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Transmission dynamics of *Toxoplasma gondii* on a pig farm

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Abstract

Transmission of *Toxoplasma gondii* infection on a pig farm in New England was investigated using genetic and ecological methods to (i) determine if infection of pigs was a result of a single source, such as in an epizootic situation (e.g. outbreak) or of multiple sources, such as in an enzootic situation, (ii) identify the main source species of infection to pigs and (iii) evaluate the role of the environment surrounding the farm as the source of infection on the farm. Genetic characterization of 25 *T. gondii* isolates from market pigs revealed three distinct genotypes with no evidence of recombinants. These data imply that at least three distinct exposure events occurred during the 7-month lifespan of these pigs. This genotype diversity is consistent with enzootic transmission of *T. gondii* on the farm. Cats were suspected as the main source of pig infection based on the high seroprevalence (>95%) in pigs. The presence of the two most common *T. gondii* genotypes in eight isolates from free ranging chickens on this farm corroborated the role of cats because chickens were probably infected through ingestion of oocysts in the soil. The seroprevalence of toxoplasmosis in 163 wild mammals and birds captured around the pig sties (overall 13.1%) increased with proximity to the pig sties. Thus, transmission of *T. gondii* was higher near the pig sties than in the surrounding environment probably because of increased density of oocysts there. We propose that the farm does not simply reflect its surroundings in terms of strain composition and risk of infection, but that it acts as a reservoir of strains from which the outflow of new infections into its surrounding environment is higher than the inflow.

Keywords: Ecological genetics; Farm; Microsatellite; Molecular epidemiology; Pig; *Toxoplasma gondii*

1. Introduction

Toxoplasmosis is a common zoonotic disease worldwide (Dubey and Beattie, 1988). Over 20% of adults in the US are chronically infected with *Toxoplasma gondii* (Jones et al., 2001a) and the prevalence is higher in many other countries. Recognized as the third leading cause of death among food-borne diseases in the US (Jones et al., 2001b) and as an important opportunistic disease of the immunocompromised (Belanger et al., 1999), effective prevention of toxoplasmosis is a public health priority. Human infection occurs primarily through ingesting food or drink contaminated with *T. gondii* oocysts shed into the environment in feces of felids or by ingesting undercooked meat containing the tissue cysts. However, little information is available on the relative importance of these routes of infection. Among meat animals, pigs are considered to be the most important meat source of human infection in the US (Dubey, 1986 and references therein). Prevalence of *T. gondii* in market pigs appears to have declined in the US with the advent of improved sanitation in large production facilities (Davies et al., 1998). However, a serological survey of pigs of variable age from 85 New England farms showed an overall prevalence of 47%, with 91% of the herds having at least one seropositive pig, and within-herd prevalence varied between 4 and 100% (Gamble et al., 1999). The latter study reveals that high infection rates persist in some small pig farms under some conditions and highlights the need for better understanding of the epizootology of toxoplasmosis on pig farms.

Several studies have attempted to elucidate the sources of pig infections with *T. gondii* based on serological information and parasite isolation from feed, soil, and animals living in and around pig farms. Ingestion of oocysts as the

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main route of infection was supported by a high prevalence of infection in cats on pig farms (Smith et al., 1992; Weigel et al., 1995) coupled with the isolation of parasites from soil, feed, and cat feces (Dubey et al., 1995). Eating infected rodents was suggested as the main source of infection in pigs on two farms in Georgia (Lubroth et al., 1983). Cannibalism was experimentally shown to be another possible route of infection (Dubey et al., 1986). These studies demonstrate that there are at least three possible sources of pig infections, but most studies suggest that oocysts shed by cats are the most common source.

Discrimination between these sources on the basis of seroprevalence data is problematic. For example, it was assumed that the source is from the animal group (e.g. cats, rodents) showing the highest seroprevalence; an assumption that is not justified. Furthermore, a high correlation is expected between infection rate in cats and in rodents. Several questions regarding the epidemiology of *T. gondii* infection on pig farms remain to be answered. These include: (i) whether the infection in pigs is the result of a single infection event, as in an outbreak or of multiple events, as in an enzootic situation? (ii) what animal species serve as the main source(s) of infection in pigs? and (iii) is the farm located in an area of unusually high transmission, so that the environment surrounding the farm is the primary source of pig infections?

