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EVALUATION OF TWO *TOXOPLASMA GONDII* SEROLOGIC TESTS USED IN A SEROSURVEY OF DOMESTIC CATS IN CALIFORNIA

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ABSTRACT: We evaluated the sensitivity (Se) and specificity (Sp) of an IgG enzyme-linked immunosorbent assay (ELISA) and IgG indirect fluorescent antibody test (IFAT) for detection of *Toxoplasma gondii*-specific antibodies in sera from 2 cat populations using a Bayesian approach. Accounting for test covariance, the Se and Sp of the IgG ELISA were estimated to be 92.6% and 96.5%, and those of the IgG IFAT were 81.0% and 93.8%, respectively. Both tests performed poorly in cats experimentally coinfected with feline immunodeficiency virus and *T. gondii*. Excluding this group, Se and Sp of the ELISA were virtually unchanged (92.3% and 96.4%, respectively), whereas the IFAT Se improved to 94.2% and Sp remained stable at 93.7%. These tests and an IgM ELISA were applied to 123 cat sera from the Morro Bay area, California, where high morbidity and mortality attributable to toxoplasmosis have been detected in southern sea otters. Age-adjusted IgG seroprevalence in this population was estimated to be 29.6%, and it did not differ between owned and unowned cats. Accounting for Se, Sp, and test covariances, age-adjusted seroprevalence was 45.0%. The odds for *T. gondii* seropositivity were 12.3-fold higher for cats aged >12 mo compared with cats aged <6 mo.

Toxoplasma gondii is a protozoan parasite of mammals and birds, and it is found in more than 300 species of warm-blooded animals worldwide (Tenter et al., 2000). The parasite has a complex life cycle, which may involve predator–prey (trophic) relationships, and it is maintained on land in cycles involving felids, carnivores, rodents, and birds (Dubey, Weigel et al., 1995; Frenkel et al., 1995; Lehmann et al., 2003). Felids most readily acquire *T. gondii* infection when they consume the tissues of infected intermediate hosts. In the felid intestine, the parasite undergoes sexual reproduction resulting in the production of environmentally resistant oocysts that are shed into the lumen of the gut and defecated into the environment (Dubey et al., 1970). Herbivores, nonfelid carnivores, and birds acquire infection by accidental ingestion of oocysts in soil and water or by consuming infected muscle (Tenter et al., 2000; Dubey, 2002). Vertical transmission may occur in mammals, including humans, if the mother is exposed to *T. gondii* during gestation (Jones et al., 2001; Freyre et al., 2003; Basso et al., 2005; Williams et al., 2005). After disseminating throughout the host via blood and lymph, *T. gondii* zoites encyst in muscle and other tissues (notably the brain), where they may remain viable for the life of the host (Dubey, 1994).

Until the 1970s, *T. gondii* infection was most frequently reported in terrestrial mammals and birds, but more recently, it has been recognized in a variety of marine mammals (reviewed by Dubey et al., 2003). Toxoplasmosis causes particularly deleterious effects in the southern sea otter population along the central California coast, and it was recognized as a primary cause of death in 16% of sea otters from 1998 to 2001 (Kreuder et al., 2003). Morro Bay was identified as an area where sea otters were 9-fold more likely to be infected with *T. gondii* than otters anywhere else on the California coast (Miller et al., 2002). Thus, an investigation of *T. gondii* seroprevalence in domestic cats (*Felis catus*) from this region was undertaken to determine whether a high prevalence of *T. gondii* existed in the local cat population, perhaps explaining the high prevalence of infection detected in southern sea otters from adjacent coastal areas. Other factors that could play a role in *T. gondii* infection of sea otters are long-term survival of oocysts in sea water (Lindsay et al., 2003), the diet specialization of sea otters, and seasonal rain events that flush large quantities of pathogens into the ocean over a short time.

