RESEARCHREPORT

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PORK SAFETY

Title: Development of a sensitive molecular assay to detect *Toxoplasma gondii* DNA in biological samples **NPB #00-132**

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I. **Abstract**: A quantitative molecular technique was developed and standardized to detect *Toxoplasma gondii* (Tg) DNA in biological samples. This real time fluorogenic Tg assay is a highly sensitive and specific method to reproducibly detect and quantitate Tg burden in animal products. Assay specificity was confirmed against a panel of DNA samples from different isolates of Tg, from other common protozoa, as well as from host animal tissues. The real time fluorogenic Tg assay uses polymerase chain reaction (PCR) primers proven to be specific only for Tg with detection based on a fluorogenic probe using the real-time TaqMan PCR technology. Specificity, or lack of cross reactivity, was checked by testing DNA samples from closely related parasites and from muscle tissues prepared from uninfected pigs and mice.

The sensitivity limits for this real time fluorogenic Tg assay were defined in several ways: 1) test serial dilutions of Tg DNA alone; 2) assess detection sensitivity for Tg DNA in pig tissues artificially infected with different doses of Tg, and 3) compare fluorogenic Tg assay results versus Tg bioassay in mice. All results were statistically analyzed. The real time fluorogenic Tg assay was able to detect as little as 0.1 pg of Tg genomic DNA, which is equivalent to 1 Tg bradyzoite. The assay has a dynamic range of detection over 6-7 log of Tg DNA concentration (from 100 ng to 100 fg). Tissues from Tg experimentally infected mice and pigs, as well as bradyzoite-spiked pig muscle samples, were used to test and standardize this technique. Positive signals were obtained with Tg parasite concentrations, ranging from as few as 4 parasites to $3.7x10^5$ parasites per gram of spiked pig tissue, with excellent linearity (R^2= 0.9776). All Tg infected animals were correctly identified using this technique. Results indicate this assay is applicable to testing swine carcasses and commercial pig products, is compatible with automation technology for potential slaughterhouse usage, and will enable scientists to diagnose and quantitate Tg in animal tissues. Such a sensitive fluorogenic Tg assay will speed results to producers and regulators, and will enable researchers to directly test pork products for possible Tg contamination.

II. **Introduction**: Infection of swine with the protozoan parasite, *Toxoplasma gondii* (Tg), continues to be a food safety concern. The parasite can be transmitted to humans by ingesting Tg oocysts in food or water contaminated with infected cat feces or by consuming tissue cysts in raw or undercooked pork or lamb meat. Pork is considered the most important meat source of Tg in the US (1). Infection with this parasite causes potentially serious consequences in humans, like birth defects, perinatal mortality and toxoplasmic encephalitis in immunocompromised individuals, e.g., transplant recipients, AIDS patients and the elderly (2). Thus, preventing Tg contamination of pork is a food safety priority for pork producers.

Although under current production conditions toxoplasmosis does not heavily affect profitability in pig farms, clinical, or more often subclinical, infections may provoke reproductive failures (3). However, from a consumer's point of view, Tg contamination in swine carcasses or pork products poses a food safety problem and thus can have a negative impact on pork consumption. Moreover, Tg-free status for US pork products will be required for full access to expanding international trade markets. Most reports on Tg prevalence in pigs are based on serology (MAT test or ELISA) from market and breeding age animals. Surveys show that the infection rate at the individual pig level is in steady decline. However, the rate of infected farms is still quite alarming, with values ranged from 22% to 90% in breeding herds (4, 5), and 7% in finisher herds (6). Very few Tg prevalence reports are based on detection of the parasite itself in field samples.

Pork producers have established a Toxoplasmosis Working Group to actively assess and address the Tg problem. However, it is still not clear whether they have the 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 of sera with other organisms (7, 8), and false negative reaction due to tests of animals early in Tg infection, i.e., before they elicit an antibody response. Infectious Tg tissue cysts can appear as soon as 2-7 days post infection and persist lifelong. Identification of viable Tg by 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 (bradyzoites), present in tissue samples. Molecular techniques should be more sensitive and quantitative; they should help overcome the difference between bioassay and serologic results.