High resolution strain typing (Blackston et al., 2001; Ajzenberg et al., 2002) may help answer these questions. Estimating the number of distinct strains (genotypes) infecting pigs would be useful in answering the first question, and identifying the same genotype in both, the pigs and in only one of many putative sources could substantiate the importance of this source. In this study, we used genetic data of *T. gondii* isolates and ecological data on the distribution of infection in wild animals around a pig farm to address these questions. Although our data are limited to a single pig farm, this study provides new insights into the transmission dynamics of *T. gondii* in such settings and raises issues that should be addressed in future studies.

2. Materials and methods

2.1. Study area

All field work was conducted on the premises of a single pig farm in New England that was selected based on the high seroprevalence in its pigs as determined in a previous survey (Gamble et al., 1999). It is a farrow-to-finish production facility (i.e. new pigs born on the farm are added continuously to the herd after weaning until market age at 6–8 months) of approximately 500 pigs. Approximately 50 sows (adult female breeders) and 2 boars were maintained on open dirt lots. Weaned pigs were fed and finished in enclosed buildings on concrete slabs. All animals were fed cooked garbage consisting of restaurant and processed food waste, as well as produce and bakery waste. Finished pigs were marketed at approximately 100 kg, generally between 7 and 8 months of age. Feral cats, mice and rats were occasionally observed around the pig sties and control measures against rodents and cats were minimal.

The farm buildings housing the pigs were located on a small hill. Within 500 m of the buildings were cow pastures, a pond, marshy areas, a number of large and medium size woodpiles, and a lake surrounded by a forested area (Fig. 1). Old and broken machinery, such as trucks and washing machines were present in various parts of this area.

2.2. Sampling of domestic and wild animals

A total of 55, 6–8-month-old pigs, 11 adult domestic chickens (*Gallus domesticus*), and 163 wild animals (Table 1) were examined for *T. gondii* infection. A full description of the pig samples was given previously (Dubey et al., 2002) and only a brief description is provided here. Two lots of pigs were obtained. The first lot, taken on November 2001 included 30 pigs that tested positive by modified agglutination test (MAT) (Dubey and Desmonts, 1987). The second lot, taken on January 2002 included 25 pigs from the oldest group of finishers that were arbitrarily selected without prior serological testing.

The eleven adult chickens were free to roam on the farm. Six of these chickens originated on this farm. Five additional chickens of similar age were purchased from neighboring farms and were brought to the pig farm 3 months before they were killed (January 2002). No previous serological information was available for the chickens.

Wild animals were captured over a 6-day period in April 2002. A total of 200 rodent traps (mostly Sherman traps), 20 Tomahawk traps, and 10 mist nets were placed at various locations on the farm property to represent all the main habitats including pig housing and feed-storage structures (Fig. 1). Traps were baited with a mixture of birdseed, oats and powdered sugar or with peanut butter. Traps were placed in rows of variable length and variable spacing to maximize capture rate by concentrating traps near signs of rodent activity (such as droppings). If a given trap had not captured an animal in 48 h, it was relocated. Traps were checked every morning and every afternoon and captured rodents were transported in these traps for further processing. Mist nets were set before sunrise for several hours until the capture rate declined and they were deployed again every afternoon until sunset. Nets were relocated if the number of birds captured was low. Nets were checked every 20–30 min and captured birds were removed from nets, identified, and placed in cloth bags or released if they were a non-target species. Target species included species that forage on the ground. Two road-kill cats and two dead rats were also included among these specimens. Every capture site was mapped using a Garmin e-map global positioning unit. With this system, our measurements of latitude, longitude, and elevation had a standard error of 5 m. Distances between mapped points were computed based on the arc distance between two points on a sphere.

