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Detection of *Toxoplasma gondii* in cured meats

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Abstract

Congenital toxoplasmosis is associated with acute maternal infection acquired during or shortly before the pregnancy. The mother's infection is initiated by the ingestion of one of the life forms of the parasite but the relative importance of the different sources of toxoplasmosis are not established. Recent epidemiological studies have confirmed ingestion of raw meats as a risk factor but also identified consumption of cured meats as being associated with acute toxoplasmosis in pregnancy. There is little existing information concerning the efficiency of commercial curing processes for inactivating *Toxoplasma gondii*. We sought to detect the presence of *T. gondii* in ready-to-eat cured meat samples by amplification of the parasite's P30 gene using the polymerase chain reaction (PCR). In addition, tissue culture was used in order to isolate viable parasites. Laboratory inoculated specimens were used to assess the sensitivity of each method. PCR was able to detect parasite contamination down to a level of 5×10^3 trophozoites/g while viable toxoplasma could be detected in tissue culture at a level of 10^3 trophozoites/g cured meat. The high salt content of some cured meats limited sensitivity of the PCR assay by inhibition of the polymerase enzyme and reduced the sensitivity of tissue culture due to osmotic pressure causing cytopathic effect. However viable *T. gondii* was detected in one out of 67 ready-to-eat cured meat samples. Our results highlight the need for improved methods for detecting toxoplasma contamination of food. Health implications of consuming cured meats in pregnancy require careful consideration.

Keywords: Congenital toxoplasmosis; Pregnancy; Cured meat

1. Introduction

Congenital infection by the protozoan parasite, *Toxoplasma gondii* is a recognised cause of human abortion and neonatal death as well as physical and mental handicap. The greatest risk of transplacental transmission occurs when the mother acquires toxoplasma infection just before or during her pregnancy. Maternal infection is acquired by ingestion of one of the life-forms of the parasite; sporocysts in soil and unwashed, raw vegetables contaminated by cat faeces or tissue cysts in raw or undercooked meats, notably pork or lamb. The relative importance of these different sources of infection is not defined and may vary from one region to another, depending on diet, culinary methods, prevalence of infected cats and farming techniques (Remington et al., 1995).

Studies of pregnant women in Norway found that eating raw or undercooked meat was the major risk

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factor for acute toxoplasma infection (Kapperud et al., 1996). However the frequency of this dietary habit is uncertain. An epidemiological study of risk factors for recent toxoplasma infection in pregnant women in Southern Italy found a strong association with eating cured pork and raw meat (Buffolano et al., 1996). Viable *T. gondii* has been isolated from a variety of raw meats and serological studies have found evidence of widespread infection amongst food animals. Consequently health education aimed at reducing the incidence of congenital toxoplasmosis routinely includes advice to the pregnant woman to avoid eating raw or undercooked meats. No such warning is given with regard to cured meats as it has been assumed that *T. gondii* cysts are destroyed by the curing process. However, a comprehensive review of the literature by Smith (1992) concluded that there was no real information available concerning the effect of the commercial curing process on cysts of *T. gondii* and emphasised the need for further research into this subject.

Other immune compromised patients, including HIV infected individuals, organ graft recipients and those with malignancy are at risk of life-threatening toxoplasmosis. In these groups, disease usually results from reactivation of the patient's long standing, previously quiescent infection but recently acquired, acute infection can occur. Ocular toxoplasmosis is thought to result as a sequelae of congenital infection in most cases but instances of eye problems associated with post-natal, acute infection are documented; reducing human exposure to *T. gondii* would be expected to reduce morbidity and mortality in each context.

In view of the lack of information regarding the efficiency of meat curing for inactivating *T. gondii* and epidemiological studies identifying ingestion of cured meat as a major risk factor for acquiring acute toxoplasma infection during pregnancy, we sought to detect this parasite in ready-to-eat cured meat samples.

2. Materials and methods

2.1. Specimen collection

Specimens selected for inclusion in this study were those collected by the Environmental Health Officers in South West London between 1st July and 31st December 1996. Specimens, 67 in total, included dried and semi-dried sausages, fermented sausages and cured (country style) hams. The pH and water activity of each meat sample was measured.

