SMALL ANIMALS

Detection of *Toxoplasma gondii*-like oocysts in cat feces and estimates of the environmental oocyst burden

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Objective—To estimate the analytic sensitivity of microscopic detection of *Toxoplasma gondii* oocysts and the environmental loading of *T gondii* oocysts on the basis of prevalence of shedding by owned and unowned cats.

Design—Cross-sectional survey.

Sample Population—326 fecal samples from cats.

Procedures—Fecal samples were collected from cat shelters, veterinary clinics, cat-owning households, and outdoor locations and tested via $ZnSO_4$ fecal flotation.

Results—Only 3 (0.9%) samples of feces from 326 cats in the Morro Bay area of California contained *T gondii*–like oocysts. On the basis of the estimated tonnage of cat feces deposited outdoors in this area, the annual burden in the environment was estimated to be 94 to 4,671 oocysts/m^2 (9 to 434 oocysts/ft^2).

Conclusions and Clinical Relevance—Despite the low prevalence and short duration of *T gondii* oocyst shedding by cats detected in the present and former surveys, the sheer numbers of oocysts shed by cats during initial infection could lead to substantial environmental contamination. Veterinarians may wish to make cat owners aware of the potential threats to human and wildlife health posed by cats permitted to defecate outdoors. (*J Am Vet Med Assoc* 2007;231:1676–1684)

Toxoplasma gondii is an ubiquitous protozoal parasite that infects 30% to 40% of the world's human population, as well as most warm-blooded animals.[1] The parasite has a life cycle involving definitive felid hosts that shed oocysts in their feces and intermediate hosts that include mammals and birds. Intermediate hosts develop cysts in their tissues (notably, muscle and brain) after ingesting oocysts or eating the uncooked flesh of infected animals. The environmentally resistant oocysts are shed only in the feces of domestic cats and other members of the Felidae.[2] Related coccidian parasites with morphologically identical oocysts are *Hammondia hammondii* and *Besnoitia darlingi*.[3] These parasites also infect cats, and their oocysts may be misclassified as *T gondii* oocysts.

Humans can acquire *T gondii* from the environment by contact with oocyst-contaminated soil, water, or cat feces; from the food supply by eating undercooked meat or inadequately washed vegetables; or by transplacental transmission.[1,4] In recent years, increasing concern has developed about waterborne toxoplasmosis. Three large human outbreaks of toxoplasmosis have been epidemiologically linked to oocyst-contaminated surface water.[5-7] There is also strong evidence linking *T gondii* infection in California's southern sea otter population to land-sea runoff. Otters off California's central coast in locations where freshwater outflow exceeded 100,000 acre-ft/y were almost 3 times as likely to be seropositive for *T gondii* as otters found in locations of low outflow.[8] Furthermore, sea otters that became stranded along the coastline between San Simeon and Morro Bay were 5 times as likely to be seropositive for *T gondii* as otters from the more remote and rocky Big Sur area.[9] The detection of a high prevalence of *T gondii* infection in sea otters from the Morro Bay area indicated that this was an ideal site for an epidemiologic investigation of definitive and intermediate hosts in the nearshore terrestrial environment. The most abundant felid host in this region was likely to be the domestic cat because 38% of households in the Morro Bay area were estimated to own cats.[10] Esti-

Abbreviations

C_T	Threshold cycle
OR	Odds ratio
CI	Confidence interval

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mates of outdoor fecal deposition by free-roaming cats from the 3 communities in the immediate vicinity of Morro Bay have been reported.[10] The purpose of the next phase of that study, which is the study reported here, was to test cat feces for the presence of *T gondii* oocysts to help determine the proportion of cats shedding oocysts and calculate the number and density of oocysts entering the coastal environment. Additional goals were to determine the analytic sensitivity of light microscopy following double centrifugation and flotation in $ZnSO_4$ and 2 PCR protocols for detecting *T gondii* oocysts in cat feces to determine the threshold at which oocysts could be detected, compare detection in fresh and dry feces (placed in cat litter for 2 to 4 days), and compare 2 assessors with different levels of experience.

Materials and Methods

Oocyst spiking in cat feces—*Toxoplasma gondii* oocysts were harvested from feces of a tiger euthanized at the Center for Animal Health and Food Safety, University of California, Davis in the fall of 2005. Oocysts were determined to be *T gondii* type II by use of described protocols[11] and were harvested by use of a double centrifugation technique. Briefly, feces were mixed to a thin slurry with 0.2% Tween-80 in distilled water and poured through a tea strainer into a series of 50-mL centrifuge tubes. Samples were centrifuged at 1,000 × g for 10 minutes, and the supernatant was decanted. The remaining pellet was thoroughly mixed with 25 mL of NaCl solution (specific gravity, 1.2) and centrifuged as before. The top 10 mL of NaCl solution was harvested and washed 3 times; the first wash was performed with 0.2% Tween-80, and the second and third washes were performed with deionized water. Harvested oocysts were sporulated in 2% H_2SO_4 and stored at 4°C until used. Oocyst concentration was determined by counting in a hemocytometer, and oocysts were diluted so as to supply volumes of $\leq$ 100 µL for any fecal sample. Immediately prior to spiking feces, oocysts were inactivated by suspending 1.5-mL aliquots in boiling water for 5 minutes.

