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Determination of the Viability of *Toxoplasma gondii* in Cured Ham Using Bioassay: Influence of Technological Processing and Food Safety Implications

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ABSTRACT

Toxoplasmosis is a zoonotic disease caused by the protozoan *Toxoplasma gondii* and distributed worldwide. Ingestion of viable cysts from infected raw or undercooked meat is an important route of horizontal transmission of the parasite to humans. Little information is available concerning the effect of commercial curing on cysts of *T. gondii*. This study is the first in which the influence of processing of cured ham on the viability of *T. gondii* has been evaluated, using bioassay to assess the risk of infection from eating this meat product. Naturally infected pigs were selected for the study, and a mouse concentration bioassay technique was used to demonstrate viable bradyzoites of *T. gondii* in porcine tissues and hams. No viable parasites were found in the final product (14 months of curing) based on results of the indirect immunofluorescence assay and histological and PCR analyses. Our results indicate that the consumption of hams cured as described here poses an insignificant risk of acquiring toxoplasmosis. However, additional studies are required to evaluate the safety of ham products cured under different conditions of curing time, salt, and nitrite concentration.

Infection by the protozoan *Toxoplasma gondii* is one of the most common parasitic zoonoses worldwide. In most adults, infection does not cause serious illness, but severe disease may occur in immunocompromised people, and abortions may occur in pregnant women. Other adverse effects are perinatal death, fetal abnormalities, and significantly reduced quality of life in children who survive a prenatal infection *(4, 10, 14)*.

Congenital infection, ingestion of infected tissues, and ingestion of oocysts are the three main modes of transmission of *T. gondii (10)*. Consumption of raw or undercooked meat products containing tissue cysts is a major risk factor associated with toxoplasmosis *(9, 14–16, 27)*. In Europe, three large case-control studies have pinpointed uncooked meat as the most important risk factor for pregnant women *(1, 4, 24)*.

In meat-producing animals, tissue cysts of *T. gondii* are most frequently found in pigs, sheep, and goats and less frequently in poultry *(31)*. Cysts in pork are persistent and an important source of infection for humans *(6, 12)*. Seropositive status in general is a good indicator of the presence of viable parasites *(10)*; however, prevalence of *T. gondii* varies greatly among types of pigs (market pigs versus sows and indoor pigs with a biosecurity system versus free-range pigs). Pigs used for unprocessed pork products (feeder pigs, market pigs) are mostly raised indoors in well-managed facilities to prevent access to rodents and cats. In these well-managed facilities, prevalence of *T. gondii* has greatly declined in the last decade *(10, 23)*. However, increasingly popular animal-friendly production systems with increased risk of exposure to *T. gondii* may cause a reemergence of pork meat as an agent of toxoplasmosis *(25, 28, 32)*.

Demands of consumers for pathogen-free meat products have focused the attention of the meat industry on food safety and the necessity to produce meat that is wholesome, safe, and of high quality, using the appropriate technological treatments. *T. gondii* tissue cysts in meat are susceptible to various physical interventions such as heat treatment, freezing, or irradiation *(25)*. Curing treatments are used to preserve meat. Some researchers have suggested that tissue cysts are killed during commercial curing procedures with salt, but relatively few studies have been conducted to examine the efficiency of the curing process for the inactivation of *T. gondii*. One of the first experiments describing the inactivation of *T. gondii* tissue cysts was conducted by Sommer et al. in 1965 *(30)*. These authors found that encysted *T. gondii* survived for 4 days in 8% NaCl, but neither these researchers nor Work *(35)* could find viable parasites in *T. gondii*–infected pork subjected to various curing processes. Lunden and Uggla *(26)* reported the absence of viable *Toxoplasma* in mutton after curing and smoking. Curing of lamb meat with salt and sugar for 64 h at

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4°C or smoking salt-injected meat at temperatures not exceeding 50°C for 24 to 28 h was effective for killing *T. gondii*. However, these experiments do not reflect conditions under which commercial cured pork products are produced.

The survival time of tissue cysts is highly dependent on the concentration of the salt solution and the temperature of storage. Isolated tissue cysts can survive for 56 days in a solution of 0.85% salt, 49 days in 2% salt, and 21 days in 3.3% salt *(7)*. Under laboratory conditions, Dubey *(7)* found that tissue cysts were killed in 6% NaCl solution at all temperatures examined (4 to 20°C) but survived for several weeks in aqueous solutions with lower salt concentrations. More recent data have indicated that injection of >2% NaCl and/or >1.4% lactate salt solutions into experimentally infected pig meat could kill the parasite. However, 1% NaCl solution provided variable results, and the addition of tripolyphosphate salts had no effect on parasite viability *(17, 18)*.

