Toxoplasma gondii Infection in Animal-Friendly Pig Production Systems

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Purpose. Consumption of undercooked pork meat products has been considered a major risk factor for contracting toxoplasmosis in humans. Indoor farming and improved hygiene have drastically reduced *Toxoplasma* infections in pigs over the past decades. Whether introduction of animal-friendly production systems will lead to a reemergence of *Toxoplasma* infections in pigs is not yet known. Investigating this possibility was the purpose of this study.

Methods. Blood was obtained from pigs raised for slaughter and tested for *Toxoplasma* antibodies by using latex agglutination and indirect immunofluorescence testing, with confirmation by immunoblotting.

Results. None of the slaughter pigs ($n = 621$) from conventional farms ($n = 30$) were positive, whereas 38 (2.9%) of 1295 animals from animal-friendly systems tested positive ($n = 33$ farms; 13 [39%] farms positive).

Conclusions. The following conclusions may be derived from this study: Conventionally (indoors) raised pigs are free from *Toxoplasma* infection, and (2) animal-friendly production systems may lead to a reemergence of *Toxoplasma* infections, although many of these farms remain *Toxoplasma* free. Slaughterhouse monitoring of pigs from animal-friendly production systems combined with on-farm prevention strategies should be applied to ensure safety for consumers of the meat products obtained from these animals. (*Invest Ophthalmol Vis Sci.* 2004; 45:3165–3169) DOI:10.1167/iovs.04-0326

The coccidian parasite *Toxoplasma gondii* is currently considered to be the most common cause of retinal infection throughout the world.[1] Recurrences of ocular toxoplasmosis are frequently observed. A long-term follow-up of patients recently showed that eventually 24% of the affected eyes become legally blind.[2] Although anti-*Toxoplasma* drugs are available, it is not yet clear whether they are effective in the treatment of ocular toxoplasmosis. Stanford et al.[3] reviewed the literature on this subject in immunocompetent patients and came to the conclusion that only a few well-designed studies have been performed in this field and that to date none of the trials has shown a beneficial effect of treatment.[3] Taking the above factors into account, it is obvious that more attention should be paid to the prevention of *Toxoplasma* infection. Sources of *Toxoplasma* infection include the ingestion of undercooked or inadequately cured meat containing encysted parasites or the uptake of soil, fruit, vegetables, or water contaminated with oocysts shed from infected cats.[4]

A multicenter epidemiologic study among pregnant women in Europe identified meat ingestion as the major source of *Toxoplasma* infection (30%–63% of cases).[5] Of the meat sources, pork has always been considered to be a major source of *Toxoplasma* infection, whereas beef has not been shown to contain infectious *Toxoplasma* parasites. Because of changes in pig production systems, the incidence of infection has declined rapidly over the past decades.[4] In the late 1960s, pigs were often kept outdoors, and up to 75% of animals were shown to be infected with *Toxoplasma gondii*.[4] Dubey[6] has shown that all edible parts of an infected pig may contain *Toxoplasma* cysts. Because of the indoor housing systems used today, the infection rate has dropped below 1%.[4] Indoor housing of animals is not regarded as beneficial for the animal's welfare, and due to social pressure, the bioindustry in several European countries has been urged to reintroduce outdoor housing. The effect of the introduction of animal-friendly production systems on the incidence of *Toxoplasma* infection in slaughter pigs is not yet known and was therefore the subject of this study. In our results, outdoor housing was indeed associated with a small but significant increase in the rate of *Toxoplasma*-infected animals.