Feces used for the spiking experiments were collected in sawdust < 24 hours after defecation from specific pathogen–free cats from the Nutrition and Cat Care Colony at the University of California, Davis. One-gram aliquots of feces were weighed, and each was placed in a clean sample cup. Oocysts were spiked into the 1-g fecal samples in the following quantities: 0, 100, 500, 1,000, 5,000, 10,000, and 20,000. The feces were then gently mixed with a wooden applicator stick. Three 1-g samples of feces for each spiking amount were allocated to either immediate processing (designated as fresh) or were placed on a layer of natural clay litter[a] for 2 to 4 days (designated as dry). Dry feces were covered with 2 to 4 cm of the clay litter, maintained at 22°C, and retrieved from the litter just before processing. It was important to assess the effect of feces left in cat litter for a few days because most cat owners supplying cat feces would be expected to submit samples collected from cat litter. Prior to centrifugation and flotation, 3 mL of distilled water was added to each fresh or dry fecal sample to form a fecal slurry to achieve a 3:1 ratio of water to fecal matter by weight. Five hundred microliters of slurry was aliquoted into 1.5-mL snap-cap vials and set aside for PCR assay. The proportion of water-diluted feces destined for fecal flotation was calculated as the initial weight (4 g) minus the weight of the PCR aliquot, divided by initial weight. Multiplication of this proportion by the number of oocysts spiked into each sample gave the expected number of oocysts in the portion of the sample processed by fecal flotation. Feces were processed by use of the double centrifugation technique, as described, with $ZnSO_4$ solution (specific gravity, 1.2) instead of NaCl solution because oocysts are more easily detected in $ZnSO_4$ solution. Two assessors (1 experienced and 1 inexperienced; both unaware of oocyst concentration) scanned the entire surface of the 18 × 18-mm coverslip mounted on a slide by use of a light microscope at 100× magnification and counted the number of visible 10 × 12-µm oocysts. After the counts were made, slides were washed with approximately 200 µL of distilled water and the water was saved for PCR assay.

Real-time quantitative PCR assay for oocysts—For each target gene, 2 primers and an internal, fluorescent-labeled probe[b] (5′ end, reporter dye: 6-carboxyfluorescein; 3′ end, quencher dye: 6-carboxytetramethylrhodamine) were designed with software.[c] The PCR system was designed within an exon to detect genomic DNA. The PCR systems were validated by use of defined protocols.[12] The dilutions were analyzed in triplicate, and a standard curve was plotted against the dilutions. The slope of the standard curve was used to calculate amplification efficiencies by use of the formula, $E = 10^{1/-s} - 1$, where E is the efficiency of amplification and s is the slope.

Sample collection and automated nucleic acid preparation—One hundred microliters of coverslip wash solution or fecal slurry (3:1 ratio of distilled water to fecal material) was collected in 150 µL of nucleic acid purification lysis buffer[d] and stored at –20°C until processed. Proteinase K[e] and two 4-mm-diameter stainless steel grinding beads[f] were added, and the slurry was homogenized in a grinder[f] for 2 minutes at 1,000 strokes/min. Protein digestion was done at 56°C for 30 minutes followed by a 30-minute period at –20°C to reduce foam and precipitate DNA. The DNA was extracted from the fecal lysates by use of a semiautomated nucleic acid workstation,[g] according to the manufacturer's instructions.

Real-time reaction and real-time PCR procedure—Each PCR reaction contained 20× primer and probes for the respective system with a final concentration of 400nM for each primer and 80nM for the probe,[b] and commercially available PCR mastermix[g] containing 10mM Tris-HCl (pH, 8.3), 50mM KCl, 5mM $MgCl_2$, 2.5mM deoxynucleotide triphosphates, 0.625 U of DNA[h] polymerase/reaction, 0.25 U of uracil N-glycosylase[i]/reaction, and 1 µL of the diluted genomic DNA sample in a final volume of 12 µL. Samples were placed

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in 384-well plates and amplified in an automated fluorometer[g] by use of standard amplification conditions: 2 minutes at 50°C, 10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, and 60 seconds at 60°C. Fluorescent signals were collected during the annealing temperature, and C_T values were extracted with a threshold of 0.04 and baseline values of 3 to 15. For stronger signals, the baseline was adjusted manually to 3 to 10.

Relative quantitation of gene transcription—Final quantitation was performed by use of the comparative C_T method[g] and is reported as relative transcription or the n-fold difference relative to a calibrator genomic DNA (ie, lowest target gene transcription). Briefly, a housekeeping gene for *Eubacteria* sp in feces was used to normalize the C_T values of the target genes (ΔC_T). The ΔC_T was calibrated against the weakest signal within each target gene. The relative linear amount of target molecules relative to the calibrator was calculated as $2^{-\Delta\Delta CT}$. Therefore, all gene transcription is expressed as an n-fold difference relative to the calibrator.

