-300	13	13	13	0	0
MSA5	October 2002	301-375	13	13	13	0	0
MSA6	November 2002	376-450	13	13	13	0	0
MSA7	December 2002	451-525	13	13	13	0	0
MSA8	January 2003	526-600	13	13	13	0	0
MSA9	February 2003	601-675	13	13	13	0	0
MSA10	March 2003	676-750	13	13	13	0	0
MSA1	April 2004	751-825	NT				

1							
MSA12	May 2003	826-900	NT				
MSA13	June 2003	901-975	NT				
MSA14	July 2003	976-1050	NT				
MSA15	August 2003	1051-1125	NT				
MSA16	September 2003	1126-1200	5	5	5	0	0
MSA17	October 2003	1201-1275	13	13	13	0	0
MSA18	Nov 2003	1276-1350	14	13	13	1	0
MSA19	December 2003	1351-1425	11	10	10	1	0
MSA20	January 2004	1426-1500	13	13	13	0	0
MSA21	February 2004	1501-1575	13	13	13	0	0
MSA22	March 2004	1576-1650	13	13	13	0	0
MSA23	April 2004	1651-1725	NT				
MSA24	May 2004	1726-1800	12	9	9	3	0
MSA25	June 2004	1801-1875	NT				
MSA26	July 2004	1876-1950	NT				
MSA27	August 2004	1951-2025	12	10	10	2	0
MSA28	September 2004	2026-2100	13	10	10	3	0
	Total	**420**	**236**	**226**	**226**	**10**	**0**
	%	*100%*	*56%*		*54%*	*2%*	*0%*
Pork samples represented by testing			**1180**	**1130**			

NT = not tested; *?ID = Tested, but insufficient or degraded DNA; C_t values for 18S and Tg both >40

To affirm results, individual aliquots of all pork samples from one MSA, where possible positive Tg samples were detected by bioassay, were provided for Tg molecular assay. The Tg assay was performed according to the established protocol, using the same conditions for each individual pork sample. Overall, 108 samples of pork were assayed, and all but one were negative for the presence of Tg DNA. The one positive sample when retested for Tg was negative. The results suggest the absence of

Tg in the commercial cuts of pork. This conclusion is limited in so far as a low parasite burden could result in a false negative, given the small sample tested. More pork samples, and larger aliquots of these samples, [at much higher costs] would need to be tested to absolutely prove this conclusion since such a small sample was tested for efficient sampling using molecular assays. Indeed, the definitive test results will be those collected and reported separately with the more detailed tests performed as part of Drs. Dubey and Hill's USDA NRI IFAFS project. All molecular test results will be compared to those results. When the IFAFS bioassay data is unblinded more samples will be tested. Samples that bioassay predicted to be positive will be retested and Tg DNA results compared to bioassay results. They hopefully will affirm the very low contamination of the US pork supply with Tg.

Objective 3) *Test the performance of the fluorogenic Tg molecular assays for assaying chicken and beef products*

To tailor the Tg detection assay for commercial chicken meats, three non-survey chicken samples (hearts and brains) were digested, and the DNA extracted. The chicken DNA was then spiked with Tg DNA and amplified using fluorogenic Tg PCR assay in order to determine the better testing method. It was established that extraction using the DNeasy DNA extraction kit showed the best results and PCR amplification results were positive. Once the testing of chicken samples was established, the fluorogenic Tg PCR assay was used to test chicken samples that had been brought from Brazil and confirmed to be Tg+ (22). These samples had been separately assessed for Tg burden using bioassays. DNA was successfully extracted using DNeasy from chicken samples including those that had positive results in the mouse bioassay. Every Tg positive Brazilian chicken sample tested gave positive results in the real time TG PCR assay. To date none of the chicken samples collected during the IFAFS project have been tested using the molecular assays.

Discussion: Validation of molecular diagnostic tests are required so that better tools will be available to the industry to quickly identify and isolate pork products that carry human pathogens like Tg. The fluorogenic Tg DNA assay should help to identify carcasses of pigs with this foodborne infection and eliminate them from the food supply. Because of the assay's specificity and sensitivity, it should be possible to accurately certify pig products as Tg-free. Pretest assays showed positive results for 25 mg tissue samples with a high parasite burden. Our results proved that detection of Tg DNA in tissues that had been experimentally infected with a high number of parasites was effective. However, when this assay was applied to commercial cuts of pork, the proven low parasite burden in pigs and the small (25mg) sample size must be taken into account. The results, that 226 samples representing 1180 commercial pork products were negative for Tg DNA, suggest the absence of Tg in the commercial cuts of pork. This conclusion is limited since a low parasite burden could result in a false negative, given the small sample tested. The process can be likened to trying to find a needle in a haystack. More pork samples, and larger aliquots of these samples, [at much higher costs] would need to be tested to absolutely prove this conclusion. The definitive test results will be those collected and reported separately with the more detailed tests performed as part of Drs. Dubey and Hill's USDA NRI IFAFS project. When the IFAFS bioassay data is unblinded more samples will be tested. Samples, that the bioassays predicted to be positive, will be retested and Tg DNA results compared to bioassay results. Those results hopefully will affirm the very low contamination of the US pork

supply with Tg. In summary, while the data suggests the absence of Tg, further testing must be done for confirmation.