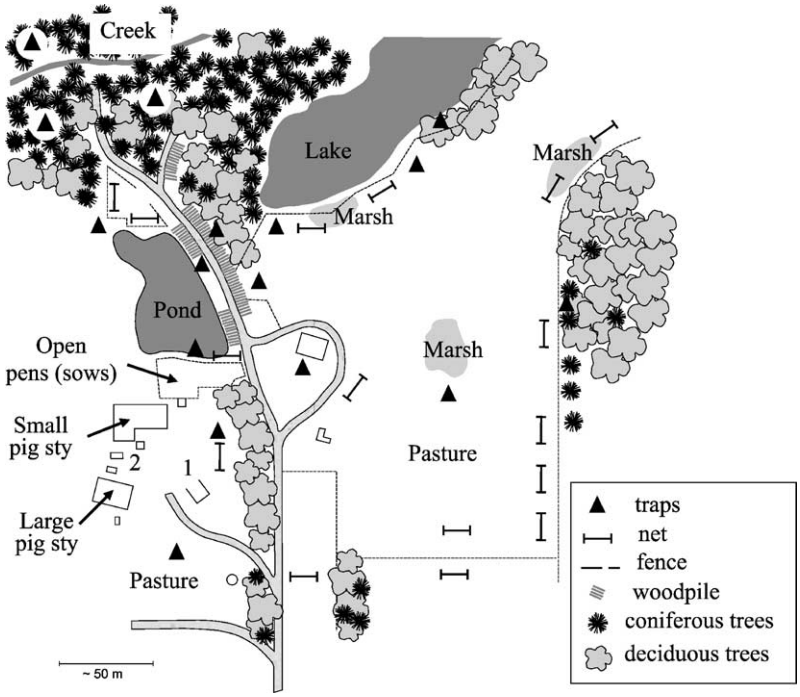

Fig. 1. Schematic showing location of the pig sties, various habitats around the farm and placement of traps and mist nets. Rodent traps were also placed in and around the pig sties (not shown). Non-meat food was stored in a semi open structure marked as '1' and in two trailers marked as '2'.

Table 1
Prevalence of *T. gondii* infection among species based on serology (MAT)

Species	*N*[a]	Serological prevalence[b]	Titer ≥10				*T. gondii* isolation
			10	20	40	80	
Mammals							
White-footed mouse (*Peromyscus leucopus*)	33	6.1 (33)				2	2
Meadow vole (*Microtus pennsylvanicus*)	8	12.5 (8)			1		0
Norway rat (*Rattus norvegicus*)	7	50 (2)				1	0
House mouse (*Mus musculus*)	3	0 (2)					0
Short tail shrew (*Blarina brevicauda*)	3	0 (2)					0
Meadow jumping mouse (*Zapus hudsonius*)	1	0 (1)					0
Eastern chipmunk (*Tamias minimus*)	1	0 (1)					0
Domestic cat (*Felis catus*)[c]	2	0 (0)					0
Birds							
European starling (*Sturnus vulgaris*)	29	**0*** (29)					1
American robin (*Turdus migratorius*)	18	**44.4***** (18)	3	3	1	1	0
White-throated sparrow (*Zonotrichia albicollis*)	15	6.7 (15)	1				0
House sparrow (*Passer domesticus*)	8	12.5 (8)		1			0
Song sparrow (*Melospiza melodia)*	7	0 (7)					0
Common grackle (*Quiscalus quiscula*)	7	**71.4***** (7)	3	2			0
Red wing blackbird (*Agelaius phoeniceus*)	6	0 (5)					0
Northern Cardinal (*Cardinalis cardinalis*)	4	25 (4)			1		0
Chipping sparrow (*Spizella passerina*)	3	0 (3)					0
Brown headed cowbird (*Molothrus ater*)	3	0 (3)					0
Carolina wren (*Thryothorus ludovicianus*)	2	0 (2)					0
Brown thrasher (*Toxostoma rufum*)	1	0 (1)					0
American Goldfinch (*Carduelis tristis*)	1	0 (1)					0
Black capped chickadee (*Parus atricapillus*)	1	0 (1)					0
Overall	163	13.1 (153)	7	6	3	4	3

Antibody titer at dilutions of 1:10 or higher was considered evidence of infection.

[a] Total specimens captured.

[b] Number of specimens tested is indicated in parenthesis. Significance is based on the binomial test using the overall prevalence (13.1%) as the expected frequency. Bolded values are significant at the multi-test level using the sequential Bonferroni test (Holm, 1979).

[c] One road kill cat was found 0.5 mile from the pig sties and another was near the barn.

Captured animals were transferred into a Ziploc bag containing halothane-soaked cotton balls for euthanasia, identified to species, and bled by cardiac puncture. The heart and head were removed and placed in numbered plastic bags on blue ice until shipment to the USDA laboratory in Beltsville, Maryland for serological tests and parasite isolation.

2.3. Seroprevalence and *T. gondii* isolation from domestic and wild animals

Sera from all animals were tested for the presence of antibodies to *T. gondii* by the MAT. Sera were diluted in a two-fold series starting at a 1:25 or a 1:10 dilution. Tissues of pigs and chickens were fed to *T. gondii* free cats, and the cat feces were subsequently examined for oocysts. The cat bioassay is very efficient because cats usually shed millions of oocysts after ingesting a few *T. gondii* tissues cysts (Dubey, 2001). The process of isolation of *T. gondii* from these pigs was described in detail previously (Dubey et al., 2002). Briefly, venous blood was obtained from every pig prior to slaughter, and heart (lot 1), and heart and tongue (lot 2) were collected and fed individually to each of 55 cats.

