Journal of Food Protection, Vol. 54, No. 9 Pages 687-690 (September 1991)

Effect of Freezing on Infectivity of *Toxoplasma Gondii* Tissue Cysts in Pork

A. W. KOTULA*, J. P. DUBEY[1], A. K. SHARAR, C. D. ANDREWS[1], S. K. SHEN[1], and D. S. LINDSAY[1]

Meat Science Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Maryland 20705

(Received for publication March 18, 1991)

ABSTRACT

To study the effect of low temperatures on infectivity of *Toxoplasma gondii* tissue cysts, pork from infected pigs was mixed with infected mouse brains and homogenized thoroughly. Twenty-gram samples of infected homogenized meat which were sealed in plastic pouches, pressed to a uniform thickness resulting in samples having the dimensions of ~.2 x 16 x 18 cm, were subjected to temperatures of -1 to -171.1°C for 1 s to 67.2 d. Treated samples were digested in HCl-pepsin solution and infectivity assayed in mice. A regression model from these data is described by the least squares linear regression: Square root of time for the inactivation of *T. gondii* (h) = 26.72 + 2.16 temperature (°C) with r = 0.77. *T. gondii* tissue cysts remained viable usually up to 22.4 d at -1 and -3.9°C and 11.2 d at -6.7°C but were usually rendered nonviable by freezing at -12.37°C. These data demonstrate that *T. gondii* tissue cysts are inactivated by freezing more readily than encysted *Trichinella spiralis* larvae.

Toxoplasma gondii can cause mental retardation, loss of vision and mortality in congenitally infected children, and encephalitis or dementia in immunocomprised adults (*5*). Humans become infected by ingesting *T. gondii* tissue cysts in meat or oocysts from cat feces. Among the ways to reduce human infection from meat are too destroy tissue cysts by cooking (*9*), irradiation (*7*), or freezing (*10*). Although there are several reports of loss of infectivity of tissue cysts by freezing between -6 and -40°C including Jacobs et al. (*12*), Work (*18*), Frenkel and Dubey (*11*), Dubey and Frenkel (*8*), Dubey (*2,4*), and Pejsova and Pejse (*16*), current commercial methods of freezing meat have not been tested nor has freezing for prolonged periods at relatively high temperatures (-1°C) been tested for loss of infectivity of cysts. We report the effect of freezing from -1 to -171.1°C, for 1 s to 67.2 d on *T. gondii* tissue cysts under defined conditions.

MATERIALS AND METHODS

Twenty-one mixed breed 8- to 10-week-old pigs from the Animal Parasitology Unit of the Beltsville Agricultural Research Center swine herd were used. They did not have detectable *T. gondii* antibodies in 1:25 dilutions of serum examined using the *T. gondii* agglutination test of Desmonts and Remington (1) and Dubey and Desmonts (*6*). In four trials, 17 pigs (4 in trial 1, 4 in trial 2, 6 in trial 3, and 3 in trial 4) were inoculated orally with mixed inocula containing approximately 10,000 *T. gondii* oocysts of each of 6 to 8 strains as described by Dubey (*4*) and Dubey et al. (*7,9*) for a total of 60,000 to 80,000 per pig; four control pigs, one for each trial, were not inoculated with *T. gondii*. The oocysts were obtained from the feces of laboratory-raised cats fed tissue cysts of *T. gondii* from the brains of mice. The mice had been inoculated with oocysts or tissue cysts of one of the 13 strains of *T. gondii* including the Me-49, ST-2, PT-1, TC-1, PT-2, and Aldrin strains used by Dubey and Frenkel (*8*) and Dubey et al (*7*). Rats were inoculated orally with 10,000 oocysts of the GT-1 strain of *T. gondii* because mice do not survive with this strain according to Dubey (*3*). Oocysts were sporulated in 2% sulfuric acid, and their infectivity was determined by bioassay in mice 3-4 weeks before inoculation of pigs. Tongues, hearts and limb muscles from infected pigs were used because more tissue cysts are present in the heart and tongue than other muscles in pigs as explained by Dubey (*4*). Although there is no evidence for strain-dependent resistance of *T. gondii*, up to 13 strains were used in the present study. This protocols was used to ensure that the times/temperatures for this inactivation of *T. gondii* determined in this study would be effective for a number of strains of the parasite.