There are many reports of molecular detection assays, i.e. Polymerase Chain Reaction (PCR), for Tg but most of them were developed for human or mouse diagnostics or for phylogenetic studies (9, 10). There are a few PCR assays for toxoplasmosis in veterinary medicine (11, 12) but only one report of PCR use for Tg diagnosis in ready-to-eat cured meats with a sensitivity limit of 10^5 trophozoites/gram meat (13). Since most field samples have low levels of Tg a very sensitive PCR protocol is necessary. In the last year, with NPPC funding we developed a "Real time fluorogenic Tg assay" (14). This assay is quick (results in <2 days), does not require the use of live animals, tissue culture or parasites, and is relatively low cost.

III. **Objectives**:

A. To develop a highly sensitive and specific molecular technique to detect Tg DNA in biological samples
B. To test the performance of this Tg assay using known infected and non-infected tissues.
C. To compare sensitivity of this Tg DNA detection assay against mouse bioassay

IV. **Procedures:**

A. <u>Development of a quantitative real time fluorogenic Tg assay</u>**:** The real time fluorogenic Tg assay is a PCR based molecular technique to detect Tg DNA using an ABI Prism 7700

Sequence Detector (Perkin-Elmer, Applied Biosystems, Foster City, CA). Primers for this assay were designed by aligning Tg 18S rRNA gene sequences from its ITS1 region (Genbank access # X75429.1, RH strain) and other coccidian parasites. This region was selected because it has been reported to have a high rate of nucleotide substitutions (15). A real time fluorogenic Tg probe sequence was designed and synthesized being dually labeled with a reporter dye (FAM, 6-carboxifluorescein) in the 5' end and a quencher molecule (TAMRA, 6-carboxitetramethyl-rhodamine) covalently coupled in the 3' end. Primer and probe sequences for detection of Tg and for normalization with housekeeping genes are shown in Table 1. The 50 µl assay mixture containing 1X TaqMan buffer A (PE Biosystems), 1.5 mM $MgCl_2$, 1 µM each ITS1 primer, 200 nM of fluorogenic probe, 200 nM of each dNTP, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer) was run with up to 1 µg of the template DNA. After activation of the Taq polymerase for 10 min at 95ºC, 50 cycles of 15 sec at 94ºC followed by 1 min at 60ºC were done an ABI Prism 7700 "TaqMan" machine. Reporter's fluorescence was acquired at the end of each annealing step. The PCR product was also electrophoresed in an agarose gel, and stained with ethidium bromide. All the TaqMan data acquisition and data analysis were performed with Sequence Detector Software (SDS version 1.7) and C_T values were recorded for statistical analysis. Once the standard curve is set, any unknown sample can be run with TaqMan technology and by knowing the C_T value the original Tg DNA concentration can be accurately estimated.

B. Infected Mouse and Pig Tissues for Testing assay: The standard curve for the real time fluorogenic Tg assay was run on DNA prepared from Tg tachyzoites harvested from RH strain Tg infected mice. Tachyzoites were produced as previously described (12). DNA from a known number of tachyzoites was extracted following classical procedures; the pellets were treated with DNA digestion buffer plus Proteinase K overnight. DNA quality and quantity was evaluated by OD 280/260. Tenfold dilutions of the Tg DNA were tested in different PCR protocols to establish the assay sensitivity. Tg cysts and bradyzoites were prepared from female Swiss-Webster mice (5-10/group) infected with different oocyst doses (10^3 to 10^{-1}) of the VEG cyst-forming Tg strain. After a 4-6 weeks period, to reach the chronic stage, the mice were bled for serum antibodies and sacrificed, their brains removed and split in two. Pig brain and tongue samples were collected from Tg-infected (n=20) and non-infected (n=6) pigs. To harvest bradyzoite Tg DNA from cysts, mouse brain or pig tissue samples were first homogenized and digested with pepsin-HCl solution by stirring at 37ºC for 90 min; and the crude extract was spun down by centrifugation. Portions of the tissues was tested by a real time fluorogenic Tg assay and the other used directly for microscopic detection of Tg cysts or for mice bioassay, as previously described (14, 16). The pellets were treated with DNA digestion buffer plus Proteinase K overnight (14). DNA was extracted by the standard phenol chloroform (PCIA) method.