Materials and Methods

Animal-Friendly Production Systems

In The Netherlands, so called "finishing pigs, " originating from regular intensive farms are housed indoors, mostly on concrete bedding, and are fed regular pig feed. In contrast, so-called free-range pigs are allowed outdoor access, have straw bedding, and are also fed regular pig feed. Pigs from organic farms are raised according to regulations set up by the European Union (EU regulation 2092/91), which includes outdoor access, straw bedding, and organic pig feed. Organic pig feed often contains the same (plant) ingredients as regular pig feed, but is grown on farms that do not use artificial chemical fertilizers or pesticides. Because of the bovine spongiform encephalitis (BSE) crisis, pigs in the EU are not allowed to be fed with products from "animal" origin. In organic pig farming the use of drugs and antibiotics is restricted and waiting times after use of these treatments is doubled compared with regular pig farming. Organic slaughter pigs are allowed only one treatment with synthetic drugs or antibiotics in their lifetime. If more treatments are needed, the animal loses its status as an organic pig and must be sold as a (cheaper) regular pig. Cutting tails or clipping teeth is not allowed in organic pig farming, and animals are weaned at a later age and are provided more living space per animal than on intensive farms.

Sampling Blood from Slaughter Pigs

Blood samples were collected from pigs weighing approximately 110 kg from different slaughterhouses in The Netherlands from April 2001

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through February 2002. The Netherlands slaughterhouses where blood samples were obtained were Bon Vivant in Didam, Compaxo in Zevenaar, Sturko/Dumeco in Apeldoorn, and Dumeco in Helmond. The animals were last fed approximately 12 hours before slaughter. The blood was collected in 10-mL plastic tubes containing clotting beads (catalog no. 26.323; Sarstedt, Nümbrecht, Germany) after the pig was electrocuted and after cardiac puncture. The farm registration number on the ear tag of each pig was noted so that individual blood samples could be linked to the farm where the pigs originated. After the blood was collected it was immediately transported to the laboratory where the tubes were centrifuged for 10 minutes at 2500*g*. From each blood sample, the serum was removed and stored in twofold in 1-mL tubes at −20°C.

Serologic Tests

Latex Agglutination Test. All porcine sera were screened for *Toxoplasma* antibody, using a latex agglutination test performed with a commercial kit according to the manufacturer's instructions (Toxo-reagent; Eiken Chemical Co., Tokyo, Japan). The kit includes a positive and a negative control serum and a buffer solution to make serum dilutions. In brief, 25 μL of a 1:64 dilution of serum was mixed with 25 μL of a suspension of *Toxoplasma*-coated latex beads into U-shaped wells of a microtiter reaction plate. After they were shaken, the latex beads were allowed to settle overnight. Agglutination patterns were read the next day.

Sabin-Feldman Dye Test. The Sabin-Feldman Dye Test is based on the fact that live *Toxoplasma* tachyzoites can actively take up methylene blue dye from the culture medium, whereas parasites that are killed because of complement-mediated lysis do not take up the dye and remain colorless. Tachyzoites were obtained by mouse inoculation. *T. gondii* tachyzoites of the RH strain were injected intraperitoneally into Swiss mice, and the animals were killed 48 hours after injection. Tachyzoites were collected by repeated flushing of the peritoneal cavity with phosphate-buffered saline (PBS; pH 7.4) and adjusted to a concentration of 25×10^6/mL. Serum samples were inactivated for 30 minutes at 56°C, and a dilution series was made from 1:1 to 1:128 in phosphate-buffered saline (PBS). Each dilution (10 μL) was transferred into a well of a flat-bottomed 96-well followed by 25 μL of human plasma as a complement source. After 10 μL tachyzoites was added, the plate was covered and incubated for 60 minutes at 37°C. After this incubation, 10 μL methylene blue solution was added to each well, and the plate was viewed under an inverted microscope at ×400 magnification. The procedures were approved by the institutional Animal Experiments Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Indirect Fluorescent Antibody Test. This test was performed using a commercially available kit (Toxo-Spot IF, catalog no. 75471; Biomérieux, Marcy-l'Etoile, France). The serum samples were tested at a dilution of 1:50, and 20 μL of this dilution was tested on each tachyzoite test spot (10 spots per slide). Slides were then incubated at 37°C for 30 minutes in a moist environment. The slides were washed in PBS for 5 minutes and dried. FITC-labeled rabbit anti-swine IgG (H+L) (RASw/IgG(H+L)/FITC; Nordic, Tilburg, The Netherlands) diluted 1:80 in PBS, was added to each spot and incubated for 30 minutes in a moist environment. The slides were washed again in PBS for 5 minutes, rinsed with distilled water, and dried. Two drops of antifade mounting medium (Fluoprep, catalog no. 75521; Biomérieux, Marcy-l'Etoile, France) were added and a coverslip was placed on the slide. The slides were viewed with a fluorescence microscope at 600× magnification. Each slide contained a positive and a negative serum control. As a positive control, a pig's serum was used which was shown to be positive in the latex agglutination test, immunoblot, and the Sabin-Feldman dye test. As a negative control, a pig's serum was used that was negative at a 1:16 dilution in the latex agglutination test. The positive control revealed a strong linear staining around the parasite. The negative control did not show a positive signal with the parasites on the glass slides. Some tested sera showed a polar staining or granular staining around the parasite. This type of staining was considered negative. Readings were performed by an observer who was not aware of the origin of the tested samples. The indirect fluorescent antibody test (IFAT) was validated against the Sabin-Feldman dye test. Fifteen IFAT positive and 13 IFAT negative sera were tested in the dye test. A perfect correlation was observed when comparing these two tests (Table 1). Based on this comparison, we chose to use the IFAT as a confirmatory test for the latex agglutination test.