Many serologic tests exist for detection of *T. gondii* infection in cats, but few have been adequately validated. Most were applied to small populations of experimentally infected or clinically ill cats; in effect, ''gold standards'' were not available (Dubey and Thulliez, 1989; Lappin et al., 1989; Lappin and Powell, 1991; Tenter et al., 1994; Kimbita et al., 2001; Huang et al., 2002; Vollaire et al., 2005). In test evaluation studies, generally a gold standard, or reference test, must be established to confirm the presence or absence of infection (Greiner and Gardner, 2000b). Test animals are then classified as infected or uninfected, the serologic results are compared with the gold standard in the 2 groups, and the sensitivity (Se) and specificity (Sp) of the new test are calculated directly. Potential gold standard tests for *T. gondii* in cats include immunohistologic examination of tissues, in vitro culture of parasites from tissues, polymerase chain reaction amplification and sequencing of parasite DNA/RNA, or bioassay in laboratory mice or cats. These confirmatory tests are expensive, may require the killing of experimental animals, and may not always indicate true infection status. Where gold standards are imperfect, or do not exist, the performance of 2 conditionally independent tests can be evaluated by testing 2 groups of animals from populations with differing disease prevalence (Hui and Walter, 1980; Pouillot et al., 2002). However, if the tests are conditionally dependent, for example, if they both measure the presence of antibodies, then a test correlation should be considered (Gardner et al., 2000). A method for estimating the Se and Sp of 2 correlated tests in 2 populations using Bayesian modeling has been described pre-

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viously (Branscum et al., 2005), and it was used in the current study.

An IgG enzyme-linked immunosorbent assay (ELISA) and IgG indirect fluorescent antibody test (IFAT) were selected for evaluation and application to serologic testing of cats from the Morro Bay area of California. The ELISA has the advantages of being nonsubjective in interpretation and easily automated, whereas the IgG IFAT may detect antibody response within 1 wk of infection (Omata et al., 1990), has shown high Se and Sp in other species (Miller, Gardner, Packham et al., 2002), and the test procedure is well established in our laboratory for protozoan serology (Conrad et al., 1993; Duarte et al., 2004).

The goals of this study were to estimate the Se and Sp of the ELISA and IFAT, to estimate inter- and intraobserver agreement, to determine *T. gondii* seroprevalence in the cat population of the Morro Bay area, and to evaluate risk factors for *T. gondii* seropositivity in cats. We hypothesized that unowned cats would be more likely to test positive for *T. gondii* antibodies than owned cats, and that older age, a diet that included raw meat, hunting mammals or birds, and being allowed outdoors would be associated with *T. gondii* seropositivity in owned cats.

MATERIALS AND METHODS

Serology

Feline blood samples were processed in the same manner for the test evaluation and field survey. Blood was collected by venipuncture, allowed to clot, and centrifuged at 1,000 *g* for 10 min. Aliquots of serum were stored at −20 C until tested. Positive and negative controls consisted of previously analyzed feline serum with titers >1,024 or titers <40, respectively.

Serum was diluted in phosphate-buffered saline (PBS), pH 7.4 (Diamedix, Miami, Florida) in 2-fold serial dilutions starting at 1:16, and 10 μl was incubated at 37 C for 1 hr on 12-well slides (Erie Scientific, St. Louis, Missouri) coated with Formalin-fixed, culture-derived *T. gondii* tachyzoites, as described previously (Miller et al., 2001). Slides were washed 3 times for 5 min each in PBS, and fluorescein isothiocyanate (FITC)-labeled goat anti-cat heavy-chain IgG antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland) diluted at 1:500 were added to detect the presence of *T. gondii* antibodies, and the slides were incubated for 1 hr at 37 C. After washing in PBS as described above, 10 μl of buffered, pH 8.5, glycerol was added to each slide well, and the slides were examined at ×200 on an immunofluorescent microscope (Karl Zeiss Optical, Inc., Chester, Virginia).

Toxoplasma gondii IgM and IgG immunoglobulins were detected in the feline sera as described previously (Brown et al., 2005). Approximately 0.73 μg of sonicated *T. gondii* RH strain tachyzoites in 100 μl of 0.06 M carbonate buffer, pH 9.6, were pipetted into wells of a microELISA plate (Immulon I; Kirkegaard and Perry Laboratories) and incubated at 4 C overnight. After 3 plate washes (200 μl/well of 0.01 M PBS solution [PBSS] plus 0.05% Tween 20 [PBSS-TW]), sera were diluted 1:64 in PBSS-TW, pipetted into triplicate wells (100 μl/well), and the plate was incubated for 30 min at 37 C. After 3 washes, 100 μl of a 1:3,000 dilution of anti-feline IgM (peroxidase-labeled, heavy chain specific; Kirkegaard and Perry Laboratories) or 100 μl of a 1:2,000 dilution of anti-feline IgG (peroxidase-labeled, heavy chain specific; Kirkegaard and Perry Laboratories) was pipetted into appropriate wells, and the plate incubated for 30 min at 37 C. After 3 washes, 100 μl of a substrate (SureBlue TMB microwell peroxidase substrate; Kirkegaard and Perry Laboratories) was pipetted into appropriate wells, and the plate was incubated for 10 min at 20 C. To stop the color reaction, 100 μl of 0.18 M H_2SO_4 was added to each well, and absorbances were then calculated by an automated microELISA plate reader using a 450-nm filter. Positive control, negative control, enzyme control, and substrate control wells were included on each plate. The mean absorbance of each serum sample was compared with a standard curve developed from negative and positive control sera and a titer assigned. Positive cutoff absorbances were defined as greater than the mean plus 2 SDs of results from 20 specific pathogen-fee (SPF) domestic cats. Titers ≥1:64 were considered positive (Lappin et al., 1989; Dubey, Lappin et al., 1995).