Conventional PCR assay for *T gondii* oocysts—A heminested PCR protocol to amplify a 193-bp segment of the *T gondii B1* gene was used.[13] Insufficient volumes of the coverslip wash solution were available for conventional PCR analysis, so only fecal slurries were tested by use of this method. One milliliter of distilled water was added to 100 µL of fecal slurry, and the sample was centrifuged at 20,000 × *g* for 3 minutes. Supernatant was discarded, and the pellet was washed and centrifuged twice in similar manner. The pellet was resuspended in 50 µL of buffer[j] after the final centrifugation, followed by a freeze-thaw cycle of 4 minutes in liquid N_2 and 4 minutes in boiling water, respectively. Then, 130 µL of buffer[j] and 40 µL of proteinase K[e] were added, and the samples were incubated overnight at 65°C. The remaining steps adhered to the protocol for extraction of DNA from tissue by use of a kit[k] with the following modifications: samples were washed 3 times with AW1 buffer (instead of once), and in the final extraction step, 50 µL of distilled water heated to 95°C was added to the sample vial prior to the final centrifugation.

PCR reaction and gel electrophoresis—Each 50-µL volume PCR reaction contained 32.5 µL of distilled water, 5 µL of 1× PCR buffer,[j] 4 µL of 2mM $MgCl_2$, 1 µL of 0.2mM dNTPs, 1 µL of 400nM forward and reverse primers, 0.5 µL of 1-unit *Taq* polymerase, and 5 µL of sample. The sequence of the *B1* gene forward primer was 5′-GGAACTGCATCCGTTCATGAG-3′ and that of the reverse primer was 5′TCTTTAAAGCGTTCGTGGTC-3′. The PCR reaction conditions consisted of 30 cycles of 3 minutes at 94°C, 1 minute of denaturation at 94°C, 15 seconds annealing at 60°C, 45 seconds extension at 72°C, and 4 minutes at 72°C. Amplified product was electrophoretically separated on a 2% agarose gel stained with ethidium bromide and viewed under UV light. *Toxoplasma gondii* oocysts in distilled water and tachyzoites harvested from culture were used as the positive control sample, and 25 mg of feces from specific pathogen–free cats served as the negative control sample.

Analysis of oocyst spiking data—The effects of assessor, spiking quantity, and freshness of feces on oocyst counts were modeled via negative binomial regression[l] to account for overdispersion in the count data.[14] Results were considered significant at $P < 0.05$. All factors were initially included in the model, and those that were not significant were removed in a backward stepwise procedure. Quantity of spiked oocysts was tested as a second-order polynomial. The final model was compared to a Poisson model with the same variables by use of the likelihood ratio test to confirm the suitability of the negative binomial distribution for the data. The percentage recovery of the assay was calculated as $e^{\beta x_i}$, where βx_i was the vector of the variables in the selected model. The probability of detecting ≥ 1 oocyst/g of feces, $S(c)$, was calculated from the parameters of the negative binomial model in the following equation:

$$S(c) = 1 - \left[\frac{1}{(1 + \alpha c_i e^{\beta x_i})}\right]^{\frac{1}{\alpha}}$$

where α is the extra-dispersion parameter for the negative binomial model, $e^{\beta x_i}$ is the percentage recovery of the assay, and c_i is number of oocysts spiked per gram of feces.[15]

Recruitment of cats for fecal survey—Samples were collected from owned cats living in Cambria, Cayucos, Los Osos, and Morro Bay, Calif, between July 2003 and August 2005. The feces of some cats that shared litter boxes (including cats in shelters that were not housed separately) were collected as pools. Owners were asked to deliver fecal samples from their cats to local veterinary clinics by use of mailings, newspaper advertisements, and posting flyers or were recruited in a door-to-door survey in 4 transects (1 in Cayucos, 2 in Los Osos, and 1 in Morro Bay). Each transect was 4 to 5 miles long, and owners of houses with accessible entrances were contacted and invited to participate in the survey if they owned a cat. Feces were also collected from the ground and from the yards of households without cats. Cat owners filled out a comprehensive questionnaire about their cats' demographics, lifestyle, and hunting activities at the time specimens were submitted. Unowned cats were sampled at 3 cat shelters during the same time period. For unowned cats, demographic information included estimated age, sex, and the location where the cat was captured (if reported).