Table 1. Comparison of Toxoplasma Serodiagnosis in Slaughter Pigs Using the Sabin-Feldman Dye Test and the Indirect Immunofluorescence Test

	Dye Test	
IFAT	**Positive**	**Negative**
Positive	15	0
Negative	0	13

Immunoblot

The immunoblot was performed using commercial strips containing size separated *Toxoplasma* antigens (R1-*Toxoplasma* WB; LDBio Diagnostics, Lyon, France). The strips were prepared by the manufacturer after SDS-PAGE size separation of a *T. gondii* tachyzoite extract followed by blotting on nitrocellulose paper. From a blot, 24 small strips measuring 0.3 × 7 cm were cut. Strips were preincubated in ELISA buffer containing 0.05% Tween-20 for 10 minutes. The porcine serum was diluted 1:50, and 2 mL was incubated with the strip for 90 minutes. The strips were subsequently washed three times for 5 minutes each in ELISA buffer and then incubated for 1 hour in 1 mL of a 1:1000 dilution of peroxidase-labeled rabbit anti-swine IgG (Fc) (RASw/IgG (Fc)/PO; Nordic). The strips were washed again three times for 5 minutes in ELISA buffer and once with distilled water. After they were washed, the blots were developed using chloronaphthol as a substrate.

To investigate the composition of the antigenic extract used to prepare the commercial strips (LDBio Diagnostics), we performed an experiment wherein the strips from one blot were incubated with a pool of *Toxoplasma* seropositive porcine serum obtained from 25 pigs and various commercially available *Toxoplasma* antibodies. The method used was slightly different from the protocol supplied by the manufacturer and is described later. An blot (LDBio Diagnostics) was cut into 4-mm strips, and nonspecific binding of antibodies was prevented by incubation for at least 1 hour at room temperature with blocking buffer (10% [vol/vol] normal rabbit serum [NRS] in PBS with 0.05% Tween-80 and 0.5 M NaCl [PBS-NT]). Subsequently, strips were incubated for 16 hours with 40 μL porcine serum in 2 mL PBS-NT containing 2% NRS. As a control, a strip was incubated with dilution buffer without the seropositive porcine serum. Separate strips from this blot were incubated for 16 hours with 10 μL monoclonal anti *Toxoplasma* ROP1 antibody (IE 10; 66 kDa; Argene, Varilhes, France); monoclonal anti *Toxoplasma* SAG-2 (II-38; 43 kDa; Argene); monoclonal anti SAG-1 (GII-9; 30 kDa; Argene), or monoclonal anti-*Toxoplasma* 24/40 kDa (T8075-40; ImmunoSource, Halle-Zoersel, Belgium) in 2 mL PBS-NT containing 2% NRS. Blots were washed three times with PBS-NT and were developed using rabbit anti-porcine IgG (P0164; Dakopats, Glostrup, Denmark) conjugated to horseradish peroxidase (HRP) 1:1000 or rabbit anti-mouse (P0260; Dakopatts) conjugated to HRP 1:1000 in 2 mL PBS-NT containing 2% NRS. Chloronaphthol (0.5 mg/mL 4-chloro-1-naphthol and 0.015% H_2O_2 in Tris-buffered saline [pH 7.4]; Sigma-Aldrich, St. Louis, MO) was used as a substrate. One hour after application of the substrate, staining was stopped by washing the strips with distilled water. All incubations were performed at room temperature, and, between all incubation steps, strips were washed three times for 10 minutes each with PBS-NT.