C. Spiked pig muscle samples: Since bradyzoites in cysts represent the chronic Tg parasite-stage found in tissues of naturally and experimentally infected pigs, we focused first on their detection. Our work on this assay showed that we can spike pig muscle samples with this parasite stage, rather than tachyzoites, because of the bradyzoite's resistance to pepsin digestion (14). To obtain bradyzoites, VEG strain Tg-infected mouse brains were dissected and processed in pepsin-HCl solution for 2-3 min., neutralized, and bradyzoites counted in hemocytometer chamber. Known numbers of bradyzoites were added to 50 g of normal pig muscle, DNA prepared and tested by both the molecular techniques and the mouse bioassay. Every cyst or bradyzoite dilution was tested by a molecular technique at least twice, on different days, in order to reduce the standard error and to assess the bias introduced by human errors.

D. Statistical analysis: Results were recorded and statistically analyzed to determine

whether the Real time fluorogenic Tg assay was as sensitive as mouse bioassay, serology and pathology findings. All data were submitted to split plot analyses, using 2 treatments (molecular technique versus mouse bioassay) and up to 10 replicates within each treatment. Differences among means were identified by ANOVA analyses, using SAS software.

V. **Results:**

A. Assay Specificity: The real time fluorogenic Tg PCR assay was developed to determine the Tg tissue burden in infected pig and mouse tissues. This assay is based on amplification of a known DNA sequence and to be most sensitive we chose ribosomal DNA gene (rDNA) as target gene because it has >100 copies per Tg genome. To enhance specificity, a set of primers was designed for the ITS1 region within rDNA gene, where major differences are found among closely related parasites, *Neospora caninum* and *Hammondia hammondi* (15) (Table 1). Specificity of these primers was confirmed in direct PCR assays (Fig. 1A, B). Positive PCR products were found with all DNA samples with universal 18S rRNA primers (Fig.1A) whereas only Tg DNA was positive with the Tg specific ITS1 region primers (Fig.1B). These results were confirmed with the real time fluorogenic Tg assay (Fig.1C). As the TaqMan PCR reaction proceeds the higher the amount of initial target gene the higher will be the reporter probe's fluorescence emission (red line, Fig.1C), thus the higher the copy target gene number leads to lower C_T values. Our specificity in the real time fluorogenic Tg assay was verified by running DNA samples of control pig and mouse DNA, and DNA from related parasites, *N. caninum* and *H. hammondi*, using our Tg rDNA primers (Fig.1C).

B. Assay Sensitivity and range of detection: Assay sensitivity limits were defined in several ways: 1) test serial dilutions of Tg DNA alone; 2) assess detection sensitivity for Tg DNA in pig tissues artificially infected with different doses of Tg, and 3) compare real time fluorogenic Tg assay results versus Tg bioassay in mice. All results were statistically analyzed. To estimate the sensitivity of the real time fluorogenic Tg assay, amplification of ten-fold dilutions, containing from 100 ng to 10 fg of Tg genomic DNA, was performed in duplicate. The fluorogenic Tg PCR assay was able to detect as little as 0.1 pg of Tg genomic DNA, which is equivalent to 1 Tg bradyzoites. The real time fluorogenic Tg assay has a dynamic range of detection over 6-7 log of Tg DNA concentration (from 100 ng to 100 fg). The PCR products generated through the real time fluorogenic Tg assay were also tested by standard gel staining techniques. In agreement with the C_T values, bands of expected size were detected down to 100 fg Tg DNA although the bands for 1 pg and 100 fg were dim (Fig. 2B lanes F, G). Figure 2C shows the standard curve of the mean C_T values from 8 replicates of the series of Tg DNA concentrations. The C_T values, ranging from 15.52 ± 0.21 for 100 ng of Tg genomic DNA to 38.41 ± 0.64 for 100 fg, showed reproducible linearity over seven orders of magnitude (R^2 = 0.9984). A significant coefficient of correlation was found for the mean C_T values and Tg DNA concentrations (r = - 0.9793). Thus, we have developed our first stage real time fluorogenic Tg PCR assay.