Inoculated pigs were killed 42, 50, 69, and 60 d postinoculation, respectively, in trials 1-4 and their tongues and hearts were removed and weighed. For each trial, skeletal muscle was added to the heart and tongue to bring the tissue mass to 6 kg. Pooled tissues were ground in a meat grinder, mixed thoroughly, and divided into three 2-kg portions. Infected mice and rat brains were mixed with pork because the number of tissue cysts in rodent brains was reported to be about 100-fold higher than in the infected pork (*7*). Brains of *T. gondii* infected rats (15 in trial 1, 62 in trial 2, 49 in trial 3, and 44 in trial 4) and mice (184 in trial 1, 140 in trial 2, 208 in trial 3, and 177 in trial 4) were removed, pooled and ground in a blender in about 100 ml of 0.85% NaCl solution (saline). Brain homogenates were divided into three equal portions. Each portion of brain homogenate was added to a 2-kg portion of infected pork and mixed thoroughly. Each 2-kg portion of infected pork-brain homogenate was processed in a high-speed food processor as described by Kotula et al. (*14*). Finally, all 6 kg of sample was mixed together. Tissues from the uninoculated (control) pigs were treated similarly using uninfected mouse and rat brains.

[1]*Zoonotic Diseases Laboratory.*

Tissues for freezing were processed using procedures described by Kotula et al. *(14)*. The mean fat content and standard error of the mean of composite samples from the ground meat-brain homogenate were 4.20% (±0.23%) on a wet basis and 19.82% (±1.14%) on a dry basis. The moisture content was 78.86% (±0.6%).

Twenty-gram samples of homogenized meat were placed individually into ~50 μm thick 240-ml heat-sealable pouches (16.5 x 20.3 cm). Bags containing samples were placed between two stainless steel plates and pressure applied to the plates to force the samples to a uniform thickness of 2 mm by placing spacer sticks between the plates. Air was expelled from the bags and the tops were heat sealed. The dimensions of the resulting samples were ~.2 x 16 x 18 cm. Several bags were filled with uninfected pork for monitoring temperature rise and decline. Prior to sealing these bags, a copper-constantan thermocouple was placed in the geometric center of the pork (thermocouple thermometer, Cole Parmer, Chicago, IL). The last 1.7 cm of the plastic coating on the thermocouple was coated with waterproof tape to a thickness of 2 mm to maintain the tip of the thermocouple within the center of the 2 mm thickness of the pork meat sample.

As shown in Table 1, four trials were performed and with a total of 11 replicates. The samples were frozen in liquid nitrogen at -171.1°C, in ethyl alcohol and dry ice at -70°C and ethyl alcohol at -17.8 to -43.1°C, and in a mixture of ethylene glycol and water at -15 to -1°C.

Temperature-treated meat samples were stored at 4°C overnight so that all samples for 1 d could be digested simultaneously. Contents of each meat-bag were transferred into a blender and the bag was rinsed with saline solution. Each sample was homogenized in a total of 100 ml of saline solution in a blender at full speed for 30 s, and the homogenate was incubated at 37°C for 10 to 15 min so that contents attained a temperature of 37°C. To each sample, 100 ml of HCl-pepsin (pepsin, Sigma, St. Louis, MO 1:10,000 activity = 5.2 g, HCl 14 ml, NaCl 5.0 g and water 1,000 ml) preincubated at 37°C was added. The pepsin-meat mixture was incubated at 37°C for 60 min on shaker. The incubated homogenate was filtered through gauze and centrifuged at 1,000 x g for 10 min in 250-ml bottles. The supernatant was discarded and the sediment was suspended in 45 ml of saline solution and centrifuged in 50-ml tubes at 1,000 x g for 10 min. The supernatant was discarded, and 2 ml of saline was added to the sedimented pellet. After thorough mixing, the mixture was drawn into a 6-ml syringe containing 3 ml of antibiotic saline solution (2,000 units of penicillin and 200 g of streptomycin/ml of saline). After 1 h at room temperature (22°C), the homogenate was inoculated subcutaneously into five mice (1.0-1.25 ml/mouse). Thus, the entire sediment from the digest of 20-g sample was bioassayed in mice. The mice inoculated were Swiss Webster albino females weighing 20-25 g. Mice were identified individually by ear tags.