Test evaluation study

The sample size was calculated following the guidelines in Greiner and Gardner (2000b). Assuming Se and Sp of the reference (ELISA) test were both 95%, and a 5% error margin on the point estimates for Se and Sp with 95% confidence, a minimum of 73 cats would be required in each population.

One hundred cats housed at the University of California–Davis (UCD) SPF cat colony were selected as the low prevalence population. On the date of blood collection, 37 cats were ≤6 mo old, 9 cats were 7 to 12 mo old, and 54 cats were >12 mo old. Median age and weight of the cats 12 mo and younger was 6 mo (range = 4–12 mo) and 2.8 kg (range = 1.8–4.9 kg), respectively. Sixteen (65%) of the 46 cats aged ≤12 mo were females. Median age and weight of the cats >12 mo old was 25 mo (range = 13–123 mo) and 4.4 kg (range = 3–6.5 kg), respectively. Forty-three (80%) of the 54 older cats were females. The sera of 94 cats had been previously tested at Colorado State University (CSU) and represented the high prevalence population. Among the CSU cats, 20 were female kittens experimentally infected with *T. gondii* and tested for antibodies 8 wk postinfection (PI), 18 were kittens co-infected with feline immunodeficiency virus (FIV) and *T. gondii*, and 56 were client-owned cats suspected to have been naturally infected with *T. gondii* based on clinical signs (Lappin, 1996). Cats co-infected with FIV and *T. gondii* were included because co-infection had been shown to affect the performance of serologic tests (Svobodova et al., 1986; Witt et al., 1989; O'Neil et al., 1991; Lappin et al., 1993). Median age for the client-owned cats (5 missing data) was 6 yr (range = 2 mo–18 yr). Twenty-two (42%) of the 53 client-owned cats with gender data were female, and the remainder were male.

Feces of the cats housed at the UCD SPF cat colony were tested to confirm that they were not shedding *Toxoplasma*-like oocysts and to detect the presence of other feline coccidian parasites that might cause serologic cross-reactions. Feces were collected ≤24 hr after defecation and <3 day of blood collection.

Fecal flotation was performed as follows. Twenty milliliters of 0.2% Tween 80 was added to 3–5 g of feces and mixed to form a slurry, which was poured through a tea strainer into a 50-ml centrifuge tube. Approximately 45 ml of 0.2% Tween 80 was washed through the sample. The resultant fecal suspension was centrifuged at 1,000 *g* for 10 min, the supernatant was decanted, and the pellet was resuspended in 12 ml of zinc sulfate (specific gravity = 1.2). The solution was decanted to a 15-ml centrifuge tube, and zinc sulfate was added to fill the tube completely, forming a convex meniscus. An 18- × 18-mm coverslip was placed on top of the meniscus, and the sample centrifuged at 600 *g* for 8 min. After centrifugation, the coverslip was transferred to a glass slide, and the area under the coverslip was examined microscopically at ×100 for the presence of parasites, including *T. gondii*-like 10- × 12-μm oocysts, *Giardia lamblia* 12- × 15-μm cysts, and *Cryptosporidium* sp. 4- × 6-μm oocysts.

To check for *G. lamblia* and *Cryptosporidium* sp. infection, the fecal slurry was collected after the first centrifugation (see above) was processed according to the manufacturer's directions using the Merifluor DFA kit (Meridian Biosciences Inc., Cincinnati, Ohio).