TABLE 2. Number of Slaughter Pigs Analyzed from Each Type of Farm

Type	Organic	Free-Range	Regular
Number of pigs	660	635	621
Number of farms	16	17	30
Mean per farm (range)	41 (17–50)	37 (16–48)	21 (17–30)

Farmer Interviews

To obtain insight into possible risk factors for *Toxoplasma* infection of pigs we conducted several on-site farm interviews. Of the farms included in our serologic survey: 18 regular, 14 free-range, and 13 organic farms were visited. At the time of the interviews, the farmers were not yet informed about the results of the *Toxoplasma* serology of their pigs. The researcher performing the interviews was also not aware of the serologic results of the study. During the interview, many questions were asked relating to farm size, number of cats, sites on the farm accessible to cats, rodent control, and source of water.

Statistical Analysis

Analysis of the statistical difference in numbers of seropositive pigs between different animal husbandry systems was performed with Yates' corrected χ^2 test.

RESULTS

In total, we collected blood samples from 1916 slaughter pigs, originating from 63 different farms. Table 2 shows how many samples were taken from each type of pig farm and how many different pig farms were tested. The number of pigs sampled per farm per occasion ranged from 16 to 50. Some farms were sampled on more than one occasion.

Sera were screened with the latex agglutination test, and positive sera were further tested with indirect immunofluorescence. Sera that showed a positive test result in both tests were subjected to immunoblot assay. Figure 1 is an example of one blot divided into 24 strips and showing a range of different positive porcine sera. Lane 21 shows the results after incubation of an immunoblot strip with the serum from a seronegative pig.

To investigate the position of various known *Toxoplasma* proteins on the commercial immunoblots, we incubated a series of strips from one blot with a pool of *Toxoplasma*-seropositive porcine serum (25 animals) and various monoclonal antibodies (Fig. 2). This experiment shows that all investigated proteins except SAG-2 were detected on the strips. The SAG-1 monoclonal antibody we used stains a protein with an approximate size of 33 kDa. This band was observed in many of the seropositive porcine sera and indicates that the animals possess antibodies to the SAG-1 protein.

Results of the *Toxoplasma* serology are shown in Table 3. As mentioned earlier a pig was considered seropositive when the specimens investigated tested positive by both latex agglutination and immunofluorescence and were also confirmed positive by immunoblotting. Of the organic pigs tested, eight (1.2%) were positive. These eight animals originated from three different farms. Thirteen (72%) organic farms were able to raise *Toxoplasma* negative pigs. Of the free-range farms, more than half (59%) had delivered one or more *Toxoplasma*-seropositive pigs for slaughter. Of the free-range pigs, 4.7% were *Toxoplasma* positive. None of the slaughter pigs raised on a conventional farm showed evidence of a previous *Toxoplasma* infection. The combined data show that 38 (2.9%) animals from a total of 1295 pigs raised under animal-friendly conditions had evidence of previous *Toxoplasma* infection.