Infected control meat samples (not temperature treated) stored at 4°C for 2 to 30 d were digested and infectivity assayed in mice in a manner identical to treated samples. After storage, the digested, centrifuged meat sediment was suspended in 5 ml of saline and five 10-fold dilutions were made in saline. Samples of each of the 10^{-1} to 10^{-5} dilutions were inoculated into two mice.

TABLE 1. *Test conditions during the four trials of freezing* T. gondii *in pork.*

Trial	Temperatures (°C)	Time s	Time min	Time h	Time d
1	-171.1, -70, -43.1, -40, -37.5, -34.4, -31.9, -28.9, -26.3, -23.3, -20.7, -17.8, -15, -12.2, -9.4, -6.7, -3.9, -1	1, 15, 30, 60	2, 4, 8, 16, 32, 64, 128, 256	8.5, 17	1.4,2.8, 5.6, 8.4, 11.2, 16.8, 22.4, 33.6, 44.8, 67.2
2	-171.1, -37.5, -34.4, -15, -12.2, -9.4, -6.7, -3.9, -1.0	1, 30, 60	2, 4, 8, 16, 32, 64, 128, 256	8.5, 17	1.4, 2.8, 5.6, 11.2
3	-15	1, 15 30			
	-12.2	1, 15 30	1, 2, 4, 8, 16		
	-9.4	1, 15 30	1, 2, 4, 8, 16, 32, 64		
	-3.9, -1				2.8, 5.6, 11.2, 16.8, 22.4, 33.6,
4	-8	1, 15 30	1, 2, 4, 8, 16, 32, 64, 128, 256	8.5, 17	1.4, 2.8, 5.6, 8.4, 11.2, 16.8, 22.4
	-6.7, -3.9, -1				1.4, 2.8, 5.6, 8.4, 11.2, 16.8, 22.4, 33.6, 44.8, 67.2

Five mice were inoculated with samples from the undiluted suspension. Pork, before mixing with rodent brain, was also assayed in mice for *T. gondii*.

Inoculated mice were examined for *T. gondii* as described by Dubey and Beattie (*5*). Impression smears of lungs and brain of the mice that died were fixed in methanol, stained with Giemsa, and examined microscopically. Blood was collected from surviving mice 30 d postinoculation. After serological examination was completed, mice were killed and the brain of each mouse was examined for *T. gondii* tissue cysts. Two squash-smears were made from 1 x 3 mm pieces of cerebrum of each mouse and examined microscopically without staining. After examination, the coverslip was removed, and the slide was fixed in methanol and stained with Giemsa stain for permanent records. Serum of each mouse was examined for *T. gondii* antibodies in the direct agglutination test as described by Dubey and Desmonts (*6*). Sera were screened at 1:25 and 1:100 dilutions. Brains of seropositive mice without visible tissue cysts were bioassayed in mice or cats as described by Dubey and Beattie (*5*). For bioassay in mice, brains of individual mice were inoculated subcutaneously into mice. For bioassays in cats, brains pooled from 10-25 mice with *T. gondii* antibody titers of <1:100 were fed to cats, and the feces of cats were examined for *T. gondii* oocysts using the procedure of Dubey and Beattie (*5*).

Data were analyzed statistically (*17*) to obtain a linear regression equation and the 99% upper confidence interval for individual values for the time at each temperature for the inactivation of *T. gondii*.

RESULTS

Tissue cysts remained viable up to 11.2 d at -6.7°C and up to 22.4 d at -3.9 and -1°C (Table 2). Tissue cysts were rendered noninfective at temperatures of -9.4°C or less for all time points, except in few instances. The exceptions were: -37.5°C for 30 s (trial 1), -34.4°C for 4 min (trials 1 and 2), and 1 s (trial 2), -12.2°C for 8.5 h (trial 1), and 1 s (trial 3). In each instance, only one of the 15 inoculated mice was positive for *T. gondii*.

Bioassays in mice indicated that in trial 1, a 10^{-2} dilution of pork and 10^{-3} dilution of pork plus rodent brains were infective for mice. In trials 2 to 4, a 10^{-5} dilution of pork plus brains was infective for mice. Tissue cysts stored at 4°C remained viable for 25 d in trial 2, 26 d in trial 3, and 24 d in trial 4. *T. gondii* tachyzoites or tissue cysts were seen in all mice with *T. gondii* antibodies in 1:100 dilutions of serum. Tissues from mice with antibody titers of 1:25 were not infective for mice and cats.