Brain, heart, and muscles from the leg and breast of each of 11 chickens were also fed individually to each of 11 cats. Feces from cats were examined for oocysts. Oocysts from the feces of cats were sporulated and fed to pairs of mice. Four days later, the mice were killed, and their mesenteric lymph nodes were homogenized in saline and examined microscopically for tachyzoites. Aliquots of tachyzoites were subinoculated into new mice and saved for DNA analysis.

Brain and heart tissues from wild animals were examined for *T. gondii* by inoculation into outbred Swiss-Webster albino mice as described previously (Dubey and Beattie, 1988). Aliquots of tissue homogenate were inoculated into two (for wild rodents) or five (for all other wild animals) mice. The mice were bled 6 weeks post-inoculation and their sera were examined for antibodies to *T. gondii* by the MAT. The mice were killed after serological tests and their brains were examined for *T. gondii* tissue cysts (Dubey and Beattie, 1988). Part of the brain of infected mice was sub-inoculated into new mice, while the rest was saved for DNA extraction.

2.4. DNA extraction and genotyping of isolates

We randomly selected 15 isolates from the first lot of pigs and 10 isolates from the second lot for genotyping. All other isolates, from chickens and wild animals, were genotyped. DNA extraction from a suspension of oocysts (pig and chicken isolates) or from infected mouse tissues (wild animal isolates) was performed using the Bio101 procedure as previously described (Lehmann et al., 2000). Lineage typing was performed using the PCR-RFLP assays of the SAG2 gene (Howe et al., 1997). High resolution genotyping at six microsatellite loci was performed as previously described (Blackston et al., 2001). All unique alleles were verified by a second round of genotyping.

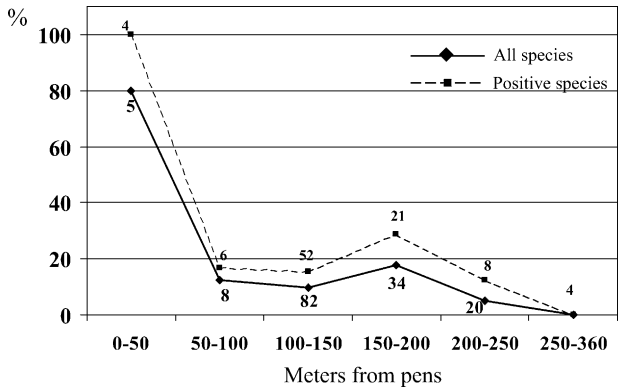

Fig. 2. The effect of distance from the pig sties on seroprevalence of wild animals. Numbers denote sample size (see text for details).

3. Results

A total of 163 wild animal specimens were collected, comprising eight species of mammals and 14 species of birds (Table 1). The overall seroprevalence of *T. gondii* infection in wild animals ($N = 153$) was 13.1%. However, prevalence differed markedly among species, with the highest values in common grackles and American robins (71 and 44%, respectively) and the lowest value in European starlings (0%). The smaller sample sizes available for most mammal species reduced the power to discern differences in infection rates between species.

To assess whether the prevalence of *T. gondii* infection in wild animals near the pig sties differed from the surrounding area, we compared the prevalence in animals collected at 50 m intervals from the pig sties (Fig. 2). The prevalence was 80% in animals collected within 50 m from the pig sties and dropped below 20% beyond this distance (Fig. 2). Logistic regression analysis showed that the probability of infection significantly decreased with distance from the pig sties (Table 2). Measuring distance in a logarithmic scale to account for the exponential increase in the area with increasing distance from the sties enhanced the significance of the distance effect. Excluding negative species, which may have confounded the effect of distance had little effect on this trend (Table 2). Moreover, unlike individual hosts, there was no indication for clustered distribution of a highly seropositive species near the pig sties (Table 3).

Table 2
The effect of distance from the pig sties on prevalence (serology) of individual hosts measured by four logistic regression models

Data set	Distance effect	*P*
All species ($N = 153$)	-0.01[a]	0.013
Positive species ($N = 95$)	-0.01[a]	0.046
All species ($N = 153$)	-1.63[b]	0.007
All species with species added as a covariate ($N = 153$)	-1.06[b]	0.033

[a] Distance measured on a linear scale (m).
[b] Distance measured on a logarithmic scale (m).