Fecal flotation of field-sampled cat feces—Feces were examined grossly for whole parasites to identify tapeworm proglottids and nematodes. Ten milliliters of 0.2% Tween-80 in double-distilled water was added to 3 to 5 g of feces and mixed to form a slurry, which was poured through a tea strainer into a 50-mL centrifuge tube. Approximately 35 mL of 0.2% Tween-80 was washed through the sample. The resultant fecal suspension was centrifuged at 1,000 × *g* for 10 minutes, the supernatant was decanted, and the pellet was resuspended in 12 mL of $ZnSO_4$ (specific gravity, 1.2). The solution was decanted to a 15-mL centrifuge tube, and $ZnSO_4$ was added to fill the tube completely, forming a slightly convex meniscus. An 18 × 18-mm coverslip was placed on top of the meniscus, and the sample was centrifuged at 600 × *g* for 8 minutes. Following centrifugation, the coverslip was transferred to a glass slide and the area under the coverslip was examined microscopically at 100× for evidence of parasite ova produced

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by helminths and protozoal cysts or oocysts, including *T gondii*, *Giardia* spp, and *Cryptosporidium* spp.

Comparison of cat demographics and owner attitudes to population-based data—Demographic characteristics of the owned cats in the fecal survey were compared to population demographics from the prior telephone survey[10] by use of the Z test to compare the difference in proportion of cats with a specific characteristic or the 2-sided Fisher exact test if counts in the contingency table were < 5, the *t* test to compare the mean number of hours spent outside per day, and the Mann-Whitney *U* test for age (to compare means from nonparametric distributions). Cats with missing data (n ≤ 19 for any variable) were omitted from summary statistics. Statistical tests were considered significant at *P* < 0.05.

Estimates of *T gondii* environmental oocyst loading—The proportion of cat feces containing *T gondii* oocysts from the fecal survey was used in combination with the estimated annual tonnage of cat feces entering the Morro Bay area from the earlier survey to calculate the annual amount of *T gondii*–infected feces entering the environment.[10] Estimates of the median number of oocysts shed by cats during initial *T gondii* infection were made by summarizing prior data for 50 cats orally infected with tachyzoites or tissue cysts from mice inoculated with isolates ME49, M7741, VEG, and TS-2 (genotypes II and III)[16-20] or cats infected with sea otter isolate No. 1340 (genotype II) at the University of California, Davis. The mean number of *T gondii* oocysts shed after initial infection for 50 cats was 84 million (median, 50 million), and oocyst shedding for the 42 cats with data available lasted for a mean of 8.3 days (median, 8.0 days). For the 6 cats at the University of California, Davis, the number of oocysts shed per infection was calculated as half the total number collected from 3 pairs of cats. Environmental loading was also estimated under a more conservative scenario, in which a cat was assumed to shed 1 million oocysts during a single infection. The same assumption was used to estimate the density of *T gondii* oocyst loading in a community in Panama.[21] The lower estimate for the total number of oocysts produced during a single feline infection was used to account for the potential overestimation of oocyst production in some experimental studies as a result of infecting young cats with extremely large doses (1,000 tissue cysts) of *T gondii*. Oocyst production in cats may be related to dose, strain, and cat age.[19,22] The median number of oocysts shed during an infection was then divided by the mean number of days cats shed oocysts after initial infection[16-20] and the mean daily fecal weight of cat feces[10] to estimate the concentration of oocysts per gram of feces. The total annual tonnage deposited outside was multiplied by the proportion of oocyst-shedding cats detected in the survey, the estimated concentration of oocysts per gram of feces, and 1.0×10^6 (to convert tonnes to grams) to determine the number of oocysts entering the environment annually. Density of annual oocyst loading was estimated by dividing the total number of oocysts entering the environment by the area[23] (3,104 ha [7,668 acres]) of the 3 communities.

Results

Oocyst spiking experiment—Estimates of the variables in the negative binomial model were determined **(Table 1)**. There was no significant (*P* = 0.15) difference in counts for the inexperienced reader, compared with the experienced reader, so this variable was excluded from the final model. Fewer oocysts were counted in dry feces (placed in cat litter for 2 to 4 days) than in fresh feces (OR, 0.26; 95% CI, 0.13 to 0.44). Quantity of spiked oocysts was best modeled as a polynomial with a small negative second-order term. The percentage recovery for fresh versus dry feces by use of the variable estimates from the negative binomial model was determined **(Figure 1)**. For fresh feces, recovery ranged from 0.4% to 1.4%, with optimal recovery occurring between 10,000 and 11,000 oocysts/g of feces. At the highest spiking quantity tested (20,000 oocysts), the number of oocysts in the field of view became too numerous to count accurately. In contrast, for dry feces, recovery was less efficient and was approximately 0.25% (range, 0.1% to 0.4%). The prob-

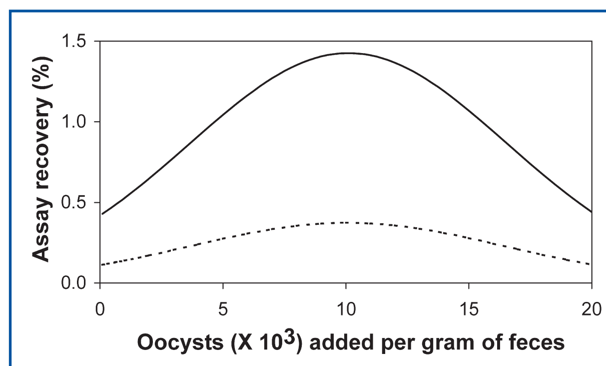

Figure 1—Percentage recovery of *Toxoplasma gondii* oocysts ($e^{\beta x_i}$) added to cat feces and detected via microscopic examination after double centrifugation and flotation in $ZnSO_4$. Feces were examined fresh (solid line), or after 2 to 4 days of being covered with clay cat litter (dotted line). Parameters were estimated from a negative binomial model (*see* Table 1).

