RESEARCHREPORT
PORK SAFETY

I. Title: Evaluation of Toxo TaqMan Assay for use in Detection of Swine Toxoplasmosis, **NPB #01-082**

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II. Abstract:

This grant was aimed at adapting technologies so that our molecular assay for detection of the foodborne parasite, *Toxoplasma gondii* (Tg), could be used for a wide range of pork tissues. Our previous data, developed by Jauregui (1) with NPB funding, proved that we could use a real time Tg polymerase chain reaction (PCR) assay, previously termed the Toxo TaqMan Assay, to detect Tg DNA in animal tissues with high sensitivity and specificity. In order to facilitate the work with a large number of samples, fast and simple commercial DNA extraction kits were evaluated and compared to the previous DNA preparation method. Two commercial kits, DNeasy and DNAzol, were tested and shown to produce good quality DNA. Using these kits real time Tg PCR assays were performed on Tg infected mouse, pig and chicken samples. Our results showed that these commercial kits could be used to produce the DNA samples to identify potential Tg contamination in different animal tissues using the real time Tg PCR assay.

III. Introduction:

Toxoplasma gondii (Tg) is a foodborne parasite that infects most mammals and is zoonotic, passing from animals to humans. It can cause serious birth defects or abortion in woman, if they are infected during pregnancy. Once infected the parasite remains dormant in the brain, this can result in encephalitis in immunocompromised persons due to parasite reemergence. The parasite is thought to be transmitted to humans by the ingestion of food or water contaminated with oocysts eliminated in cat feces or the consumption of parasite cysts present in infected tissues. Pork meat is cited as one of the most important sources of *T. gondii* infection (2). Currently, detection of Tg parasite in pig herds is based on serological tests and/or bioassay of pig tissues in cats or mice.

Under current production conditions toxoplasmosis does not heavily affect profitability in pig farms, although clinical, or more often subclinical, infections may provoke reproductive failures (3). However, the presence of Tg in meats poses a food safety problem for consumers and thus can result in a negative impact on pork consumption. Moreover, Tg-free status for US pork products may be required for full access to expanding international trade markets. Most reports of Tg prevalence in pigs are based on serology (MAT test or ELISA) from market and breeding age animals. In 1995 Dubey et al. (4) compared Tg epidemiologic study results based on bioassay in mice and cats to antibody tests; they determined Tg prevalences in Iowa sows to be 17% and 22%,

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respectively. Growing/finishing swine were much less positive (3.1%) (5). Since that time, the rate of infected pigs has been dramatically reduced to 0.58%; only 13 of 2238 samples tested were positive for Tg by MAT assay (6). Of those tested 12 of 13 positive pigs were found to be among the 63 pasture raised pigs tested, indicating the substantially higher Tg risk due to pigs from outdoor facilities (6, 7). Very few Tg prevalence reports are based on detection of the parasite itself in field samples.

Pork producers have established an NPB Toxoplasmosis Working Group to actively assess and address the Tg problem. For this, proper diagnostic tools are required. Despite many advantages, serology has some problems: false positive reactions due to cross reactivity of sera with other organisms and false negative reaction due to tests of animals early in Tg infection, i.e., before they elicit an antibody response (8). Infectious Tg tissue cysts can appear as soon as 2-7 days post infection and persist lifelong. Identification of viable Tg by cat or 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 the actual Tg tissue burden, i.e., the presence, not the number, of cysts, nor number of infectious organisms (bradyzoites), present in tissue samples. Molecular techniques have the advantage of being more sensitive and quantitative and should be an important tool for Tg detection. As part of our previous NPB funding a quantitative molecular technique was developed and standardized by Jauregui (1) to detect Tg DNA in biological samples. The real time Tg PCR assay (previously known as "TaqMan") has a high sensitivity and specificity to detect and quantitate Tg burden in animal tissues in a relatively short time.