To assess the consistency of 2 different readers interpreting IFAT slides on the same day, interobserver agreement for readers blinded to the source of the samples was determined for the 194 feline sera using unweighted and weighted kappa values. For the weighted kappa, Cicchetti–Allison weights were used for all cells representing partial agreement (Fleiss, 1981). To assess the consistency of readings for the same person on 2 consecutive days for the same sample, intraobserver agreement for the more experienced reader was calculated using unweighted and weighted kappas for a subset of 55 randomly selected samples: 30 samples from the UCD SPF cats and 25 samples from the CSU cats. All analyses were performed in SAS, version 9.1 (SAS Institute, Cary, North Carolina).

In Bayesian analysis, prior information (''priors'') about 1 or more parameters of interest is incorporated into the statistical model. In the case where 2 dependent tests are applied to 2 populations, there are 8 parameters to be estimated, i.e., 2 sensitivities, 2 specificities, preva-

TABLE I. Beta distributions specified for the Bayesian model and starting values used to evaluate the ELISA (T1) and IFAT (T2) in 2 cat populations. Disease status, + = infected, − = uninfected.

Parameter	Mode (%)	Percentile (%)	Parameters of beta distribution (α, β)	Starting value for MCMC* (%)
Prevalence in UCD SPF cats	1	5†	1.88, 88.28	5
Prevalence in CSU cats	80	66‡	27.05, 7.51	60
Se (ELISA)	95	90‡	99.70, 6.19	95
Sp (ELISA)	95	90‡	99.70, 6.19	95
Probability of T2+|D+T1+§	U (0,1)‖		1.00, 1.00	80
Probability of T2+|D+T1−#	U (0,1)		1.00, 1.00	50
Probability of T2−|D−T1−¶	U (0,1)		1.00, 1.00	70
Probability of T2−|D−T1+**	U (0,1)		1.00, 1.00	80

* Markov-chain Monte Carlo sample.
† 5th percentile.
‡ 95th percentile.
§ Probability of testing T2 positive, if T1 positive and infected.
‖ Uniform (0,1) distribution, assumes no prior knowledge of Se and Sp covariance.
Probability of testing T2 positive, if T1 negative and infected.
¶ Probability of testing T2 negative, if T1 negative and uninfected.
** Probability of testing T2 negative, if T1 positive and uninfected.

lence in each population (2 parameters), and the Se and Sp covariance (2 parameters). However, there are only 6 df available to estimate 8 parameters, so the problem is statistically unidentifiable (Branscum et al., 2005). Thus, prior information must be available for at least 2 of the parameters (Branscum et al., 2005). In this situation, the approach is usually to incorporate estimates of the Se and Sp of 1 of the tests and to model them as beta distributions. The IgG ELISA used in the present study had been in use since 1989 and applied to more than 200,000 samples at CSU (by M.R.L.), and its Se and Sp were both estimated to be 95%. These estimates were provided by the expert (M.R.L.) before the analysis. The remaining 6 parameters were estimated by WinBUGS software (http://www.mrc-bsu.cam.ac.uk/bugs/winbugs/contents.shtml). Priors input to WinBUGS were modeled using beta distributions (Table I) generated in BetaBuster, which is available at http://www.epi.ucdavis.edu/diagnostictests/betabuster.html. The code used for this analysis is in Appendix I. When test covariances are unknown, diffuse priors for the test covariances should be specified, so the test covariances were designated as beta (1,1) distributions, the equivalent of a Uniform (0,1) distribution (Branscum et al., 2005). Initial values for the 8 parameters used in the model are in Table I. Sensitivity analysis was conducted by varying the priors for prevalence in the 2 populations, ELISA Se, and ELISA Sp to determine their influence on the parameter estimates.

TABLE II. Comparison of demographics of owned cats serologically tested to cats in a 2003 telephone survey (Dabritz et al., 2006) of cat owners from the greater Morro Bay area.

Characteristic	Telephone survey (n = 263)	Cats serologically tested (n = 43)	*P* value
Female (%)	54	55	0.92
Neutered/spayed (%)	94	59	<0.001*
Visiting veterinarian <12 mo (%)	60	79	0.02*
Allowed outdoors (%)	67	74	0.30
Observed hunting (%)	57	44	0.12
Fed raw meat (%)	8	2	0.33
Always using litter box (%)	40	41	0.83
Always defecating outside (%)	36	21	0.03*
Mean time spent outside (hr)	12.8	6.5	<0.001*
Median age (yr)	7.0	2.0	<0.001*

* Denotes statistically significant differences at $P < 0.05$ between cats tested for *Toxoplasma gondii* antibodies and the population data from the 2003 telephone survey.