In contrast, several studies have indicated the potential failure of curing to inactivate *T. gondii*. In an epidemiological study of risk factors for recent *Toxoplasma* infection in pregnant women in southern Italy, a strong association was found between infection and eating cured pork or raw meat *(2)*. Warnekulasuriya et al. *(33)* detected *T. gondii* in 1 of 67 ready-to-eat cured meat samples (1.5% contamination). However, these authors did not provide information about the time of curing and final salt concentrations, which could affect the viability of the parasite.

Thus, scientific information concerning the effect of the commercial curing process on cysts of *T. gondii* is lacking. Some studies have highlighted consumption of cured meat products as a risk factor for acquiring toxoplasmosis during pregnancy *(2, 4, 24, 33)*, and the health implications of consuming cured meats in risk groups (pregnant women and immunocompromised patients) require careful consideration.

Dry-cured ham is an important food in the Mediterranean area, and Spain is one of the main producers of this product. This ham type is widely consumed in Spain and is exported to other countries. It is greatly appreciated by consumers because of its flavor and texture and for its nutritional properties. Dry-cured ham is a nonsmoked product manufactured according to basic principles: curing with salt and nitrites and stabilization through decreased water activity. The whole process takes at least 7 months, although in some cases the hams may be aged for more than 1 year. Dry-cured ham is consumed without heat treatment.

Because of the lack of information regarding the efficacy of meat curing for inactivating *T. gondii* and the inconsistency of results of epidemiological studies in which ingestion of cured meat was identified as a risk factor for acquiring acute *Toxoplasma* infection during pregnancy, the aim of the present study was to provide data that could be used to estimate the risk of *T. gondii* infection from eating cured ham.

MATERIALS AND METHODS

Pigs seropositive for *T. gondii* infection were selected to study the influence of technological processing of curing on the viability of *T. gondii* bradyzoites. A mouse concentration bioassay technique was used, and the presence of the parasite in mice was determined by indirect immunofluorescence assay (IFA) at different stages of processing (fresh meat, middle of curing process, and cured ham). Results in cured hams were confirmed by histology and using PCR techniques.

Sampling of naturally infected pigs. Three farms of Landrace × Large White × Duroc male hybrid pigs were selected for this study. Farms housed 3,000 to 4,000 finisher pigs in total confinement with controlled feed supply. All farms were maintained under good hygienic conditions, but cats were observed occasionally.

Serum samples from 30 pigs 5 to 6 months of age were tested for antibodies to *T. gondii* by IFA (bioMérieux, Marcy l'Etoile, France; DakoCytomation, Copenhagen, Denmark). Three milliliters of blood was collected from the punctured neck vein into sterile 5-ml tubes (BD Vacutainer, no additive, BD, Franklin Lakes, NJ) and centrifuged at 3,500 rpm for 10 min (Hettich Universal, Germany) and the serum was collected. Serum dilutions of 1:20, 1:40, 1:80, 1:160, and 1:320 were prepared from each sample to be tested. All prepared slides were examined with an Olympus BH2 fluorescence microscope (Olympus, Melville, NY). Titers of 1:80 and above were considered positive for *T. gondii*. Six seropositive pigs (four with a titer of 1:80 and two with a titer of 1:160) were selected to evaluate the effect of curing on meat from naturally infected animals.

Selection and sampling of hams. The selected pigs (6 to 7 months old, and 120-kg body weight) were slaughtered in a commercial abattoir. Both haunches were obtained for subsequent curing as is normal industry practice. At day 0 (before the curing process began), samples (~300 g) were obtained from the external surface of six haunches (one from each pig) to avoid quality loss of the final product. After 7 months of curing, the whole ham was analyzed for viable forms of *Toxoplasma*. The six remaining hams continued the curing process until 14 months, when samples were collected. In both cases (at 7 and 14 months), meat samples were collected with a hollow punch that allowed us to collect external and internal samples, thus obtaining a representative sample. After homogenization, we obtained the analytical sample (50 g) to be tested with the mouse bioassay.