IV. Objectives:

a. Improve the utility of the real time Tg PCR assay to detect Tg DNA from known infected and non-infected pigs.
b. Develop faster methods to produce Tg DNA for the assay
c. Test the performance of the assay using commercial meat products

V. Procedures:

a. Tissue Processing:

Infected Mouse and Tissues for Testing assay: DNA preparations were produced from Tg tachyzoites, harvested from RH strain Tg infected mice. Tg cysts and bradyzoites were obtained from brains of female Swiss-Webster mice infected with oocyst of the VEG cyst-forming Tg strain.

Pig samples: Muscles samples from VEG strain oocyst infected pigs were collected from pig ham muscle when killed at 14 days of infection. DNA was prepared directly from the muscle samples using manufacturer's directions.

Chicken samples: Chicken samples collected in Brazil (9) were bioassayed in mice after pepsin digestion of the samples. Positive digested samples were kept at –20°C for DNA extraction and real time Tg PCR assay evaluation.

b. DNA purification: DNeasy™ (Qiagen) and DNAzol® (Life Technologies) were tested for DNA extraction using manufacturer's directions. DNA quality and quantity were compared to those generated using the standard Jauregui method (1) where the tissue pellets were treated with DNA digestion buffer plus Proteinase K overnight followed by DNA extraction by the standard phenol chloroform method. DNA concentration was estimated by spectrophotometry at 260nm.

c. Real time PCR assay: Real time Tg PCR assay was performed with extracted DNA using 1) universal 18S rRNA primers to confirm DNA presence and 2) Tg specific ITS1 region primers to confirm Tg contamination. Results were analyzed using ABI 7700 Sequence Detector System

(SDS) and SDS software. As the real time PCR reaction proceeds the primers produce more product that then binds the reporter probe and causes fluorescence emission to increase.

VI. Results:

A. Comparisons of different commercial DNA extraction methods

The purpose of these preliminary studies was to develop sample preparation procedures that were adaptable to a broad range of laboratory situations and that involved no hazardous chemicals. For these studies we tested two different commercial DNA extraction methods and compared them to our previous standard DNA extraction method. The DNA extraction protocol that we published as a result of our previous NPPC grant #00-132 (1) was a standard method that required an overnight incubation with enzymatic (proteinase K) digestion of the tissues followed by DNA extraction with hazardous chemicals (phenol-chloroform-isoamyl alcohol and precipitated in Na citrate and ethanol).

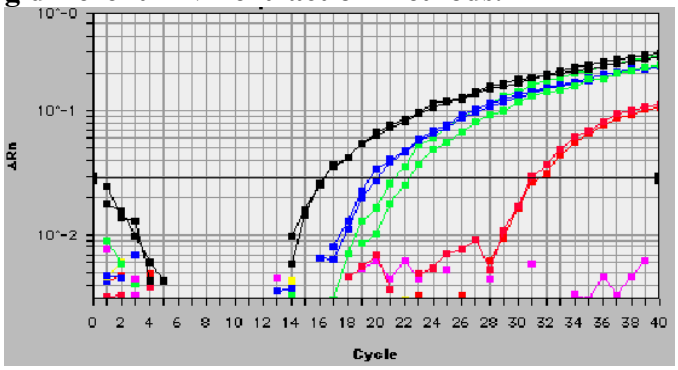
The Gibco Life Technologies DNAzol® reagent uses a guanidine-detergent lysing solution that permits the selective precipitation of DNA from the cell lysate using ethanol treatment. It is a fast protocol that permits isolation of DNA from large number of samples and can be completed in 40 minutes. The Qiagen DNeasy™ Tissue kit uses proteinase K digestion and a silica-gel membrane for DNA recovery, without organic extraction and ethanol precipitation. The procedure can be completed in 20 minutes after the tissue digestion, which depending on the type of the tissue tested, can take 1-3 hours. The DNeasy™ protocol does not involve hazardous chemicals, e.g., guanidine used in DNAzol. The digestion and DNA recovery was first evaluated using only the purified Tg parasites (tachyzoite stage). Once those data proved fruitful then tissues from Tg infected mice were tested.