Table 3
Spatial distribution of seropositive species with respect to distance from the pig sties, showing that, unlike individual hosts, there was no clustering of positive species around the pig sties

Species	0–50 m	50–100 m	100–150 m	150–200 m	200–250 m	250–360 m
White-footed mouse	1 (1)	0	14	10 (1)	4	4
Meadow vole	0	0	4	3 (1)	1	0
Norway rat	1 (1)	0	0	1	0	0
American robin	2 (2)	2 (1)	9 (2)	4 (3)	1	0
White-throated sparrow	0	2	12 (1)	0	1	0
House sparrow	0	1	7 (1)	0	0	0
Common grackle	0	0	6 (4)	0	1 (1)	0
Cardinal	0	1	0	3 (1)	0	0
Total	4 (4)	6 (1)	52 (8)	21 (6)	8 (1)	4 (0)

Number of seropositive individuals is shown in parenthesis.

Toxoplasma gondii was isolated from tissues of 51 of 55 pigs (Dubey et al., 2002). However, only 25 isolates were selected for genotyping (see Section 2). Isolates were obtained from seven of eight seropositive chickens and from one of four seronegative (at a 1:10 dilution) chickens. Although, parasite isolation was attempted from brains and hearts of all 163 wild animal specimens, only three isolates were obtained. Unexpectedly, one isolate was obtained from a seronegative starling, while the others were derived from white-footed mice (Table 1). Isolation from seronegative animals indicated that infection was higher than estimated by serology. Mice inoculated with tachyzoites or tissue cysts from the 11 chicken and wild animal isolates remained asymptomatic.

Among the 25 pig isolates obtained from the two lots, three distinct strains (multi-locus genotypes) were identified (Table 4). The multi-locus genotype provides near fingerprinting resolution as indicated by identifying 14 distinct genotypes among 17 isolates from sows slaughtered in Iowa (all of lineages II or III, not shown), and all were distinct from the three multi-locus genotypes described here. Thus, the probability that we have substantially underestimated the diversity of strains is unlikely. The most common genotype (named 3a throughout this paper) predominated in both lots of pigs (Table 4). Similar strain composition was found among the eight chicken isolates, with genotype 3a comprising 63%. The three isolates from wild animals had the same genotype as that predominating in the pigs (3a, see Table 4). Two isolates were from white-footed mice trapped 20 and 215 m from the pig sties and one isolate was from a starling captured 120 m from the sties. There were no recombinant genotypes among the 36 isolates obtained from the various hosts on and around the farm.

4. Discussion

Genetic and ecologic data were used to assess the transmission dynamics of *T. gondii* infection on a New England pig farm that had previously been reported to have a high rate of infection in pigs (Gamble et al., 1999). Genetic characterization of 25 *T. gondii* isolates from pigs that were born and raised on this farm revealed three distinct genotypes. Since no recombinant genotypes were observed, this information implies that there were at least three independent exposure events, each involving a distinct individual animal as a source and occurring during a 7-month period (the age of the pigs). This inference relies on the assumption that a host typically is infected with only one parasite strain because only one strain has been isolated from naturally-infected individual hosts (Howe et al., 1997). The actual number of sources of infection may have been higher if different source animals harbored the same genotype (see below). Such a diversity of strains is consistent with stable, enzootic

Table 4
Multi-locus genotypes observed in various samples based on six microsatellite loci (Blackston et al., 2001) and the RFLPs assay at the SAG2 locus (Howe et al., 1997)

Multi-locus genotype	Pigs			Chickens ($N = 8$) (%)	Wildlife[a] ($N = 3$) (%)
	Lot 1 ($N = 15$) (%)	Lot 2 ($N = 10$) (%)	Total ($N = 25$) (%)		
3a[b]	13 (86)	7 (70)	20 (80)	5 (63)	3 (100)
2a[c]	1 (7)	0	1 (4)	0	0
2b[d]	1 (7)	3 (30)	4 (16)	3 (37)	0

Unique multi-locus genotypes were named arbitrarily (e.g. 3a) for simplified reference in the context of this publication.

[a] Comprising of two isolates from white-footed mice and one from an European starling.

[b] Multi-locus allele composition (bp) were M6 = 200, M33 = 167, M48 = 213, M102 = 191, M163 = 174, M95 = 402, and lineage (SAG2) = III.

[c] Multi-locus allele composition (bp) were M6 = 216, M33 = 171, M48 = 249, M102 = 175, M163 = 160, M95 = 218, and lineage (SAG2) = II.