Lay Interpretation: Preventing pork associated foodborne diseases is important to pork producers. Infection with the parasite, *Toxoplasma gondii* (Tg), is a known risk factor associated with pork consumption. Thus assays that help producers find and eliminate such infections are needed. Current methods for Tg detection are based on serology and bioassays. These assays are dependent on time for serology, to get antibodies that are detectable, and on live animals for the expensive bioassays. The sensitive molecular assay for Tg DNA was developed to help producers and regulators to more easily test pork products for possible Tg contamination. Using high throughput techniques and improving this molecular assay could easily make the fluorogenic Tg DNA assay adaptable to work with miniaturized monitors to perform ante-mortem Tg tests. However, sensitivity and specificity of such assays, at the whole animal level, and in the final pork products, must be addressed.

Pork products were collected in markets nationwide in a statistically valid sampling under a separate USDA IFAFS grants by Drs. Hill and Dubey and their collaborators. Data reported here used the fluorogenic Tg DNA assay to check for Tg contamination of small aliquots from 226 pools representing 1180 pork products. The data showed 0/226 samples as positive for Tg DNA, indicating no or low level Tg contamination of these products. Further testing, directly comparing these DNA assay results with the well-established Tg bioassay in cats, and the meat juice antibody assay, is in process and should determine the validity of this molecular test. The combined results should show the actual level of Tg contamination in the nation's pork products. Results using all of these assays should help affirm the actual low risk to the consumer of getting a foodborne Tg infection from the US pork supply. They will help assure our international trading partners of the high quality and safety of US pork products.

References:

1. Dubey JP. 1994. Toxoplasmosis. J Am Vet Med Assoc. 205:1593-1598
2. Dubey JP. 2004. Toxoplasmosis - a waterborne zoonosis. Vet Parasitol. 126:57-72.
3. Jones JL, Kruszon-Moran D, et al. 2001. *Toxoplasma gondii* infection in the United States: seroprevalence and risk factors. Am J Epidemiol. 154:357-365.
4. Center for Disease Control (CDC). "Toxoplasmosis." http://www.cdc.gov/ncidod/dpd/parasites/toxoplasmosis/default.htm
5. Lindsay DS, Collins MV, et al. 2003. Sporulation and survival of *Toxoplasma gondii* oocysts in seawater. J Eukaryot Microbiol. 50 Suppl: 687-688.
6. Montoya JG, Liesenfeld O. 2004. Toxoplasmosis. Lancet. 363: 1965-1976.
7. Jones JL, Kruszon-Moran D, Wilson M. 2003. *Toxoplasma gondii* infection in the United States, 1999-2000. Emerg Infect Dis. 9: 1371-1374.
8. Lopez A, Dietz VJ, Wilson M, Navin TR, Jones JL. 2000 Preventing congenital toxoplasmosis. MMWR Recomm Rep. 49(RR-2):59-68.
9. Jones JL, Ogunmodede F, et al. 2003. Toxoplasmosis-related knowledge and practices among pregnant women in the United States. Infect Dis Obstet Gynecol. 11: 139-145.
10. Tenter AM, Heckeroth AR, Weiss LM. 2000. *Toxoplasma gondii*: from animals to humans. Int J Parasitol. 30:1217-58.
11. Kijlstra A, Eissen OA, et al. *Toxoplasma gondii* infection in animal-friendly pig production systems. 2004. Invest Ophthalmol Vis Sci. 45: 3165-3169.

12. Gresham CS, Gresham CA, Duffy MJ, Faulkner CT, Patton S. 2002. Increased prevalence of *Brucella suis* and pseudorabies virus antibodies in adults of an isolated feral swine population in coastal South Carolina. J Wildl Dis. 38: 653-656.
13. Davies PR, Morrow WE, Deen J, Gamble HR, Patton S. 1998. Seroprevalence of *Toxoplasma gondii* and *Trichinella spiralis* in finishing swine raised in different production systems in North Carolina, USA. Prev Vet Med. 36: 67-76.
14. Villard O, Filisetti D, et al. 2003. Comparison of enzyme-linked immunosorbent assay, immunoblotting, and PCR for diagnosis of toxoplasmic chorioretinitis. J Clin Microbiol. 41: 3537-3541.
15. Jauregui L, Higgins J, Zarlenga D, Dubey JP, Lunney JK. 2001. Development of a Real-Time PCR Assay for Detection of *Toxoplasma gondii* in Pig and Mouse Tissues. J. Clin. Microbiol. 39: 2065-2071.
16. Lunney J, Jauregui LH, Higgins J, Zarlenga D, Dubey JP. 2004. Polymerase chain reaction assay for detection of *Toxoplasma gondii*. United States Patent # 6,770,440 issued August 3, 2004.
17. Bastien P. 2002. Molecular diagnosis of toxoplasmosis. Trans R Soc Trop Med Hyg. 96 Suppl 1:S205-15.
18. Buchbinder S, Blatz R, Rodloff AC. 2003. Comparison of real-time PCR detection methods for B1 and P30 genes of *Toxoplasma gondii*. Diagn Microbiol Infect Dis. 45: 269-271.
19. Warnekulasuriya MR, Johnson JD, Holliman RE. 1998. Detection of *Toxoplasma gondii* in cured meats. Int J Food Microbiol. 45: 211-215.
20. Aspinall TV, Marlee D, Hyde JE, Sims PF. 2002. Prevalence of *Toxoplasma gondii* in commercial meat products as monitored by polymerase chain reaction--food for thought? Int J Parasitol. 32: 1193-1199.
21. Hill D, Dubey JP. 2002. *Toxoplasma gondii*: transmission, diagnosis and prevention. Clin Microbiol Infect. 8: 634-640.
22. Dubey JP, Graham DH, et al. 2002. Biological and genetic characterisation of Toxoplasma gondii isolates from chickens from Sao Paulo, Brazil: unexpected findings. Int J Parasitol. 32: 99-105.

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