The starting material was replicate pellets (samples 1 and 2) of tachyzoites obtained from centrifugation of peritoneal lavage of Tg infected mouse. After the extraction of Tg tachyzoites, the concentration and the quality of the DNA were estimated by spectrophotometry absorbance (A) at 260 and 280nm (A_{260}/A_{280}) wavelength and the Tg DNA content evaluated by the fluorogenic real time *T. gondii* detection assay (Table 1 and Figure 1). Positive PCR products were found with all DNA samples with the Tg specific ITS1 region primers. As the real time PCR reaction proceeds the reporter probe's fluorescence emission increases, the higher the amount of initial target gene, e.g., Tg DNA, leading to lower C_T values.

Table 1. Results from the DNA recovered from Tg tachyzoites:

Method	Sample	Concentration (µg/ml)	A_{260}/A_{280} ratio	C_T value
Jauregui	1	140	1.2	30
	2	122	1.4	19
DNAzol	1	16	2.1	21
	2	56	1.8	16
DNeasy	1	92	2.0	18
	2	102	1.9	16

Figure 1. Amplification in the fluorogenic real time *T. gondii* detection assay of DNA prepared from Tg tachyzoites using different DNA extraction methods.



Legend for colors: Positive control, Jauregui protocol, DNAzol, DNeasy, No template control (NTC), No amplification control (NAC)

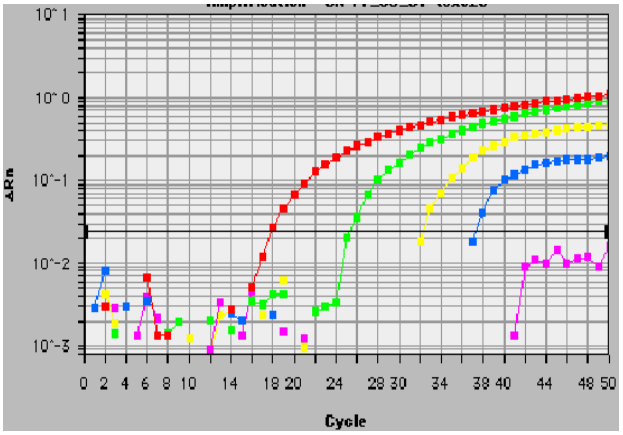
The Jauregui DNA extraction protocol is the most time consuming and, although the spectrophotometric readings indicated the highest concentration values in µg/ml, the purity of the samples was low as determined by the A_{260}/A_{280} ratio of <1.6 (Table 1). In comparison both the DNAzol and DNeasy kits resulted in good DNA quality (A_{260}/A_{280} ratio 1.6-2.0). The DNAzol method is the fastest technique but the final DNA recovery is better with the DNeasy method (more µg/ml DNA). Results from the real time Tg detection assay indicated that the best amplification results were obtained with the DNA recovered from tachyzoites using either the DNAzol and DNeasy kits (Figure 1).

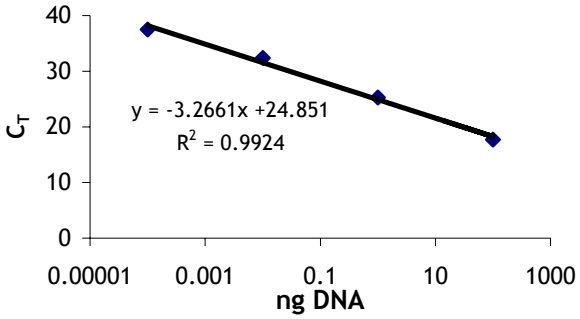
Extraction methods were next tested using tissues from Tg infected mice. As with the Tg tachyzoites, the Jauregui protocol resulted in the highest concentration, but lowest quality, of DNA. That low quality was confirmed by the low A_{260}/A_{280} ratio and weak positive signal in the real time Tg PCR assay (Table 2). The DNeasy kit eluate had the best DNA purity estimated at µg DNA recovered and the best signal in the real time Tg detection assay (lowest C_T value). Good quantities of DNA were prepared from infected mouse brains using the DNAzol procedure but the A_{260}/A_{280} ratio was lower and the C_T value higher than those obtained with the DNeasy kit (Table 2).

