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Short communication

Toxoplasma gondii tissue cyst formation and density of tissue cysts in shoulders of pigs 7 and 14 days after feeding infected mice tissues

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ABSTRACT

Among the meat sources of *Toxoplasma gondii*, pork is considered important in the epidemiology of toxoplasmosis in the USA. How soon after infection *T. gondii* forms tissue cysts in pork is unknown. In the present study, eight serologically negative ~3 months old pigs were fed mouse tissues infected with VEG (Type III) strain of *T. gondii* and euthanized 7 (4 pigs) and 14 days (4 pigs) post-inoculation (p.i.). Meat from the right shoulder of each pig was bioassayed in mice for *T. gondii* tissue cysts by peptic digestion. From each pig, the shoulder muscle was cut at random spots into 5 g, 10 g and 50 g portions. Extreme care was taken to use different scalpels and forceps to minimize cross contamination among 17 samples (6 replicates of each 5 g and 10 g portions and 5 replicates of 50 g). From the four pigs euthanized at 7 days p.i., a composite of ~200 g of leftover meat from each shoulder was bioassayed in cats and their feces were tested for oocyst excretion. All eight pigs developed *T. gondii* antibodies (modified agglutination test, MAT, 1: 80 or higher) and viable *T. gondii* was isolated from shoulder meat of each pig. All four cats fed pork from excreted *T. gondii* oocysts. The density of *T. gondii*, based on mouse infectivity, varied within 5–50 g samples each pig, and between pigs within the same group, day 7 versus day 14 p.i. There were no significant differences in mouse bioassay results obtained with day 7 versus day 14 infected pigs. Overall, the rate of isolation of *T. gondii* increased with sample size of meat bioassayed. Results demonstrate that tissue cysts are formed early in infection and they are unevenly distributed.

1. Introduction

Toxoplasma gondii infection is widely prevalent in humans and animals, and toxoplasmosis continues to be a public health concern worldwide, including the USA (Dubey, 2010). Humans become infected postnatally by ingesting infected uncooked/undercooked meat or food and water contaminated with oocysts excreted by cats (Dubey and Jones, 2008). Among the meat sources of *T. gondii*, pork is considered important in the epidemiology of toxoplasmosis in the USA as pigs are highly susceptible to *T. gondii* infection with a minimum infective dose of 1 oocyst (Dubey et al.,1996). Beef and commercially raised poultry are rarely infected with *T. gondii* (Dubey et al., 2005). Oocyst-induced infections are considered more severe clinically than tissue cyst-induced infections (Dubey, 2010).

After the ingestion of *T. gondii* (oocysts or tissue cysts), *T. gondii* invades, multiplies, and persists in many tissues of pigs. Tissue cysts have been demonstrated in muscle of pigs up to 875 days after feeding oocysts (Dubey, 1988). The parasite has been isolated from all commercial cuts of pork, including bacon, ham, spare ribs, tenderloin, Boston butt, and shoulder picnic of both naturally infected and experimentally-infected pigs (Dubey et al., 1986). However, there are no quantitative data on distribution of *T. gondii* in pork for risk assessment (Guo et al., 2017). How soon after infection *T. gondii* forms tissue cysts in pork is also unknown.

The objective of the present study was to qualitatively assess formation and distribution of *T. gondii* tissue cysts in pigs 7 and 14 days post-inoculation (p.i.). Of the pigs euthanized on days 7 and 14 p.i., whole right shoulder of eight pigs was used for the present report. This

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report is confined to only results of *T. gondii* tissue cysts in shoulder picnic.