Table 1—Negative binomial model predicting the number of *Toxoplasma gondii* oocysts visible via microscopy in 1 g of cat feces.

Parameter	Estimate	OR	95% CI	*P* value
Intercept	–5.4752	—	—	< 0.001
Freshness of feces				
Fresh	—	1.00	—	—
Dry	–1.3288	0.26	0.13–0.50	< 0.001
Quantity of oocysts spiked	2.4×10^{-4}	—	—	0.009
(Quantity of oocysts spiked)^2	-1.20×10^{-8}	—	—	0.01
Dispersion parameter*	1.2886	—	0.76–1.81	—

*No *P* value is provided from the software output for this parameter of the negative binomial distribution (estimated by maximum likelihood).
— = Not applicable.

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ability of detecting ≥ 1 oocysts for the spiking quantities tested in fresh and dry feces was determined (**Figure 2**). For fresh feces, oocysts could be detected microscopically 50% of the time when approximately 300 oocysts/g were present, whereas for dry feces, 1,000 oocysts/g had to be present for a 50% chance of detection.

PCR detection of *T gondii B1* gene DNA—All coverslip and fecal slurry samples yielded negative results by use of PCR assay.[a] One fecal slurry sample taken from feces spiked with 20,000 oocysts yielded positive results by use of conventional PCR assay. That sample was estimated to contain 500 oocysts because it represented approximately 25 mg or 2.5% of the total mass (1 g) of spiked feces.

Cat demographics—Median age of the 203 owned cats in the fecal survey (which included data from cats whose feces were pooled) was 6.0 years (range, 2 months to 20 years [age not reported for 5 cats]). Of the cats with data, 99 of 184 (54%) cats were female and 167 of 186 (90%) cats were neutered or spayed. Two of the 99 (2%) female cats had given birth to a litter within the previous 12 months. One hundred twenty-six of 194 (65%) cats were allowed outside, spending a mean of 7.4 h/d outside (95% CI, 5.8 to 8.6 h/d), and 68 of 197 (35%) cats had been observed hunting. Four of 197 (2%) cats were fed raw meat, and 135 of 187 (72%) cats had been examined by a veterinarian within the last 12 months. Information about disposition of feces was provided for 199 cats. For 104 (52%) cats, feces were always collected in litter boxes and 13% always defecated outside, with 22% using the outdoors > 75% of the time. For the 173 cats that used litter boxes at least some of the time, 84% of fecal material was disposed of in the garbage, 13% in the toilet (including 1 cat trained to defecate directly into the bowl), and 3% in the owner's yard.

Cat fecal survey—Contact was attempted at 1,811 households (between 385 and 524 per transect), and 1,050 (58.0%) were reached. Four hundred eighty-five (46%) households were estimated to own cats on the basis of contact with the householder, contact with a neighbor, or personal observation by the first author (HAD) in the yard or on the porch of the residence. Cat ownership was confirmed by direct contact with 365 (75%) households. One hundred twenty-four transect households supplied cat fecal samples, including 7 that did not own cats but allowed for fecal sample collection in their yards. Thus, 117 of 365 (32%) cat-owning transect households contacted directly participated in the cat fecal survey. Seventy-four of the 248 (30%) cat-owning transect households that declined to participate in the survey did so because they did not know where to locate their cat's feces. The remaining samples were collected from the ground outside the transects, from animal shelters, or through clinics held in the offices of local veterinarians. A total of 326 fecal samples were examined for evidence of parasites. Of these, 153 were from owned cats, 107 were from unowned or feral cats, and 66 were found outdoors. *Toxoplasma*-like oocysts were seen in 3 of the 326 (0.9%) samples. The first specimen was a pooled sample from 2 owned cats (male, 6 years old; female, 7 years old) that were allowed outside for < 3 h/d, and the second was from an owned 3-year-old female cat that spent 50% of the day outdoors. Both were collected in the door-to-door survey in January 2005. The third specimen was from a feral cat with an anti-*T gondii* antibody titer of 10,240 that was being spayed in a trap-neuter-return program. In all cases, few oocysts were visible and the oocysts could not be confirmed to species by use of DNA amplification because the low concentration of oocysts precluded PCR testing.