Table 2. Results for DNA recovery from Tg infected mouse brain samples:

Method	Sample	Concentration (µg/ml)	A_{260}/A_{280} Ratio	C_T values (2µl)
Jauregui	1	173	1.12	39
	2	139	1.01	40
DNAzol	1	174	1.17	40
	2	97	1.74	40
DNeasy	1	144	1.63	34
	2	72	2.05	38

Figure 2. A. Linearity of real time Assay for Tg DNA prepared using commercial kit. A. Tg DNA was successfully extracted from tachyzoites using DNeasy kit and detected by Real time PCR. DNA was diluted at different concentrations (**red = 0.1mg**; **green = 1ng**; **yellow = 10 pg**; **blue = 100 fg**). **B.** Real time PCR efficiency test. The slope value of -3.3 indicates 100% efficacy. The linearity of the assay was 0.99 (DNA range from 0.1 mg to 100 fg).





In summary, comparisons of the two commercial DNA extraction methods (DNAzol® and DNeasy™) to our previous standard Jauregui DNA extraction method for both purified Tg parasites (tachyzoite stage) and Tg infected mice tissue confirmed that the commercial kits gave good quality DNA (Tables 1,2) and clear Tg detection results using the real time PCR assay (Figure 1, 2). The kits were faster and simpler to use when compared to the standard Jauregui method. The DNeasy™ method was chosen for continue on objective C.

B. Determination of optimal DNA concentration to use for the real time Tg detection assay

Most infected animals have relatively low amounts of Tg in their tissues. Thus we expected that there would be relatively low amounts of Tg DNA recovered from infected mouse brains or pig tissues. Therefore, before we started testing pig tissues we tested different quantities of DNA from infected mouse brains in the real time Tg PCR reaction to determine the optimal amount of DNA to test in each reaction.

Table 3. Comparison of increasing amounts of DNA from Tg infected mouse on results of real time Tg detection assay

Method			**Input 2 ul**			**Input 5 ul**			**Input 0.5 ug DNA**	
		(µg/ml)	**ug DNA**	**C_T**	**ΔR_n**	**ug DNA**	**C_T**	**ΔR_n**	**C_T**	**ΔR_n**
Jauregui	**58**	173	0.346	38.2	0.225	0.865	35.7	0.096	36.1	0.177
	60	149	0.298	34.6	0.260	0.745	35.2	0.228	33.5	0.224
DNAzol	**58**	174	0.348	27.2	0.427	0.870	30.5	0.259	28.7	0.392
	60	70	0.140	34.5	0.115	0.350	29.8	0.269	35.8	0.076
DNeasy	**58**	144	0.288	24.4	0.538	0.720	25.5	0.421	25.6	0.424
	60	63	0.126	30.0	0.430	0.315	30.2	0.367	30.4	0.278
	Tg	control		25.4	0.515					

	DNA									
	NTC		0.000	0.02	0.038					

NTC = No template control

DNA extracted from Tg infected mouse tissues showed best results when lower volumes (0.2 µl not 0.5 µl) of extracted DNA were used, as shown by the lower C_T values and the higher ΔR_n values (Table 3). Because the concentration of DNA was different for each sample and extraction method, for one set of data the volumes were adjusted to set the total DNA content in the reaction at a constant 0.5 µg total DNA per reaction. Based on the results in Table 2 we determined that for most of the samples tested the best C_T and ΔR_n values, and least interference, would be obtained when the sample DNA concentration was </= 0.3 ug.

Because of indications that too high DNA content was causing poor Tg DNA detection, tests were performed on different amounts of DNA. Tg infected mouse DNA was added to set amounts of purified Tg DNA. If the added DNA did not interfere in the reaction then the C_T values should be higher for the Tg DNA alone than for the infected mouse DNA + Tg DNA. Since the infected mouse DNA already contained Tg DNA then adding the Tg DNA should result in lowered C_T values.