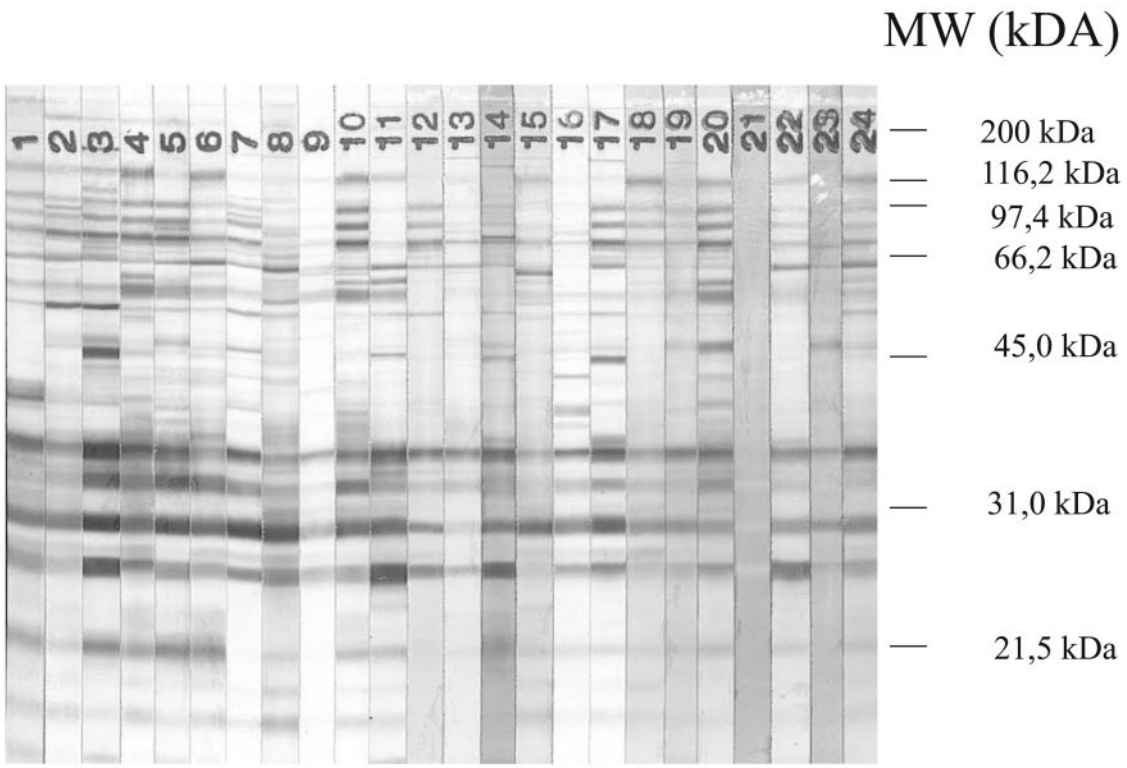

FIGURE 1. Representative immunoblot showing the profile of 23 sera samples obtained from different pigs with a positive test result in both the latex agglutination test and the indirect immunofluorescence test. Lane 21 is serum from a seronegative pig.