2.2. Preparation of specimens and isolation of DNA for PCR

Working under a Class II cabinet, meat specimens were separated into 10-g portions and ground in a mortar using 5 ml of antibiotic saline solution (physiological saline 0.85% containing 100 U/ml of penicillin and streptomycin and 12.5 U/ml heparin). Approximately 5 g of the suspension were removed and placed into a sterile container. The remainder of the sample was stored in sterile containers at 4°C.

Duplicate 100-mg portions were mixed by vortexing with Tris EDTA buffer (pH 8.0). Emulsified specimens were digested with 200 μg/ml proteinase K in equal volumes of TEN buffer (50 mM Tris–HCl, pH 8, 150 mM NaCl, 25 mM EDTA) containing 2% SDS for 3 h at 50°C. DNA was purified by phenol/chloroform extraction, recovered by ethanol precipitation and redissolved in 20 μl, 10 mM Tris–HCl, 1 mM EDTA, pH 8 and stored at −20°C.

2.3. Amplification and detection of toxoplasma DNA by PCR

Toxoplasma DNA was amplified by means of a previously described nested PCR procedure with primers based on the toxoplasma P30 gene (Burg et al., 1989) the outer primers generating a 914 base pair fragment (bpf) and the nested primers a 522 bpf (Savva et al., 1990). Duplicate volumes of 10 μl extracted DNA from each sample were amplified in a 50-μl reaction after denaturation at 98°C for 5 min with 30 cycles for each primer set at 95°C for 1 min, 60°C for 1 min and 74°C for 3 min. A volume of 10 μl PCR product from the first set of primers was used for nested PCR under the same conditions.

PCR products were analyzed by 1.0% agarose gel electrophoresis and ethidium bromide staining (Savva et al., 1990). The presence of the 522 bpf indicated a positive result, a negative result was defined by the absence of this 522 bpf.

DNA from RH strain toxoplasma trophozoites was

used as a positive control along with 1 μl of the amplification product of the first PCR reaction to quality control the second round of PCR in the nested cycle. Reagent blanks were processed throughout all procedures as negative controls. Contamination with PCR product was avoided by use of dedicated pipettes, gloves, clothing, solutions and equipment at every stage with spatial separation of DNA extraction, PCR amplification and PCR product analysis. A positive PCR reaction was confirmed by repeat testing the original specimen. When the controls for a PCR batch did not give the expected results, the entire batch was repeated.

2.4. Tissue culture for T. gondii

For cell culture, human embryonic lung (HEL) fibroblast cell lines were used (Hughes et al., 1986; Johnson et al., 1993) as previously described. In order to assess the viability of any *T. gondii* cysts present, duplicate 100-mg portions of the meat suspension were inoculated into a 24-well plate containing HEL cells. The culture medium was changed after 1 h absorption at 37°C. The plates were re-incubated and maintained by daily medium replacement using a 10% fetal calf serum in Eagle's MEM. Wells were examined twice weekly for typical cytopathic changes. Cultures showing cytopathic effect (CPE) and all cultures intact at 14 days incubation were examined microscopically for the presence of *T. gondii*. A positive cytopathic effect (CPE) with the presence of identifiable parasite was defined as a positive isolation of viable *T. gondii*.

2.5. Evaluation of the sensitivity of PCR and tissue culture

In order to evaluate the sensitivity of the assays used, meat samples were 'spiked' with known concentrations of tissue cysts and trophozoites of *T. gondii*. A meat sample proven to be PCR and tissue culture negative was selected and further emulsified in antibiotic saline. Trophozoites were obtained by intra-peritoneal inoculation, 0.5 ml/mouse, of a 5×10^5 trophozoites/ml suspension of *T. gondii* in viral transport medium. Mice were left for an optimum of 3 days after which time the peritoneal fluid was harvested into antibiotic saline solution. The number of trophozoites present per ml was calculated and adjusted to give an initial concentration of 3.6×10^5 trophozoites/g of meat suspension. Sequential dilutions down to 0.5 and 0 trophozoites/g of the emulsified negative meat sample were performed.

Tissue cysts were obtained by intra-peritoneal inoculation of 5 and 10 cysts in a 0.5 ml suspension of mouse brain homogenate. After 42 days incubation the mouse was killed and the brain extracted, homogenised and examined microscopically to calculate the number of tissue cysts present per ml. An initial concentration of 140 cysts/g was then sequentially diluted down to 10 and 0 tissue cysts/g, again using the emulsified negative meat sample.