[d] Multi-locus allele composition (bp) were M6 = 210, M33 = 171, M48 = 215, M102 = 175, M163 = 164, M95 = 218, and lineage (SAG2) = II.

transmission of *T. gondii* on the farm, rather than with an epizootic event. The consistently high seroprevalence in pigs on this farm, since it was first studied in 1999, and the high seroprevalence among other pig farms in the New England states (Gamble et al., 1999) also indicate that infection is not a rare event as is implicit in an epizootic. Considering the small area of the pig sties and associated structures (approximately 1500 m^2), the diversity of strains there was high.

The role of oocysts as the main source of pig infection was suspected based on the high prevalence of *T. gondii* in the pigs (>95%), because an infected rodent, bird, and even pig carcass cannot expose over 200 pigs. The role of oocysts was corroborated by the presence of the two most common genotypes found in the pigs (3a and 2b) in the eight chickens kept on the farm. It is more likely that the chickens would be exposed through ingestion of oocysts in the soil than by eating infected rodents or infected tissues from carcasses of pigs (Ruiz and Frenkel, 1980).

Only three isolates were obtained from wild animals captured at the various environments surrounding the pig sties. This small sample size precluded comparisons of genotype composition between various natural host species, environments, and the pigs. However, all three isolates were identical to the most common genotype found in the pigs (3a). Whether these white-footed mice and the starling were infected on the farm from the same source(s) of infection as the pigs or whether this strain predominated in the area surrounding the farm remains unanswered. The correspondence between the location where an animal was infected with *T. gondii* and the site of its capture is weaker the larger the animal's foraging area. Thus, this relationship is weaker in birds than in rodents. Among seropositive wild animals, robins were the most numerous (Tables 1 and 3). We believe that the capture location of robins is indicative of their infection site because robins are territorial for a large part of the year (February–September) and the size of a territory ranges between 200 and 1300 m^2 (Bent, 1949). Territories of such sizes are not much larger than the home range of many rodents and their area is equivalent to that of circles with radii of 8–20 m.

The seroprevalence of wild animals around the farm increased with proximity to the pig sties. The marked differences in prevalence persisted after excluding negative species from the analysis, which potentially could confound the observed pattern. These results suggest that transmission of *T. gondii* near the pig sties was considerably higher than in the surrounding environment, despite its diverse habitats and larger area, and thus, the farm can be considered a hyper-endemic focus of infection with this parasite. According to this view, such farms do not simply reflect their immediate surroundings in terms of strain composition and risk of infection, but serve as reservoirs of infection, from which transmission to the surrounding environment is greater than transmission from the environment to the farm. The mechanisms contributing to the higher transmission and strain diversity near the pig sties may include (1) an increased density of host species facilitating greater likelihood of contact with oocysts and increased cat density resulting in higher density of oocysts, (2) a high likelihood of introduction of new strains by "concentrating" migratory and far-ranging birds and mammals, (3) a greater longevity of oocysts in the moist, shaded, and temperature-regulated environment of the pig sties, and (4) a dissemination of oocysts into a greater area by the constant mixing of the soil and feed as the pigs move and forage. Considering these possibilities, a single introduction of *T. gondii* into a pig farm by any source (an infected rodent, bird, or cat) could result in amplification of infection on the farm and subsequent high rates of transmission, even in environments where original *T. gondii* transmission has previously not occurred. More information is needed to test whether farms such as the one described here act as reservoirs of *T. gondii* strains that, in turn, increase the risk of infection of wild, feral, and domestic animals (besides meat animals) over that of the surrounding environment and consequently increase the risk of human infection.

Foci of high transmission associated with farms may help explain the low genetic diversity of *T. gondii* (Lehmann et al., 2000 and references therein). Serving as superior habitats for *T. gondii*, early farms might have amplified a small subset of ancient strains and spread them world wide with the spread of farming technology within the past 10,000 years (Cavalli-Sforza et al., 1994). Contemporary strains of *T. gondii*, may be the result of a recent population expansion of this small subpopulation. A corollary hypothesis is that infection with *T. gondii* prevails at the domestic and peridomestic environment and it is relatively rare in many pristine wild settings.

Strategies to prevent exposure of pigs to *T. gondii* must focus on cat and rodent control as previously recommended (Dubey, 1996). In addition, drainage of water (during heavy rains) into and from the pig sties should be prevented or controlled to minimize exposure of hosts to oocysts from the soil. A pressure wash of cement floors with steam (Dubey, 1998) between each group of pigs, may further reduce the density of viable oocysts in the area.

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