A freeze-death curve for the inactivation of *T. gondii* tissue cysts in pork is shown in Fig. 1. The curve is based on the presence or absence of infective *T. gondii* in pork exposed to temperatures from -12.2 to -1°C for time periods of 17 h to 33.6 d. The data for colder temperatures and longer times were not utilized because the *T. gondii* were not infective after such exposure. The three spurious bioassay positive samples after exposures at -37.5 and -34.4°C were not used in the analysis because their use would have resulted in a less conservative analysis. Those three positive mice are not readily explained since all samples between -31.9 and -15°C were negative. The least squares linear regression analysis resulted in the equation: Square root of time for inactivation of *T. gondii* (h) = 26.72 + 2.16 temperature (°C).

The correlation coefficient was r = 0.77. When the equation was solved for 0 time, a temperature of -12.4°C was obtained as the theoretical temperature at which *T. gondii* would be inactivated instantaneously. Fig. 1 provides the curve for this regression of times with freezing temperatures. A curve describing the 99% upper confidence interval for the individual values is also provided.

DISCUSSION

Tissue cysts were generally killed by freezing at -12.4°C. The results of the present study generally support published results except in six isolated instances where an occasional *T. gondii* survived at -12.2, -34.4, and -37.5°C. Because the Aldrin strain of *T. gondii* originated from the heart of a monkey stored at -20°C for 16 d in the study by Dubey and Frenkel (*8*), we also included the Aldrin strain

TABLE 2. *Ratio of positive mice to total mice sampled in bioassay for* T. gondii *in infected pork frozen for various times and temperatures.*

Time	Temperature (°C) -1.0	-3.9	-6.7	-8.0
64 min	15/15	15/15	14/15	ND*
128 min	15/15	15/15	10/15	ND
256 min	15/15	15/15	15/15	ND
8.5 h	15/15	15/15	14/15	ND
17 h	15/15	15/15	15/15	ND
1.4 d	27/30	30/30	26/30	1/15
2.8 d	41/45	42/45	20/45	0/15
5.6 d	45/45	45/45	19/45	0/15
11.2 d	45/45	33/45	4/45	0/15
16.8 d	12/30	10/30	0/30	0/15
22.4 d	3/30	2/30	0/30	0/15
33.6 d**	0/30	0/30	0/30	0/15

*ND=not done.

**All mice inoculated with samples of meat frozen for 44.8 and 67.2 d were negative for *T. gondii*.

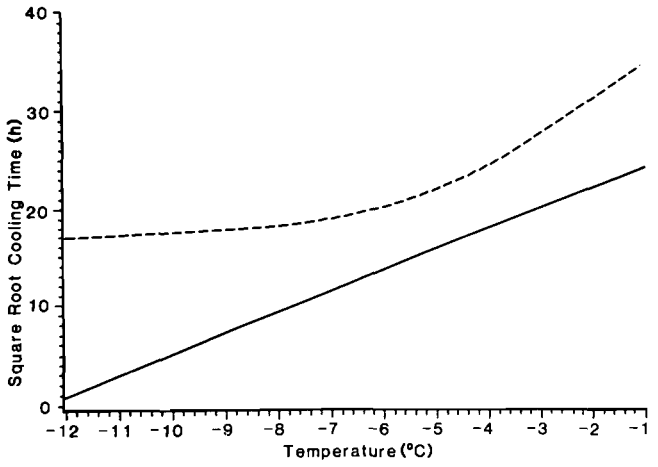

Figure 1. *The least squares linear regression of freezing times and temperatures for the inactivation of* T. gondii *(solid line) expressed by the equation: Square root of time (h) = 26.72 + 16 (°C), with r = 0.77 and the 99% upper confidence interval for individual values (broken line).*

in the present study. The results indicated that survival of *T. gondii* in the heart of the monkey was probably related to chance rather than to the strain of *T. gondii*.

This study determined the thermal death curve of *T. gondii* under well-defined conditions. All infected meat samples were prepared at one time, to uniform thickness and sealed, thus reducing variability among samples. Furthermore, temperature inside the meat was recorded for each time-treatment sample. Previous reports either used a variety of tissues or did not record temperature inside the frozen samples *(2,4,8,11,12,18)*. Although *T. gondii* tachyzoites can be cryopreserved as explained by Eyles et al. *(11)* and Jeon et al. *(13)*, nothing is known of cryopreservation of tissue cysts.