2. Materials and methods

2.1. Experimental infection of pigs with T. gondii

Eight mixed breed, 31–35 kg, 12 weeks old mixed-sex pigs were each fed one ground mouse carcass in a cookie dough to enhance palatability. Pigs were housed in a climate controlled facility as per procedures approved by the Institutional (Beltsville) Animal Care and Use Committee Protocol #18-012. The mice were inoculated 4 weeks previously with 100 VEG strain oocysts (Dubey et al., 1996). The mice were euthanized, skinned, eviscerated, and their brains were examined microscopically by removing a small snip (1–2 mm). The rest of the brain and the carcass was ground in a blender, mixed in a ball of raw cookie dough (~50 g total weight of cookie and carcass) that was placed on the floor of the pen where each pig was observed for consumption of the preparation within 2 h.

2.2. Serological examination

Pigs were bled 7 days before the experiment to confirm them to be *T. gondii* negative and 1 day before planned euthanasia (7 and 14 days p.i.). Their sera were tested for antibodies to *T. gondii* using the modified agglutination test (MAT) as described previously (Dubey and Desmonts, 1987). For this, sera were diluted 2-fold starting a 1:10 dilution.

2.3. Testing of meat for T. gondii

Four pigs were euthanized at each time point (7 and 14 days) with a lethal injection of Euthasol (Virbac AH, Inc., Fort Worth, TX, USA) and necropsied. For this study, the right shoulder from each of eight pigs was removed and stored at 4 °C for processing within 48 h. The shoulders were washed with cold water to remove surface debris and wiped with clean tissue papers. The shoulder muscle was cut at random spots into 5 g, 10 g, and 50 g portions. Extreme care was taken to use different scalpels and forceps to minimize cross contamination among 17 samples (6 replicates of each 5 g and 10 g portions and 5 replicates of 50 g samples for mouse bioassay). From the 4 pigs euthanized at 7 days p.i., a composite of ~200 g of leftover meat from each shoulder was collected for bioassay in cats.

All 17 samples from each pig shoulder were bio-assayed in mice. For this, 5 g, 10 g and 50 g portions of muscles were homogenized in saline, and incubated in acidic pepsin solution (final muscle to pepsin ratio 1:10) at 37 °C for 1 h in a shaker water-bath as described previously (Dubey, 2010). The digested samples were filtered through two layers of gauze, centrifuged, suspended in saline, neutralized with 1.2% sodium bicarbonate, and the sediment was suspended in 1–5 ml of antibiotic saline solution (1000 units of penicillin and 100 µl of streptomycin/ml of saline solution). After 1 h, samples were inoculated subcutaneously into Swiss Webster (SW) mice (2 mice each for 5 g and 10 g meat samples and 5 mice for 50 g samples - in total 49 mice per shoulder).

The mice inoculated with pig tissues were observed for 7–8 weeks and tested for *T. gondii* infection as described previously (Dubey, 2010). Mice were bled after 6 weeks p.i. and a 1:25 dilution of serum from each mouse was tested for *T. gondii* antibodies by MAT. All mice were euthanized 7–8 weeks p.i. and their brain smears were examined microscopically for tissue cysts, irrespective of serological results.

For bioassay in cats, 200 g of meat from the 4 pigs euthanized 7 days p.i. were fed to 4 cats (1 cat per pig), for 1–2 days. Cats were ~3 months old and were raised in captivity at the Beltsville Agricultural Research center as reported in detail previously (Dubey, 1995). The cats had no detectible antibodies to *T. gondii* in a 1:10 serum dilution tested by MAT prior to feeding of pig meat. All feces from each cat were collected daily, 3–14 days after feeding pork and examined for *T. gondii* oocysts as described (Dubey, 2010).

2.4. Statistical analysis

Differences among test groups were compared by chi-square ($\chi 2$) test with Yates correction, using EpiInfo 7.2 statistical package. A P value of < 0.05 was considered as significant.

2.5. Ethics

Infected pigs, mice, and cats were housed at the USDA's Beltsville Agricultural Research Center and cared for in accordance with the Animal Welfare Act, Guide for the Care and Use of Laboratory Animals (https://www.nap.edu/search/?term=Guide+or+the+Care+and+Use+of+Laboratory+Animals+) and with the approval of the USDA/ARS Beltsville Area Institutional Animal Care and Use Committee (BAACUC Approval #18-012, #18-013, #18-014).