Serosurvey of domestic cats

Collection of sera: The required sample size (2-sided calculation) was estimated in MINITAB®, release 14 (Minitab, Inc., State College, Pennsylvania) to be 82 cats in each group (owned and unowned) to detect a difference in seroprevalence of 20% versus 40%, with 95% confidence and 80% power.

Owned cats were recruited from Cambria, Cayucos, Los Osos, and Morro Bay through local veterinarians from 2003 to 2005, or they were invited to attend blood collection clinics in June and October 2004, and August 2005 by mailings, newspaper advertisements, and posting flyers. Cat owners completed a comprehensive questionnaire about their cat's demographics, lifestyle, and hunting activities. Unowned cats were sampled at a privately owned cat shelter, or blood was drawn from feral cats in trap–neuter–return programs, while they were being spayed or neutered by local veterinarians in 2003 and 2004. For unowned cats, demographic information included weight (if available), estimated age, sex, and the location where the animal was captured (if known). The cutoff point for a positive IFAT was determined by maximizing the estimates of Se and Sp in the Bayesian model, and the cutoff for a positive IgG or IgM ELISA test was 1:64.

Data analysis: Demographic characteristics of the 43 owned cats sampled (Table II) were compared with population demographics from a prior telephone survey (Dabritz et al., 2006), to determine whether the owned cats tested for *T. gondii* antibodies differed significantly from the cat population for putative predictors of *T. gondii* seropositivity. Z-tests were used for proportions (Fisher's exact test if any cell count was <5), the *t*-test for the mean number of hours spent outside per day, and the Mann–Whitney *U*-test for difference in median age. Chi-square tests were used to determine whether unowned cats (n = 80) differed significantly by age strata from the population, and from the owned cats sampled. Fisher's exact test (2-sided) was used to determine whether a risk factor was associated with a positive result for the IgM-ELISA or any serologic test for the 43 owned cats, using the test cutoffs specified above (Stokes et al., 2001). The risk factors assessed were cat age (categorized as <6 mo, 6–12 mo, or >12 mo), gender, neuter/spay status, whether the cat was ever allowed outdoors, whether the cat was ever observed hunting, whether raw meat was fed in the diet, and city of residence. The Mann–Whitney *U*-test with modified rank scores was used to determine whether differences existed in the median age and hours allowed outside for infected versus uninfected owned cats. Logistic regression was used to assess risk factors for being IgG seropositive on the IFAT or ELISA test, for being IgM-ELISA seropositive, or for being seropositive on any test for all the cats sampled. In the combined analysis (123 cats), factors tested included ownership status

TABLE III. Distribution of IgG ELISA and IFAT titers (reciprocal of dilution) for *Toxoplasma gondii* in 194 cats.

ELISA	IFAT <16	16	32	64	128	256	512	1,024	2,048	4,096	8,192	16,384	32,768	131,072	Total
<64	115	3	5	13	8	3									147
64	7			1											8
128	1						3	1							5
256								2							2
512	1				1		1		4	2	1	1			11
1,024									3	3		1			7
2,048										4	2		2		8
4,096								1			2		1	1	5
8,192													1		1
Total	124	3	5	14	9	3	4	4	7	9	5	2	4	1	194

(owned or unowned), age (categorized as <6 mo, 6–12 mo, or >12 mo), and gender. Risk factors with a $P < 0.20$ also were entered into a multivariate model. Models were compared using the likelihood ratio test, and model fit was assessed by the Hosmer–Lemeshow goodness-of-fit statistic (Agresti, 2002). $P < 0.05$ was considered significant for all statistical analyses.

Because the proportions of sampled cats in the 3 age strata differed significantly from the population, age-adjusted prevalence for the population was calculated by multiplying the proportion of cats testing IgG positive in 3 age strata (defined above) by the estimated number of cats in each age stratum from the population-based telephone survey (Dabritz et al., 2006). The 95% confidence intervals (CI) of the estimate were calculated as for a proportion under stratified random sampling with finite population correction (Scheaffer et al., 1996). The Rogan–Gladen estimator (Rogan and Gladen, 1978) was then applied to the joint estimates of test Se, Sp, and covariances (Gardner et al., 2000) to estimate the age-adjusted prevalence in each stratum incorporating test performance. The variance of this estimator was calculated according to variance 1 (var_1) of Greiner and Gardner (2000a) with the addition of the stratified random sampling component described above.