The 'spiked' samples were then analyzed using tissue culture and PCR on neat DNA and 1:10 dilutions.

3. Results

Examination of laboratory inoculated meat samples demonstrated the limit of sensitivity of tissue culture and PCR methods to be 10^3 and 5×10^3 trophozoites/g, respectively. Corresponding results for parasite cyst studies were 5×10^1 and $> 7 \times 10^1$ per g. Microscopic examination revealed that intact tissue cysts each contained between 10^2 and 10^3 trophozoites.

Tissue culture was found to be affected by the intrinsic cytopathic activity of the cured meat (Table 1) leading to early destruction of the cell monolayer or CPE in the absence of detectable parasite. A single sample of cured ham produced a positive PCR reaction as well as parasite-associated cytopathic effect on tissue culture indicating the presence of viable *T. gondii*.

The pH of the meat samples ranged from 6.3 to 7.1 (median 6.8) while water activity ranged from

Table 1
Tissue culture of cured meat for the isolation of viable *Toxoplasma gondii*

Monolayer destroyed within 48 h of inoculation	16
No cytopathic effect 14 days after inoculation	44
Cytopathic effect observed 14 days after inoculation — no parasite detected	6
Cytopathic effect observed and parasite detected 14 days after inoculation	1
Total samples	67

0.77 to 0.97. The cured ham contaminated with viable *T. gondii* had a pH of 6.98 and a water activity of 0.945.

4. Discussion

The established reference assay for the isolation of *T. gondii* from foodstuffs is gavage or inoculation into animals, usually mice. This approach has the advantage of optimum sensitivity and specificity but is limited by ethical and financial considerations. Tissue culture is less sensitive than animal inoculation but produces results in a shorter period of time at a lower cost (Holliman, 1990). A number of assays based on PCR amplification of different DNA sequences of *T. gondii* have been developed. These molecular methods that detect DNA fragments cannot readily distinguish viable from non-viable parasites (Holliman, 1994).

In our study, the sensitivity of both tissue culture and PCR for the detection of *T. gondii* in cured meats was relatively low. Constituents of the food, notably the high salt content, exerted a cytopathic effect on cell cultures and produced significant inhibition of the polymerase enzyme. Further studies are required to enhance the sensitivity of 'in-vitro' methods for detecting *T. gondii*. Parasite cysts in food could be concentrated by centrifugation (Kotula et al., 1991) with percoll density-gradient separation (Omata et al., 1997). Pepsin digestion of the meat samples prior to examination has been shown to increase sensitivity (Sharma and Dubey, 1981) while alternative methods of DNA extraction could be investigated in an attempt to reduce polymerase inhibition. The application of an RNA-based PCR using reverse transcriptase would allow the detection of viable, as opposed to non-viable parasites. Studies utilising these approaches are in progress. The highest concentration of toxoplasma cysts tested (7×10^1/g) could not be detected by PCR. However, given that each cyst contained 10^2 to 10^3 trophozoites, the sensitivity limits established by inoculation of meat samples with free trophozoites or cysts were comparable.

We were able to detect viable *T. gondii* in one out of 67 ready-to-eat cured meat samples indicating a failure of the commercial curing process. Larger studies are required to establish the frequency of cured meat contamination with viable parasites. However, the demonstration of contaminated cured meat supports earlier epidemiological studies which highlighted consumption of this food as a significant risk factor for acquiring toxoplasma infection (Kapperud et al., 1996; Buffolano et al., 1996). Given the sensitivity of our assays, the degree of *T. gondii* contamination of the cured meat was $\geq 5 \times 10^3$ trophozoites/g. The human infective dose for *T. gondii* is not established but extrapolation from animal studies suggest a figure of less than 10^4 organisms (Remington et al., 1995). The detected level of parasite contamination would be sufficient to establish human infection following the consumption of a typical meal portion of cured meat.