Curing the hams. Cured ham production included three steps. For salting, the fresh haunches (approximately 12.5 kg) were surface salted with 100 g of a commercial mixture of salt, sugar, sodium citrate (E-331), sodium ascorbate (E-301), potassium nitrate (E-252, 4.5 g), and sodium nitrite (E-250, 2.5 g) and covered with sea salt, which remained on the hams approximately 1 day for each kilogram of ham in chambers at 0 to 3°C and 85 to 95% relative humidity. The resting period began after the salt was cleansed from the ham surface. The hams were kept for at least 40 days at 3 to 6°C and 80 to 90% relative humidity to allow salt penetration and enhance dehydration. During drying and maturation, the hams continued to lose moisture under conditions of increased temperature and decreased relative humidity. The final flavor and aroma were developed during this stage.

The curing process was complete at 14 months. The final mean composition of dry-cured hams at the end of the curing process was 47.8% water content, 18.2% fat, 30.5% protein, 3.9% NaCl, 25 mg/kg nitrate, and <3 mg/kg nitrite.

Mouse bioassay of tissues for *T. gondii*. A concentration bioassay technique was used to demonstrate viable bradyzoites of *T. gondii* in hams and dry-cured hams as described previously *(8)*. Each analytical sample (~50 g) was homogenized in a blender with 250 ml of saline solution (0.85% NaCl). After homogeniza-

tion, 250 ml of pepsin solution (pH ~ 1) (2.6 g of pepsin, 1:10,000 NF, Panreac Quimica Sau, Barcelona, Spain; 5.0 g of NaCl; 7.0 ml of 37% hydrochloric acid; enough distilled water to make 500 ml) was added and incubated at 37°C for 1 h. The homogenate was filtered through two layers of gauze and centrifuged at 2,800 rpm for 10 min. The supernatant fluid was discarded, and the sediment was resuspended in 20 ml of phosphate-buffered saline (pH 7.2); 15 ml of 1.2% sodium bicarbonate (pH 8.3) was added, and the mixture was centrifuged at 2,800 rpm for 10 min. The supernatant fluid was again discarded, and the sediment was resuspended in 3 to 5 ml of antibiotic saline solution (1,000 U/ml penicillin and 100 μg/ml streptomycin; Sigma-Aldrich, St. Louis, MO); 0.5 ml of this solution was inoculated intraperitoneally into each of five mice. The digestion procedure and inoculation were done in triplicate for each sample (150 g total). All experiments included one negative control mouse within each group, which was analyzed at the end of the process.

The mice were NIH Swiss females (20 to 25 g) and obtained from Harlan Laboratories (Indianapolis, IN). Mice were maintained at the Biomedicine and Biomaterials Service of the University of Zaragoza (Spain) under conditions that complied with international rules of good laboratory practices in the care of experimental animals.

IFA of mouse sera. Blood samples were drawn from mice that survived 60 days after inoculation. Serum from each mouse was diluted 1:10, 1:20, 1:40, 1:80, 1:160, and 1:320 and tested for *T. gondii* antibodies with an IFA (Toxo-Spot IF, bioMérieux) with polyclonal rabbit anti-mouse immunoglobulins (DakoCytomation). The slides were immediately viewed at ×400 total magnification with an Olympus BH2 fluorescence microscope. A positive result was recorded when a clear whole-perimeter tachyzoite fluorescence was observed at dilutions higher than or equal to 1:20.

Positive and negative murine control sera were provided by the Unit of Pathological Anatomy of the Department of Animal Health (Faculty of Veterinary Medicine, University of Zaragoza).

Histological analysis. Mouse brain samples were fixed for light microscopy by immersion in 10% buffered formalin. Formalin-fixed brains were routinely processed for paraffin embedding, sectioned at 5 μm, stained with hematoxylin and eosin, and examined at ×100 to ×400 total magnification. In the brain slides, we searched for cysts and/or lesions compatible with *T. gondii* infection, included scattered necrotic foci surrounded by inflammatory cells, focal gliosis, and hemorrhage.

DNA extraction and ITS-1 nested PCR. We used the GenomicPrep cell and tissue DNA isolation kit (Amersham Biosciences, Uppsala, Sweden) to extract DNA from *T. gondii* ME49 tachyzoites and 10 to 20 mg of mouse brain tissue following the manufacturer's protocols. Five samples from each mouse brain were analyzed by PCR assay.