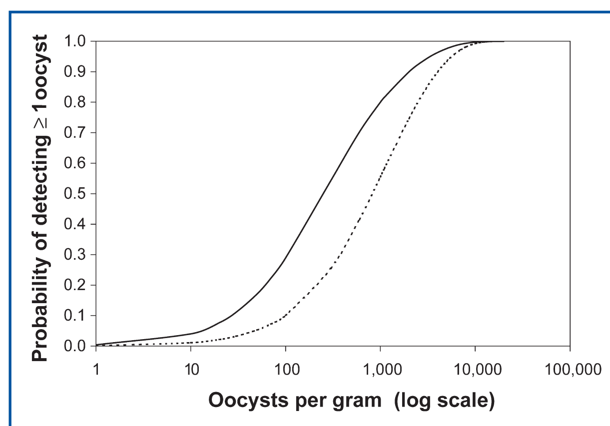

Figure 2—Analytic sensitivity [*S*(*c*)] of detection of *T gondii* oocysts in cat feces via microscopic examination after double centrifugation and flotation in $ZnSO_4$. Parameters were estimated from the negative binomial model (*see* Table 1) in the equation,

$$1 - \left[\frac{1}{(1 + \alpha c_i e^{\beta x_i})}\right]^{\frac{1}{\alpha}}$$

where α is the extra-dispersion parameter, $e^{\beta x_i}$ is the percentage recovery of the assay, and c_i is number of oocysts spiked per gram of feces.[15] *See* Figure 1 for key.

Comparison of cat demographics and cat owner attitudes in the fecal survey to the population-based survey—Comparison of demographics for the owned cats in the fecal survey with the population-based survey was performed (**Table 2**). The populations were similar in age, sex, and neuter-spay status, but they differed substantially for other characteristics. The percentage of cats in the fecal survey allowed outdoors (65%) was similar between the 2 groups, but the cats in the fecal survey spent significantly fewer hours outside than cats in the population-based survey (7.4 hours vs 12.8 hours). Fewer cats in the fecal survey had been seen hunting (35% vs 57%), were fed raw meat (2% vs 8%), or were exclusively defecating outside (13% vs 36%). There was a corresponding increase in the proportion of cats sampled whose feces were always collected in litter boxes (52%). More of the cats in the fecal survey had been examined by a veterinarian in the past 12 months (72%) than cats in the population (60%). For the 107 fecal samples representing 182 unowned or feral cats (including pooled samples), age was provided for 174 cats. Sixty-four (37%) were < 6 months old, 39 (22%) were 6 to 12 months old, and 71 (41%) were > 12 months old. For 69 of these cats, exact age was supplied (median, 6 months; range, 2 months to 16 years). For the 168 unowned or feral cats with sex information, 97 (58%) were female.

Environmental loading of *T gondii* oocysts—Based on our own and reported experimental *T gondii* infec-

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Table 2—Comparison of demographics of owned cats in a fecal survey for *T gondii* with demographics obtained during a 2003 telephone survey of cat owners.

Characteristic	Telephone survey, 2003 (n = 263)	Cats in fecal survey (n = 203 including pooled feces)	*P* value
Female (%)	53	54	0.99
Neutered-spayed (%)	92	90	0.13
Visited veterinarian < 12 mo (%)	60	72	0.014*
Allowed outdoors (%)	67	65	0.75
Mean time spent outside (h)	12.8	7.4	< 0.001*
Observed hunting (%)	57	35	< 0.001*
Fed raw meat (%)	8	2	0.01*
Always uses litter box (%)	40	52	0.01*
Always defecates outside (%)	36	13	< 0.001*
Median age (y)	7.0	6.0	0.14

*Significant ($P < 0.05$) difference between groups.

Table 3—Estimates of residential environmental loading with *T gondii* oocysts.

Fecal deposition	Mean oocyst density/g (/oz)*	Total oocysts/y†	Oocysts/ha (/acre)	Oocysts/m² (/ft²)
High‡	1.5×10^5 (4.2×10^6)	1.45×10^{11}	4.7×10^7 (1.9×10^7)	4,671 (434)
Low§	3.0×10^3 (8.4×10^4)	2.9×10^9	9.4×10^5 (3.8×10^5)	94 (9)

*Based on the total number of oocysts shed per infection divided by the mean daily fecal weight (40.2 g for an adult cat) and mean oocyst shedding duration of 8.3 days. †Based on 107.6 metric tonnes (105.9 tons) of feces deposited outdoors annually by feral and owned cats, with 0.9% of the annually deposited fecal mass containing *T gondii* oocysts. ‡Based on 50 million oocysts shed by a cat following initial infection with *T gondii*. §Based on a conservative estimate of 1 million oocysts/cyst-induced infection in cats.

tion of cats, we calculated that 145 billion oocysts (46.8 million oocysts/ha [18.9 million oocysts/acre]) or 4.7×10^3 oocysts/m² (434 oocysts/ft²) were expected to be defecated into the environment annually (**Table 3**). Under the more conservative scenario of 1 million oocysts shed/feline infection, the estimate was 2.9 billion *T gondii* oocysts entering the environment/y for a density of approximately 9.4×10^5 oocysts/ha (3.8×10^5 oocysts/acre) or 94 oocysts/m² (9 oocysts/ft²).