C. Precision of real time fluorogenic Tg assay: The real time fluorogenic Tg assay precision intra-assay (white bars) (Fig. 3) was measured on 10 replicates of three Tg DNA concentrations and spiked pig samples tested on the same day, and expressed as mean C_T values. Results showed low variability with a CV of <1.75% for the lowest Tg genomic DNA concentration (10 pg) and <1% for the Tg-spiked sample. The precision inter-assay (black bars) (Fig. 3), expressed as mean C_T values from 20 replicates (4 replicates on 5 different days), presented a CV of 4.5% for Tg genomic DNA and a CV of 1.7% for the Tg-spiked sample.

D. Real time fluorogenic Tg PCR assay results on bradyzoite spiked pig tissues and

samples from animals experimentally infected with Tg. Tissues from Tg experimentally infected mice and pigs, as well as bradyzoite-spiked pig muscle samples, were used to test and standardize this technique. Positive signals were obtained with Tg parasite concentrations, ranging from 4 parasites to $3.7x10^5$ parasites per gram of spiked pig tissue, with excellent linearity (R^2= 0.9776). All Tg infected animals were correctly identified using this technique. The capability of our Real time fluorogenic Tg assay to correctly identify Tg-infected animals with a high degree of sensitivity was demonstrated for both parasite infected mice and pigs, and was shown to work on multiple stages of infection. These results were corroborated by both serological response of all infected animals and presence of tissue cysts in the brain of infected mice.

E. Discussion:

The real time fluorogenic Tg assay has been developed to determine the Tg tissue burden in infected pig and mouse tissues. Our results indicate this real time fluorogenic Tg assay is applicable to testing swine carcasses and commercial pig products, is compatible with automation technology for potential slaughterhouse usage, and will enable scientists to diagnose and quantitate Tg in animal tissues. In the future such analyses could be carried out using multiplex TaqMan assays with different fluorogenic probes. A sensitive real time fluorogenic Tg assay will prevent use of the bioassay, i.e., use of live animals for accurate Tg detection, will speed results to producers and regulators, and will enable researchers to directly test pork products for possible Tg contamination.

With a combined tissue digestion, fast DNA preparation, and real time fluorogenic Tg assay it is expected that Tg detection could be completed within 24 hrs, if not less time. This newly developed assay will thus help to monitor the contamination of Tg in commercial meat products, and ultimately reduce foodborne transmission of this harmful parasite. Moreover, this real time fluorogenic Tg assay will provide an objective tool for quantitating Tg burden for vaccination studies and clinical trials for therapeutic treatments.

VII. References:

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Table 1: Sequences of primers and probes used in this study.

Gene	Primers /Probe sequences
Universal 18S rRNA	5' CGGCTACCACATCTAAGG 3' (sense) 5' TATACGCTATTGGAGCTGG 3' (anti-sense)
Toxo ITS1 primers	5' GATTTGCATTCAAGAAGCGTGATAGTA t 3' (sense) 5' AGTTTAGGAAGCAATCTGAAAGCACATC 3' (anti-sense) 5' 6-FAM-CTGCGCTGCTTCCAATAT TGG-TAMRA 3' (probe)
Mouse (Hu) beta-actin	5' TCACCCACACTGTGCCCATCTACGA-3' (sense) 5' CAGCGGAACCGCTCATTGCCAATGG 3' (anti-sense) 5' 6-FAM-ATGCCC-x (TAMRA)-CCCCATGCCATCCTGCGT 3' (probe)

Figure 1: Comparison of amplification of parasite and host DNA samples using direct PCR and *Toxoplasma gondii* fluorogenic assay.