Toxoplasma DNA in all samples was detected using the four oligonucleotides described by Hurtado et al. *(21)*. The primary reaction included DNA equivalent to one tachyzoite of *T. gondii* as a positive control, and 300 to 400 ng of genomic DNA extracted from mouse brain samples was used as template in each reaction. Primary DNA amplification was performed in a 25-μl reaction containing 1× PCR buffer, 2 mM $MgCl_2$, 200 μM concentrations of each deoxynucleoside triphosphate, 10 pmol of each primer, and 1 unit of DNA polymerase (BioTaq, Bioline, Luckenwalde, Germany). After the primary amplification, 5 μl of a 1:5 dilution of the PCR product was added to the secondary amplification reaction mixture. Secondary DNA amplification was performed in a 25-μl reaction containing 1× PCR buffer, 2 mM $MgCl_2$, 200 μM concentrations of deoxynucleoside triphosphates, 400 μM concentrations of deoxyuridine triphosphates, 5 pmol of each primer, 1 unit of uracyl-DNA-glycosylase (UNG, Eurogentec, Seraing, Belgium), and 1 unit of DNA polymerase. Samples were subjected to one cycle of 94°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final cycle of 72°C for 5 min for the primary amplification. Secondary amplification conditions were one cycle of 50°C for 2 min, 35 cycles of 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min, and a final cycle of 72°C for 5 min.

Different rooms and aerosol-resistant pipette tips were used at each step of the procedure to avoid the carryover of contaminating nucleic acids. A positive control was included in each batch of PCR amplifications. To identify false-positive results, negative control reactions (reactions without template and extractions of DNA from negative brain) were added to each set of PCRs. The secondary amplification product was visualized after electrophoresis as a 227-bp band in a 1.5% agarose–ethidium bromide gel in 0.5× Tris-borate-EDTA buffer.

RESULTS

Table 1 shows results of the serological analysis obtained from pig samples. These results allowed the selection of naturally infected pork meat. Antibodies against *T. gondii* were detected in 9 (30%) of the 30 pigs evaluated, with antibody titers ≥80 in 7 animals (77.7%) and titers ≥160 in 2 animals (22.2%).

Data from inoculated mice used to evaluate the effect of curing on meat from naturally infected animals are shown in Table 2. No serological response was observed in mice inoculated with homogenates of raw meat. However, viable *T. gondii* was detected from all hams after 7 months.

After curing hams for 14 months, no inoculated mice produced sera containing detectable *T. gondii* antibody titers ≥20 (IFA results). None of the mouse brains microscopically examined had cysts or lesions compatible with *T. gondii* infection. *T. gondii* DNA was not detected by nested PCR in mouse brains.

The parasite was not detected in the six control mice by any of the techniques used (IFA, histology, and PCR). All inoculated mice except one survived.

DISCUSSION

To the best of our knowledge, this study is the first to evaluate the influence of processing of cured ham on the viability of *T. gondii* using bioassay and naturally infected pigs. Most published studies have been conducted with experimentally infected pigs or tissue cysts isolated from the brains of infected mice *(7, 17, 18, 22)*.

TABLE 1. *Serological analysis for* Toxoplasma gondii *infection in pigs from three farms*

Farm no.	No. of pigs tested	No. of pigs with serum titers ≥20			
		20	40	80	160
1	10	1	3	4[a]	2[a]
2	10	4	4	2	0
3	10	6	3	1	0

[a] Pigs selected for this study.

TABLE 2. *Detection of viable* Toxoplasma gondii *in starting material (raw ham), semifinished product (dry cured ham after 7 months), and finished product (dry cured ham after 14 months)*

Ham sample no.	*T. gondii* titer in pig	No. of mice positive by IFA/no. of mice inoculated (antibody titers)		
		Raw ham	7-mo ham	14-mo ham[a]
1	≥80	0/5	1/5 (20)	0/5
2	≥80	0/5	1/5 (20)	0/5
3	≥160	0/5	2/5 (160, 320)	0/5
4	≥80	0/5	1/4[b] (320)	0/5
5	≥160	0/5	2/5 (160, 320)	0/5
6	≥80	0/5	3/5 (160, 320, 320)	0/5

[a] Results confirmed by histology and PCR assay.
[b] One mouse died after inoculation.