Discussion

The proportion of cats shedding *T gondii* oocysts in their feces was low (0.9%), as has been determined in other surveys.[24-32] This estimate of oocyst shedding prevalence is not precise and may lead cat owners and the public to underestimate the health risks of soil contamination with *T gondii* oocysts. Estimates of the oocyst burden suggested that the annual density of oocyst loading was 94 to 4,671 oocysts/m² (9 to 434 oocysts/ft²). Although the oocysts detected microscopically in the 3 samples with positive results were not confirmed as *T gondii* oocysts by use of other methods, the microscopically identical parasites *H hammondii* and *B darlingi*[3] are even more rarely identified than *T gondii* oocysts in fecal surveys. For example, in a study[33] of 1,000 cats from Columbus, Ohio; of 9 samples with *Toxoplasma-Hammondia*–like oocysts detected microscopically, 7 were confirmed as *T gondii* and 2 as *H hammondii* by use of a mouse bioassay. *Toxoplasma gondii* oocysts can also be distinguished from *H hammondii* oocysts via PCR testing,[34] but in the present study, insufficient numbers of oocysts were present for PCR amplification. Although 2 of the samples were from owned cats that always defecated outside and were not serologically tested for *T gondii* antibodies, the third specimen originated from a feral cat with an anti-*T gondii* antibody titer of 10,240. Although many cats are seronegative when shedding *T gondii* oocysts, an earlier study[35] found that 36% of naturally infected kittens shedding oocysts had detectable *T gondii* titers. We therefore concluded that the result for the aforementioned feral cat was most likely a true positive, particularly in light of the fact that few oocysts were present. If the estimates of *T gondii* oocyst loading in the Morro Bay area were based on 1 of 326 (0.3%) cats in the fecal survey shedding oocysts, the density of oocysts would still be 31 to 1,557 oocysts/m² (3 to 145 oocysts/ft²).

Other studies[34-36] have detected oocyst shedding prevalence of much greater magnitude. Ruiz and Frenkel[35] found that 23% of cats were shedding oocysts in a study of Costa Rican cats, and 17% of fecal pools from queens with kittens in southern Germany contained *T gondii*–like oocysts.[37] Although light microscopy following fecal flotation is commonly used to detect *T gondii* oocysts in feces, it is less sensitive than mouse bioassay.[35,38] The results of our spiking experiment indicated that microscopy is likely to detect moderate to heavy oocyst shedding by cats, but could miss low quantities of oocysts shed towards the end of the patent period. In a prior study,[38] the analytic sensitivity (detection limit) of microscopic evaluation was determined to be 250 oocysts/g of feces, whereas mouse bioassay was much more sensitive (2 oocysts/g). That estimate for microscopy agrees with the output from our negative

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binomial model, which predicted that *T gondii* oocysts would be detected 50% of the time when 300 oocysts/g were present. In contrast, PCR detection of *T gondii* DNA was unsuccessful for all but 1 sample, even when feces were spiked with high quantities of oocysts. In part, this was attributable to the small amount of sample that can be tested in a single PCR reaction, but could also be a result of oocyst loss in the centrifugation steps prior to PCR assay and the presence of PCR inhibitors in fecal matter. Another explanation for the low detection rate of oocysts in the present study is that many of the samples were collected from litter boxes or the ground. The spiking experiment revealed significantly poorer detection of *T gondii* oocysts in feces allowed to dry out in cat litter for 2 to 4 days (OR, 0.26 for dry vs fresh feces).

Cats that spent most of their time outdoors and were most likely to be at risk for contracting toxoplasmosis[39-41] were undersampled because owners of cats exclusively defecating outside did not know where to locate their cat's feces. To participate in the survey, owners of cats that usually defecated outside had either to confine their cat for 24 hours and supply a litter box, locate their cat's feces in their yard, or see their pet in the act of defecation. In fact, 30% of cat owners who declined to participate in the survey indicated that they could not find their cats' feces. This estimate was supported by the data collected in the earlier telephone survey,[10] in which 36% of owned cats were found to be defecating outside all the time.

The relatively low prevalence of oocyst shedding in the present survey could also be related to the fact that the owned cats spent fewer hours outside (7.4 h/d) than the general cat population (12.8 h/d), were less likely to be observed hunting (35% of owned cats vs 57% in the general population), and fewer (2%) were fed raw meat than cats in the general population (8%). The cats in the fecal survey may therefore have had a lower risk for exposure to *T gondii*. The low proportion of samples with positive results from feral cats may also have been attributable to the fact that 41% of the 107 cats tested had been confined in a rescue facility for several days to > 1 week at the time of sampling. Cats in rescue facilities would be unlikely to be exposed to *T gondii* parasites in food because they were fed dry or canned food exclusively, were housed in cages, and were restricted from hunting. Although cats may shed *T gondii* oocysts for 2 to 3 weeks, the median duration[16-20] was 8 days, with peak shedding between 1 and 3 days after the 3- to 5-day prepatent period following consumption of tissue cysts.[19] Therefore, peak shedding would most likely occur < 8 days after admittance to a shelter, and the low quantities of oocysts produced in the latter part of the 2- to 3-week shedding period may not be microscopically detectable. Furthermore, cats previously infected with *T gondii* are unlikely to reshed oocysts unless they are deprived of food,[35] superinfected with other coccidian parasites,[16] administered high doses of corticosteroids,[42] or reexposed to *T gondii* years after their initial infection.[43]