3. Results

All eight pigs developed *T. gondii* antibodies (MAT, 1:80 or higher) and viable *T. gondii* was isolated from shoulder meat of each pig. All four cats fed pork from the pigs euthanized 7 days p.i. excreted many (not counted) oocysts (data not shown).

Mouse bioassay data are summarized in Table 1 and in detail in the supplementary Table S1. All mice inoculated with pig tissues survived. Mouse infectivity data in Table 1 are based on finding tissue cysts in the brains of mice considered infected with *T. gondii*; all parasitologically-positive mice were serologically positive and tissue cysts were not found in the brains of serologically negative mice.

The rate of infection of *T. gondii*, based on mouse infectivity, varied within 5–50 g samples from each pig, and between pigs within the same group, day 7 versus day 14 p.i. Overall, the rate of isolation of *T. gondii* increased with sample size of meat bio-assayed (Table 1). No statistical differences ($P > 0.05$) were observed in the detection of *T. gondii* from all infected pigs of day 7 and day 14 p.i. by bioassay. However, when the sample sizes were compared within the same group, differences were observed between 5 g and 50 g as well as 10 g and 50 g samples in day 7 p.i. group ($P < 0.05$) (Table 1).

4. Discussion

Results of this investigation confirm findings by others that pigs can be readily infected by feeding *T. gondii* tissue cysts (Boch et al., 1964a, b; Janitschke and Wormuth, 1970; Work et al., 1970; de Meuter et al., 1978; Wingstrand et al., 1997a,b). In these studies, pigs were

Table 1
Comparison of mouse bioassay results of pigs euthanized day 7 and 14 post-inoculation.

Day pig euthanized	Mouse bioassay		
	Sample size (g)	No. of pork samples positive/No. tested (%)	No. of mice *T. gondii* positive/ No. inoculated (%)
7	5	6/24 (25. 0)[#a]	9/48 (18. 7)[c]
	10	9/24 (37. 5)[b]	14/48 (29. 1)[d]
	50	15/20 (75.0) [a,b]	63/100 (63. 0)[c,d]
14	5	10/24 (41.6)	17/48 (35.4)
	10	13/24 (54.1)	25/48 (52.0)
	50	15/20 (75.0)	60/100 (60.0)

[#] Same letter = Groups differ significantly (chi-square (χ^2) test with Yates correction, $P < 0.05$).

inoculated orally with homogenized mouse brains containing tissue cysts. In the present study, pigs were fed mouse carcasses mixed in cookie dough to simulate natural ingestion, avoid spillage, and prevent inadvertent infection of the research staff. Natural ingestion has the advantage that *T. gondii* are released slowly from infected mouse tissues as the digestion proceeds, thus allowing more time for bradyzoites to infect host tissue.

Previous studies have revealed that the density of *T. gondii* in pork and other meats is low (Dubey, 2010). Therefore, bioassay methods were used to detect low numbers of *T. gondii* in meat. Of all the methods, available for the detection of *T. gondii* in meat, the cat and the mouse bioassay are the most efficient (Dubey, 2001, 2010), but these methods are qualitative. Therefore, only qualitative data were obtained here.

Results of the present study revealed that *T. gondii* can encyst in muscles of pigs as early as 7 days p.i. and the overall detection of tissue cysts by mouse bioassay was not different than at day 14 p.i. In the USA, around 100 million pigs are slaughtered annually. Although the prevalence of *T. gondii* in pigs raised in biosecure facilities has declined drastically, even a 0.1% prevalence of *T. gondii* amounts to 1000 infected pigs. Market pigs (feeder pigs) weigh around 100 kg, thus resulting in more than 600 helpings of infected meat. Results of the present study demonstrate that even 5 g samples can be infected and that even recently infected pigs harbor tissue cysts and can be source of infection for humans.

Declaration of interests

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2019.04.004.

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