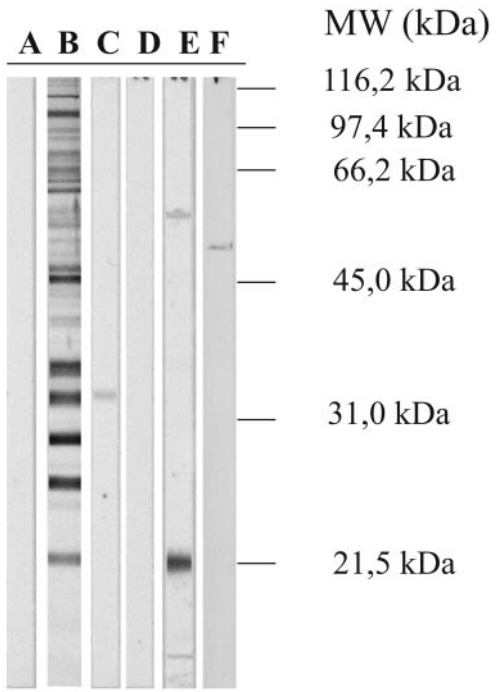

FIGURE 2. Commercial *Toxoplasma* immunoblot strips incubated with (*lane A*) conjugate control (omitting primary seropositive porcine serum), (*lane B*) a pool of *Toxoplasma*-seropositive porcine sera, (*lane C*) monoclonal anti-SAG-1 antibody, (*lane D*) monoclonal anti-SAG-2 antibody, (*lane E*) monoclonal anti-24/40 kDa antigen, and (*lane F*) monoclonal anti-ROP-1 antibody.

Farmer interviews showed that the regular farms were markedly larger (mean number of pigs: 729) than the free-range (455 pigs) or the organic farms (337 pigs). None of the regular farms had an outdoor area for the pigs, whereas all free-range and organic farms had an outdoor area for their animals. Interviews with the farmers concerning rodent control showed that all conventional farmers used chemical rodenticides, whereas 69% of the organic and 86% of the free-range farmers used rodenticides. Farmers not using rodenticides reported the use of farm cats as a method to control rodents. The number of cats on organic (mean number: 4.9) and free-range farms (mean number: 6.3) was significantly higher than on conventional farms (mean number: 2.2). Cats had access to the outdoor area of the pigs as well as to the areas were the feed and straw bedding was kept. The water source did not seem to be a risk factor. None of the farms provide water to their pigs originating from an open natural source such as a canal or local ditches. Many farmers including the regular farmers use a deep well to obtain water. The fact that none of the regular farms had *Toxoplasma*-positive animals indicates that these wells are not a source of infection. Of the 27 animal-friendly farms interviewed, 13 were *Toxoplasma*-positive and 14 were negative. The number of pigs on the *Toxoplasma*-positive farms was lower (328 pigs) than on the *Toxoplasma*-negative farms (464 pigs). Furthermore the *Toxoplasma*-positive farms had a mean number of 6.8 cats compared with 4.6 cats on the negative farms. No differences were observed between rodent-control strategies when comparing the *Toxoplasma*-positive and -negative farms.

DISCUSSION

The results of this study show that an animal-friendly way to produce pigs is associated with a reemergence of *Toxoplasma* infections. Although only a small percentage of the pigs from either a free-range or organic system were shown to be seropositive, these findings are certainly reason for concern. It has been estimated that the meat of one pig is eaten by approximately 300 to 400 hundred individuals,[7] and Dubey[6] has stated that all edible parts of a seropositive pig should be considered infectious. The general public should be made aware of these findings and should practice proper kitchen hygiene. Retailers should include advice on the labels of these products warning their customers to heat the products adequately before consumption. The meat industry should also be aware of the possible presence of the parasite *T. gondii* in the raw materials used for the preparation of sausages and various other products. Dubey et al.[8] have already warned that several methods of curing meat may not result in the killing of infectious cysts of the parasite. Recently, an investigation of various meat products showed that many products containing ingredients of porcine origin contained detectable *Toxoplasma* DNA.[9] The presence of DNA shows that the meat originates from a *Toxoplasma*-infected animal but does not necessarily mean that the product contains infectious organisms. Whether *Toxoplasma*-seropositive pigs harbor infectious tissue cysts in their meat has been addressed extensively by Dubey et al.[10] To validate *Toxoplasma* serology they fed cats and mice pig meat and studied these animals for the appearance of a *Toxoplasma* infection (bioassay). A good correlation was observed between the antibody titer in the pigs and the subsequent isolation of *Toxoplasma* in the cats and mice.[10] These findings indicate that *Toxoplasma*-seropositive pigs contain viable cysts in their meat and form a potential risk for the human consumer.