A calculation that may support the 0.9% oocyst shedding prevalence would be to estimate the number of *T gondii* cases in owned cats per cat-year by use of the population size determined in the earlier telephone survey.[10] The median survival time for the cat population was incorporated to estimate the number of years at risk and the age-adjusted seroprevalence (29.6%) from a concurrent survey[44] to estimate the total number of *T gondii* cases in the population. A total of 2,156 *T gondii* infections would be expected to occur in the 7,284 owned cats over 50,988 cat-years at risk (median survival time × No. of cats in the population at risk) for a mean incidence of 0.04 *T gondii* infections/cat-year. This represents approximately 4% of owned cats in the population becoming infected each year or a shedding prevalence in a cross-sectional survey of 4%. This estimate is higher than the 0.9% shedding prevalence detected in the fecal survey and suggests that the 0.9% estimate is conservative.

Despite the low prevalence of *T gondii*–like oocyst shedding in the present survey and the difficulty of obtaining fecal samples from high-risk cats, the estimated annual environmental burden of oocysts in the region was 3 to 145 billion in an area of 3,104 ha (7,672 acres). Cats can shed large numbers of oocysts during initial *T gondii* infection, but some studies[16-20] have used high doses to initiate infection, which can lead to a longer shedding period and greater production of oocysts. Cats used in experimental studies are also younger (2 to 3 months old), and kittens shed more oocysts than adult cats.[22] Therefore, estimates of the oocyst burden based on the results of experimental studies may overestimate the magnitude of environmental loading. For this reason, our estimates also used a conservative value of 1 million oocysts shed for each cat infected with *T gondii* for the first time, as used by others to estimate the environmental burden of *T gondii* oocysts.[21] In the present study, when cats were infected with *T gondii*, only one third produced extremely high numbers of oocysts (approx 50 million/cat), whereas 4 cats produced a mean of 8.25 million oocysts/infection. An estimate of 1 million oocysts produced/cat infection may be more appropriate for naturally acquired *T gondii* infections. In a prior study in Panama,[21] 50 to 200 cats in an area of approximately 7.7 ha were estimated to be capable of seeding the environment with 193 to 774 oocysts/m^2. Our estimate of the density of oocyst shedding in the 3 communities (94 oocysts/m^2), based on the same number of oocysts shed for a single feline infection, was comparable. Genotypes of *T gondii* that were prevalent in terrestrial animals in the Morro Bay area were unknown. Predominance of specific genotypes with increased or decreased capacity for infecting cats and wild felids in this geographic region could result in different patterns of oocyst dissemination in the local environment.

The low prevalence of *T gondii*–like oocyst shedding in cat fecal specimens in the present and previous studies may lead scientists, veterinarians, and the public to underestimate the health risk posed by cats defecating outside. We estimated that 3 to 145 billion oocysts (94 to 4,671 oocysts/m^2 [9 to 434 oocysts/ft^2]) could be entering the environment annually in the Morro Bay area of California, depending on the estimate used for the total number of oocysts shed by a cat during initial *T gondii* infection. This amount of pathogen dissemination into the environment is cause for concern. Gardening frequently and working in soil-related occupations have been associated with *T gondii* infection in several human studies,[45-48] although research among pregnant women to assess knowledge about risk fac-

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tors for acquiring toxoplasmosis indicated that many were unaware that they could acquire toxoplasmosis from contaminated soil or water.[49] In countries like the United States, where meat such as lamb and pork is well-cooked before human consumption, the principal source of *T gondii* transmission to humans is likely to be from the environment. If animals destined for the food chain are appropriately managed and careful food hygiene is practiced, the greatest challenge in decreasing the incidence of toxoplasmosis in humans will be to reduce environmental sources of infection.

a. Oildri Corp, Alpharetta, Ga.
b. TaqMan, Applied Biosystems, Foster City, Calif.
c. Primer Express, Applied Biosystems, Foster City, Calif.
d. 1X ABI TransPrep buffer, Applied Biosystems, Foster City, Calif.
e. Invitrogen, Carlsbad, Calif.
f. SpexCertiprep, Metuchen, NJ.
g. Applied Biosystems, Foster City, Calif.
h. AmpliTaq Gold DNA, Applied Biosystems, Foster City, Calif.
i. AmpErase UNG, Applied Biosystems, Foster City, Calif.
j. ATL buffer, Qiagen, Valencia, Calif.
k. Qiagen, Valencia, Calif.
l. SAS, version 9.1, SAS Institute Inc, Cary, NC.

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