Table 4 shows that amplification of Tg DNA occurred when mouse DNA was added, but that it did not decrease the C_T value as expected. It appears that high amounts of mouse DNA interfered with the real time Tg detection reaction. Further tests are needed to determine how best to prevent this interference, e.g., to determine if highly concentrated DNA solutions should be diluted prior to testing in the Tg detection assay. For this, a mouse and a pig housekeeping gene, the 18S RNA, will be used for normalization of animal tissue DNA relative to Tg DNA in any given sample.

Table 4. Results from real time Tg detection assays of Tg DNA mixed with and without infected mouse DNA.

		0.25 µg mouse DNA		0.5 µg mouse DNA	
		C_T	**ΔR_n**	**C_T**	**ΔR_n**
Mouse DNA	65	21.6	0.395	23.1	0.393
	69	27.2	0.203	-	-
Mouse DNA + Tg	65	18.4	0.427	19.7	0.397
	69	20.7	0.359	21.6	0.206
Tg DNA Alone		18.6	0.433		
NTC		50	0.013		

C. Testing assay with known infected pork and chicken samples:

Our plan is to compare the utility of the real time Tg PCR assay with serologic testing and bioassays using commercial meat products. A major survey of market meats was approved in 2001 to collect pork samples for these comparisons. However, no samples were available for these tests until the 2002 grant testing period. These are being analyzed under our NPB #02-101 grant.

However, chicken samples had been brought from Brazil to assess the Tg burden using bioassays. Thus we decided to test aliquots of these chicken samples that had positive results in the mice bioassay for reactivity using the real time TG PCR assay. DNA was successfully extracted using DNeasy; 0.25 µg DNA was then tested by real time Tg PCR. Every positive chicken sample tested gave positive results in the real time Tg PCR assay (Table 5). For pork sample testing, pig muscle samples were collected from deliberately infected pigs as part of an ongoing experiment.

DNA was successfully extracted using DNeasy from the ham muscle. These pigs were serologically positive by MAT at 1:50; 0.25 μg of sample DNA was shown to be positive by real time Tg PCR (Table 5).

Table 5. Real Time PCR results on Tg positive pork and chicken samples:

	C_T values
Chicken	26.0 23.3
Pork	39.6 35.7
Tg DNA	18.6

Each infected pork and chicken sample tested gave positive results in the real time Tg PCR assay (Table 5). These preliminary tests on market cuts of meat serve as a positive verification of the utility of the real time Tg PCR assay for planned scans of commercial meat products. These scans are now underway.

D. Conclusions and Future Plans:

Detection and elimination of foodborne diseases is essential for a safe meat supply. This grant supported our comparisons of molecular methods to improve Tg detection. There are many advantages of the use of molecular methods in market meat surveys. The real time Tg PCR assay has these advantages: 1) high specificity and sensitivity, 2) adaptability to being performed on a large scale, 3) detection of presence of the Tg parasite even in early stages of infection, 4) actual quantitation of parasite burden and 5) alternative method to the use of live animals for bioassays.

Since the commercial DNA extraction methods gave good results with control Tg tachyzoites, experimentally infected mouse tissues, and actual chicken meat samples they will now be tested with tissues collected from Tg infected pigs and commercial pork samples. Experiments are underway to test the effectiveness of the different extraction methods for DNA recovery from muscle, lymph nodes and liver of naturally and experimentally Tg infected pigs. These known Tg-infected and uninfected pig samples will be tested to evaluate the assay sensitivity and reproducibility. Additionally preliminary tests have already been performed on market meat samples collected as part of the larger USDA and NPB funded Tg meats survey (Hill, Dubey, et al.). As more samples are collected in this extensive national survey they will be tested for Tg DNA using the real time Tg PCR assay.

VII. Impact for Pig Producers:

Preventing pork associated foodborne diseases is important to pork producers. Infection with the parasite, *Toxoplasma gondii* (Tg), is a known pork risk factor. 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.

This grant funded improvement of our sensitive molecular technique, the real time Tg PCR assay. This assay will enable producers, diagnostic labs, and potentially packing plants to develop faster Tg screening tests. Results using this assay will assist in eliminating this foodborne contaminant from the US pork supply. It will help assure our international trading partners of the high quality and safety of US pork products.

VIII. References:

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