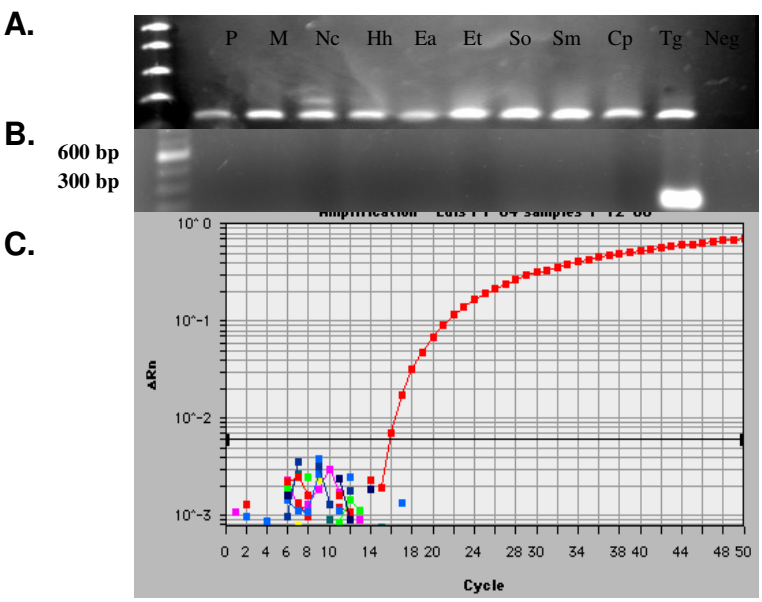
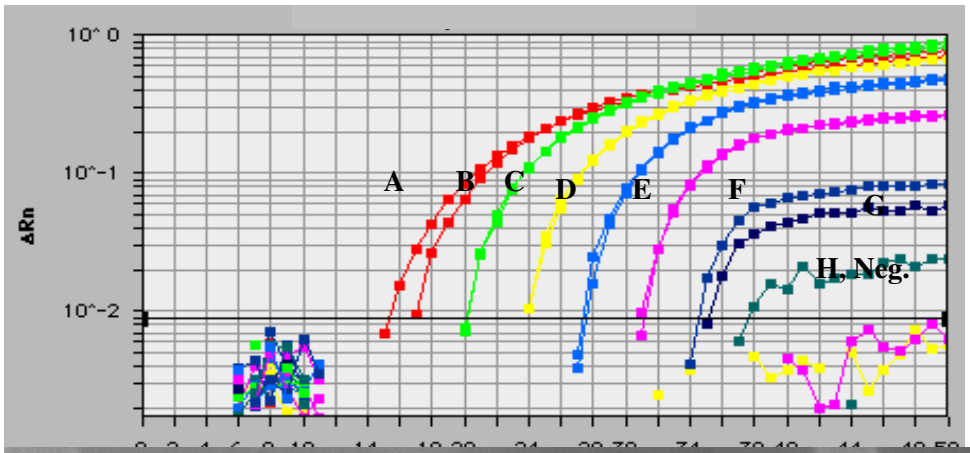


Fig.1. Analyses were performed by direct PCR using universal 18S rRNA primers (**A**), or *Toxoplasma gondii* ITS1 primers, Tg primers (**B**). The PCR products were electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. For the fluorogenic assay (**C**), samples were assayed on an ABI Prism 7700 Sequence Detector using the SDS version 1.7 software. Reporter dye fluorescence is plotted on the Y-axis (ΔRn) and cycle number (C_T) on the x-axis. The amplification plot shows test results from Tg DNA through 50 cycles (red); other samples are below the threshold line, indicated in bold. Specificity with the Tg primers was confirmed by assaying approximately 150 ng of each DNA sample was tested; P (pig), M (mouse), and protozoan parasite DNA samples: Nc (*Neospora caninum*), Hh (*Hammondia hammondi*), Ea (*Eimeria acervulina*), Et (*E. tenella*), St (*Sarcocystis tenella*), Sm (*S. muris*), Cp (*Cryptosporidium parvum*), Tg (*T. gondii*), and Neg (no DNA control).

Figure 2: Sensitivity determination of the *Toxoplasma gondii* fluorogenic assay.