The data also demonstrated that *T. gondii* tissue cysts are inactivated by freezing more readily than are encysted *Trichinella spiralis* larvae in pork as reported by Kotula et al. *(15)*.

ACKNOWLEDGMENTS

The authors thank Diane S. Hawkins, Eva Kovacs, L. M. Spriggs, K. R. Goodson, R. E. Billhimer, and E. M. Sanders for technical assistance and Mary Camp for statistical analysis of the data.

REFERENCES

1. Desmonts, G., and J. S. Remington. 1980. Direct agglutination test for diagnosis of *Toxoplasma* infection: method for increasing sensitivity and specificity. J. Clin. Microbiol. 11:562-568.
2. Dubey, J. P. 1974. Effect of freezing on the infectivity of *Toxoplasma* cysts to cats. J. Am. Vet. Med. Assoc. 165:534-536.
3. Dubey, J. P. 1980. Mouse pathogenicity of *Toxoplasma gondii* isolated from a goat. Am. J. Vet. Res. 41:427-429.
4. Dubey, J. P. 1988. Long-term persistence of *Toxoplasma gondii* in tissues of pigs inoculated with *T. gondii* oocysts and effect of freezing on viability of tissue cysts in pork. Am. J. Vet. Res. 49:910-913.
5. Dubey, J. P., and C. P. Beattie. 1988. Toxoplasmosis of animals and man. CRC Press, Inc., Boca Raton, FL. p. 220.
6. Dubey, J. P., and G. Desmonts. 1987. Serological responses of equids fed *Toxoplasma gondii* oocysts. Equine Vet. J. 19:337-339.
7. Dubey, J. P., R. J. Brake, K. D Murrell, and R. Fayer. 1986. Effect of irradiation on viability of *Toxoplasma gondii* cysts in tissues of mice and pigs. Am. J. Vet. Res. 47:518-522.
8. Dubey, J. P., and J. K. Frenkel. 1973. Experimental *Toxoplasma* infection in mice with strains producing oocysts. J. Parasitol. 59:505-512.
9. Dubey, J. P., A. W. Kotula, A. Sharar, C. D. Andrews, and D. S. Lindsay. 1990. Effect of high temperature on infectivity of *Toxoplasma gondii* tissue cysts in pork. J. Parasitol. 76:201-204.
10. Eyles, D. E., N. Coleman, and D. J. Cavanaugh. 1956. Preservation of *Toxoplasma gondii* by freezing. J. Parasitol. 42:408-413.
11. Frenkel, J. K., and J. P. Dubey. 1973. Effect of freezing on the viability of *Toxoplasma* oocysts. J. Parasitol. 59:587-588.
12. Jacobs, L., J. S. Remington, and M. L. Melton. 1960. The resistance of the encysted form of *Toxoplasma gondii*. J. Parasitol. 46:11-21.
13. Jeon, Y., O. O. Rhee, S. H. Kim, and H. Jang. 1988. Preservation of *Toxoplasma gondii* in liquid nitrogen. Research Reports of the Rural Development Administration, Veterinary (Korea) 30:46-50.
14. Kotula, A. W., K. D. Murrell, L. Acosta-Stein, and L. Lamb. 1983. *Trichinella spiralis:* Effect of high temperature on infectivity in pork. Exp. Parasitol. 56:15-19.
15. Kotula, A. W., A. K. Sharar, E. Paroczay, H. R. Gamble, K. D. Murrell, and L. Douglass. 1990. Infectivity of *Trichinella spiralis* from frozen pork. J. Food Prot. 53:571-573.
16. Pejsova, V., and M. Pejse. 1988. Devitalizace cyst *Toxoplasma gondii* ve svalovine jatecnych zvirat zmrazovanim. Veterinarstvi 38:127-128.
17. Snedecor, G. W., and W. G. Cochran. 1972. Statistical methods, 6th ed. Iowa State University Press, Ames. p. 156.
18. Work, W. 1968. Resistance of *Toxoplasma gondii* encysted in pork. Acta Pathol. Microbiol. Scand. 73:85-92.