Relatively few studies have examined the efficiency of the curing process for the inactivation of *T. gondii*. Sommer et al. (1965) showed that encysted *T. gondii* could survive for 4 days in 8% NaCl but neither this group nor Work (1968) could demonstrate viable parasite in *T. gondii* infected pork subject to various curing processes. Similarly Lunden and Uggla (1992) reported the absence of viable toxoplasma in mutton following curing and smoking although the parasite survived microwave cooking.

Current health education for pregnant women and immune suppressed patients does not incorporate advice with regard to the potential risk of eating cured meats (Chatterton, 1992). In view of previously published epidemiological studies and our current findings, this advice may require revision.

References

Buffolano, W., Gilbert, R.E., Holland, F.J., Fratta, D., Palumbo, F., Ades, A.E., 1996. Risk factors for recent toxoplasma infection in pregnant women in Naples. Epidemiol. Infect. 308, 347–351.

Burg, J.L., Grover, C.M., Pouletty, P., Boothroyd, J.C., 1989. Direct and sensitive detection of a pathogenic protozoan, *Toxoplasma gondii* by polymerase chain reaction. J. Clin. Microbiol. 27, 1787–1792.

Chatterton, J.M., 1992. Pregnancy. In: Ho-Yen, Do, Joss, A.W. (Eds.), Human Toxoplasmosis. Oxford University Press, Oxford, pp. 144–183.

Holliman, R.E., 1990. The diagnosis of toxoplasmosis. Serodiagn. Immunother. Infect. Dis. 4, 83–93.

Holliman, R.E., 1994. Recent developments in the diagnosis of toxoplasmosis. Serodiagn. Immunother. Infect. Dis. 6, 5–16.

Hughes, H.P., Hudson, L., Fleck, D.G., 1986. In vitro culture of *Toxoplasma gondii* in primary and established cell lines. Int. J. Parasitol. 4, 317–322.

Johnson, J.D., Butcher, P.D., Savva, D., Holliman, R.E., 1993. Application of the polymerase chain reaction to the diagnosis of human toxoplasmosis. J. Infect. 23, 147–158.

Kapperud, G., Jenum, P.A., Stray-Pedersen, B., Melby, K.K., Eskild, A., Eng, J., 1996. Risk factors for *Toxoplasma gondii* infection in pregnancy. Results of a prospective case-control study in Norway. Am. J. Epidemiol. 144, 405–412.

Kotula, A.W., Dubey, J.P., Sharar, A.K., Andrews, C.D., Shen, S.K., Lindsay, D.S., 1991. Effect of freezing on infectivity of *Toxoplasma gondii* tissue cysts in pork. J. Food Protect. 54, 687–690.

Lunden, A., Uggla, A., 1992. Infectivity of *Toxoplasma gondii* in mutton following curing, smoking, freezing or microwave cooking. Int. J. Food Microbiol. 15, 357–363.

Omata, Y., Taka, A., Terada, K., Koyama, T., Kanda, M., Saito, A., Dubey, J.P., 1997. Isolation of coccidian enteroepithelial stages of *Toxoplasma gondii* from the intestinal mucosa of cats by percoll density-gradient centrifugation. Parasitol. Res. 83, 574–577.

Remington, J.S., McLeod, R., Desmonts, G., 1995. Toxoplasmosis. In: Remington, J.S., Klein, J.O. (Eds.), Infectious Diseases of the Fetus and Newborn Infant. WB Saunders, Philadelphia, pp. 140–267.

Savva, D., Morris, J.C., Johnson, I.D., Holliman, R.E., 1990. Polymerase chain reaction for detection of *Toxoplasma gondii*. J. Med. Microbiol. 18, 665.

Sharma, S.P., Dubey, J.P., 1981. Qualitative survival of *Toxoplasma gondii* tachyzoites and bradyzoites in pepsin and trypsin solution. Am. J. Vet. Res. 42, 128–130.

Smith, J.L., 1992. *Toxoplasma gondii* in meats — a matter of concern?. Dairy Food Environ. Sanit. 12, 341–345.

Sommer, R., Rommel, M., Levetzow, R., 1965. Die Überlebensdauer von Toxoplasmazysten in fleish und fleishzubereitungen. Fleischwirsch 5, 454–457.

Work, K., 1968. Resistance of *Toxoplasma gondii* encysted in pork. Acta Pathol. Microbiol. Scand. 73, 85–92.