Antibodies against *T. gondii* were detected in 9 (30%) of the 30 pigs evaluated. Prevalence of *T. gondii* can differ dramatically among types of pigs surveyed (market pigs versus sows, indoor pigs with a biosecurity system versus free range pigs) *(10).* This variation is probably related to differences in age, animal handling, and environmental conditions that can promote the dissemination of *T. gondii* infection *(9).* The pigs used in our study were market hogs that had been raised indoors in well-managed facilities to prevent access to rodents and cats, although entry of these animals may occur occasionally. Pig *T. gondii* seropositive rates were high compared with those previous reported from studies carried out in the United States *(11, 34).* However our study was focused only on three farms selected for possible presence of seropositive pigs so that naturally infected samples could be obtained. Seropositivity in general is a good indicator of the presence of viable parasites in meat *(10).* However, in another study no association between antibody titer and isolation of the parasite was found *(5).*

Mouse bioassay has the advantage of optimum sensitivity and is the standard test to detect infection in tissues *(20).* Some authors have stated that this methodology is better than PCR for detecting *T. gondii* in tissues from pigs *(14, 29).* However, the bioassay technique is expensive, time-consuming, and hazardous for the operator *(13).*

Other assays based on PCR amplification of various DNA sequences of *T. gondii* have been developed. These molecular methods detect DNA fragments and cannot readily distinguish viable from nonviable parasites *(19).* Warnekulasuriya et al. *(33)* were able to detect *T. gondii* in ready-to-eat cured meat samples by amplifying the parasite's P30 gene with PCR and using tissue culture to isolate viable parasites. However, the authors noted that the sensitivity of both tissue culture and PCR assay for the detection of *T. gondii* in cured meat was relatively low. Constituents of the food, notably the high salt content of some cured meats, limited the sensitivity of the PCR assay because of inhibition of the polymerase and reduced the sensitivity of tissue culture because of cytopathic osmotic pressure. These authors remarked that their results highlight the need for improved methods for detecting *Toxoplasma* contamination of food.

In our study, no response was observed in mice inoculated with homogenates of fresh meat. However, viable *T. gondii* was isolated from six hams in the middle of the curing period. This discrepancy may be due to the different procedure used for sampling. In the first case and in order to avoid damage to the ham, sampling was limited to the meat surface, while samples at 7 and 14 months of curing were collected from all over the ham. These results indicated the importance of the sampling technique to detect *Toxoplasma*, since the number of cysts per gram of tissue from food animals such as pigs may be low *(9).* This could be the reason why we were not able to detect *Toxoplasma* in raw material.

In our study, the sensitivity of the mouse bioassay was high for the detection of *T. gondii* in hams in the middle of the curing period; viable *T. gondii* was isolated from hams after 7 months, and we detected the parasite in six of six hams sampled. However, at 7 months hams were insufficiently cured and all had the appearance of raw meat. At 14 months, hams were totally cured, and no evidence of infection was found in mice inoculated with homogenates of these meat products.

Hams of different compositions and curing times are available in the market; however, little information is available about the effect of processing conditions on the inactivation of the *T. gondii,* such as use of organic acids, nitrites, and nitrates or the combination of salt concentration, time, and temperature during maturation of cured ham *(27).* Previous studies were conducted with isolated cysts or in experimental animals *(7, 17, 18, 22, 26),* but the actual industrial process used to obtain commercial ready-to-eat meat products has not been investigated. For example, nitrites prevent the growth of pathogenic microorganisms, but their effect on the viability of *T. gondii* cysts is unknown *(27).*

For meat products, information on the composition and the minimum curing time is required to avoid risk to sensitive populations such as pregnant woman and immunosuppressed patients. Current health education for these sensitive populations does not incorporate advice with regard to the potential risk of eating cured meats *(3)*; the assumption has been that *T. gondii* cysts are destroyed by the curing process. However, gynecologists sometimes do advise pregnant woman to avoid eating cured meat. Therefore, it is imperative to clarify whether it is necessary to issue such a recommendation. We recognize the need for further research into this subject.

Our results indicate that curing salt concentration (3.9% NaCl, 25 mg/kg nitrate, and <3 mg/kg nitrite) and the duration of the curing period (14 months) influence the inactivation of *T. gondii*; we found no parasites in dry-cured ham (14 months) with any of the diagnostic techniques used. The curing process employed in this study appears to produce hams that do not pose a serious concern to human health and therefore could be marketed and consumed without significant health risk. However, further studies are needed to complete the risk assessment and the analysis of other products cured using technological processes other than those investigated in the present study.

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