It is not clear how the pigs reported in our study became infected with *Toxoplasma*. Earlier studies in the United States have analyzed the risk factors involved in the occurrence of *Toxoplasma* infection in pigs.[11] Major reported risk factors were farm size, presence of cats, and method of rodent control. Our farmer interviews also point to the same risk factors. Three routes of infection can be envisaged for a pig to become infected with *Toxoplasma*. The first is the transfer of infection during pregnancy. Although congenital *Toxoplasma* infection

TABLE 3. Effect of Farm Type on Toxoplasma Infection of Slaughter Pigs

Type of Pig Farm	Total Pigs (*n*)	Seropositive Pigs (*n*)	Positive Pigs (%)	Total Farms (*n*)	Farms with Seropositive Pigs (*n*)	Positive Farms (%)
Organic	660	8*	1.2	16	3	18
Free-range	635	30	4.7	17	10	59
Conventional	621	0	0	30	0	0

* χ^2 analysis $P < 0.02$ when comparing organic with conventional; $P < 0.01$ when comparing organic with free range.

has been described in pigs, no data are available concerning its incidence. A second route of infection is through ingestion of another intermediate host or through meat products in the feed of the animals. The latter option is unlikely, since pig feed in the European Union is not allowed to contain animal products. Moreover, high temperatures are used in the processing of pig feed, leading to an inactivation of the parasite. Infection due to the uptake of an intermediate host such as a rodent is a possibility that should not be ruled out. Various studies have shown that a small percentage of wild rodents are infected with the parasite.[12] Of interest is the fact that, unlike in humans, vertical transmission in mice can go on for several generations.[13] This implies that rodents can form a reservoir of *Toxoplasma* that can be very difficult to control. A third route is an infection due to the uptake of oocysts shed into the environment through the feces of infected cats. From the farmer interviews, it became clear that both free-range and organic farmers kept a large number of cats on their farms and often relied on cats for rodent control. Cats on these farms often had access to the straw bedding and the sites on the farms where feed was stored. These findings suggest that cats could be a major source of *Toxoplasma* infection on these pig farms. Despite the presence of various risk factors such as outdoor access, presence of cats, and inappropriate rodent control, the number of animals that became infected was low, often only a small percentage. This could be due to other additional factors that we did not investigate, such as the *Toxoplasma* infection status of the rodents and cats on the farm. Small changes in these risk factors may already result in a farm with pigs being free from *Toxoplasma*.

The hypothesis that cats play a role in *Toxoplasma* infection of pigs is supported by the observation that vaccination of farm cats with a live attenuated strain of *T. gondii* led to a decrease in the number of infected pigs.[14] The effects of vaccination may take a number of years to become apparent, since oocysts in the soil may stay infectious for a long period. Furthermore, as mentioned earlier, vaccination of cats may lead to a decrease in infectious oocysts from the environment but may not influence the reservoir of *Toxoplasma* cysts in the rodent population, which is maintained by vertical transmission. Further support for the role of the cat in the epidemiology of toxoplasmosis comes from studies showing a very low (0.9%) prevalence of *T. gondii* antibody in 1264 feral pigs from a remote island in the U.S. state of Georgia where there were no cats, compared with an 18% prevalence in 170 feral pigs from the mainland in Georgia.[15]

At present, there are no epidemiologic data supporting the hypothesis that animal-friendly farming will affect the incidence of human toxoplasmosis. However, the high incidence of ocular toxoplasmosis in certain areas of Brazil has been linked to the consumption of undercooked pork products (Belfort R, et al. *IOVS* 2004;45:ARVO E-Abstract 1674).

The fact that many of the farms raising pigs in animal-friendly conditions were able to grow pigs that were not infected by *T. gondii* shows that it is possible to control this parasitic infection. Development of a hazard analysis critical control point (HACCP) system in combination with serologic monitoring at slaughter should lead to the implementation of strategies to prevent *Toxoplasma* infection of pigs raised in conditions that improve their welfare.

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