2A



2B

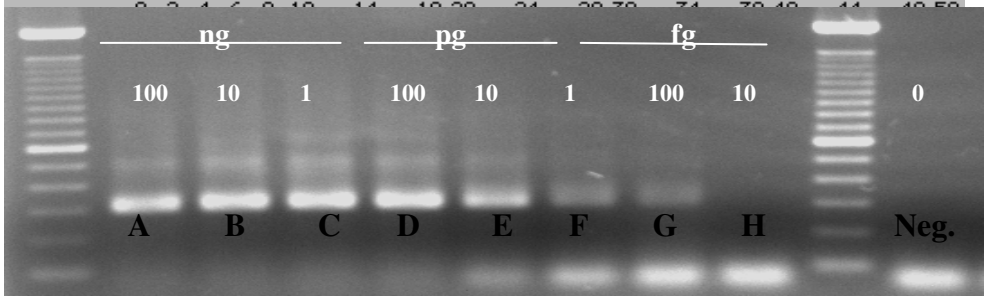


Fig.2A. Dilutions of *Toxoplasma gondii* DNA containing 100 ng (A), 10 ng (B), 1 ng (C), 100 pg (D), 10 pg (E), 1 pg (F), 100 fg (G) and 10 fg (H) plus no template controls (Neg.) were assayed. The assay threshold limit is shown as a bold line. **Fig.2B**. The PCR products were electrophoresed in 1.5% agarose gel and stained with ethidium bromide. Both assays show positive products down to 10 fg Tg DNA (G).

Figure 3: *Toxoplasma gondii* fluorogenic assay precision for detecting *T. gondii* DNA.

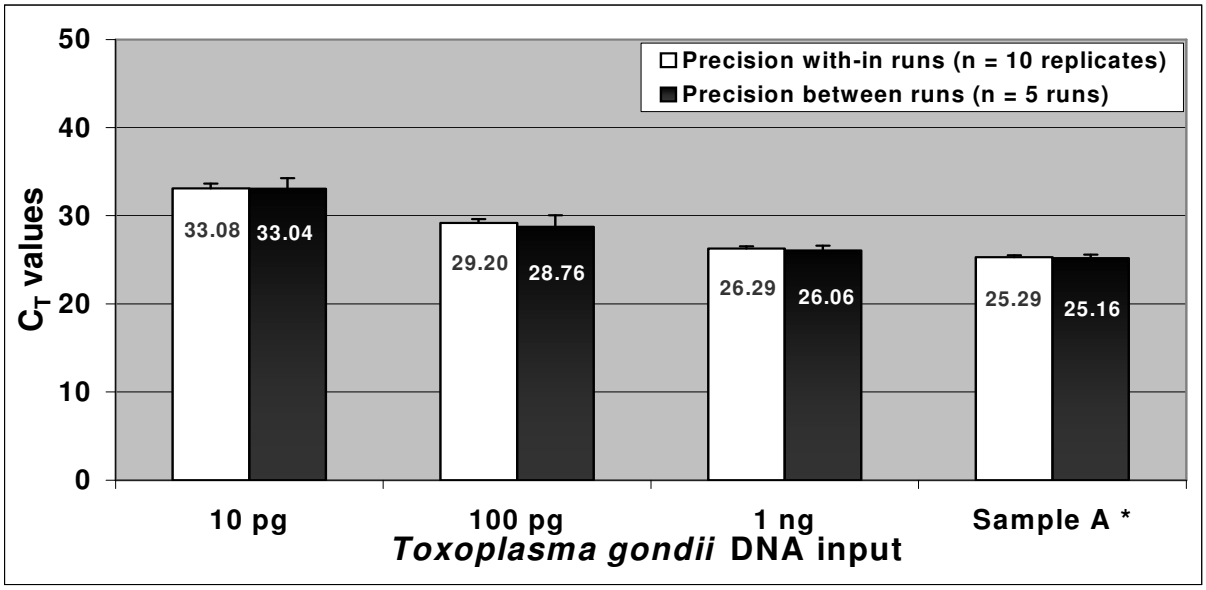


Figure 3: Precision intra-assay for the detection of genomic *T. gondii* DNA (1 ng, 100 pg, 10 pg) and a bradyzoite-spiked pig sample (sample A) is represented by the mean cycle number (C_T) value from 10 replicates tested simultaneously (white bars). Repeatability of the Toxo TaqMan assay is shown as the mean C_T values ± 1 SD from 4 replicates of same *T. gondii* DNA samples analyzed at 5 different days (black bars). *Sample A: 1g pig muscle sample spiked ~3.7 x 10^4 *T. gondii* bradyzoites.