RESULTS

Test evaluation study

Inter- and intraobserver agreement: Unweighted kappa for IFAT interobserver agreement was 0.65 (95% CI = 0.57–0.73), and weighted kappa was 0.90 (95% CI = 0.80–0.99), indicating good-to-excellent agreement between readers (Szklo and Nieto, 2000). Kappa for intraobserver agreement was 0.44 (95% CI = 0.31–0.58), and weighted kappa was 0.98 (95% CI = 0.93–1.0). Discordant results for intraobserver agreement occurred mainly at low dilutions in sera from the UCD SPF cats.

Fecal flotation: No 10- × 12-μm *T. gondii*-like oocysts were detected in the feces of the 100 UCD SPF cats. Cysts of *Giardia* spp. were detected in the feces of 37 cats by fecal flotation or DFA, and *C. parvum* oocysts were detected in the feces of 11 cats.

Estimates of Se, Sp, prevalence, and covariances of the ELISA and IFAT: The cross-tabulation of ELISA and IFAT serologic results for the 194 cats in the test evaluation study are shown in Table III, and estimates of the 8 parameters from the Bayesian model are shown in Table IV. The history, autocorrelation, and density plots for the 8 parameters estimated by the Bayesian model were reviewed to check the stability of the estimates, and no unusual patterns in the graphs were observed. For the 100 SPF cats, antibody reactions in the IFAT occurred at dilutions of 1:16 for 3 cats, 1:32 for 1 cat, 1:64 for 4 cats, and 1:128 for 4 cats. Nine of the 10 cats with titers ≥64 were ≤6 mo of age, and 4 cats were shedding *Giardia* sp. cysts in their feces, including 3 co-infected with either *Isospora felis* or *Cryptosporidium* spp. Two (2%) of the 100 SPF cats also reacted positively (at a dilution of 1:64) in the ELISA. Using 1:128 as the definition of a positive test from the Bayesian estimation procedure, Se of the IFAT was 81.0% and Sp was 93.8% (Table IV). When a cutoff of 1:64 was used to define a positive test in the IFAT, its Sp decreased to 86.3%, whereas Se marginally improved to 83.3%. Thus, a cutoff of 1:128 was selected to define cats in the field serosurvey as *T. gondii* positive in the IFAT.

For the 18 experimentally infected cats bled 8 wk PI, 2 cats

TABLE IV. Estimates of ELISA and IFAT Se, Sp, *Toxoplasma gondii* prevalence, and covariance of the tests in 2 cat populations.*

Parameter	With FIV-infected cats		Without FIV-infected cats	
	Median (%)	95% probability interval	Median (%)	95% probability interval
Prevalence in UCD SPF cats	0.9	0.1–3.2	0.9	0.1–3.0
Prevalence in CSU cats	59.6	50.0–68.0	64.8	54.5–74.7
Se ELISA	92.6	86.5–96.8	92.3	86.1–96.4
Sp ELISA	96.5	93.4–98.4	96.4	93.5–98.3
Se IFAT	81.0	68.0–91.1	94.2	84.7–98.9
Sp IFAT	93.8	88.3–97.5	93.7	87.9–97.5
Test covariance for infected cats	10.3	−12.6 to 48.5	27.4	−4.8 to 75.4
Test covariance for uninfected cats	12.8	−4.2 to 52.1	12.7	−4.2 to 51.0

* Analysis based on a Bayesian estimation procedure in WinBUGS using 50,000 Markov-chain Monte Carlo samples.

TABLE V. Values used to estimate the *Toxoplasma gondii* IgG seroprevalence in owned cats from the Morro Bay area, adjusted for age and test performance.

Age class	% Cat population*	No. estimated for cat population	SP†	No. estimated seropositive in cat population based on SP‡	TP§	Adjusted no. estimated seropositive in population
<6 mo	5.4	393	3.6	14	5.5	22
6–12 mo	3.9	284	17.4	49	26.4	75
>12 mo	90.7	6,607	31.7	2,094	48.1	3,178
Total		7,284		2,157		3,275

* Based on a telephone survey of 142 households owning 263 cats conducted in the summer of 2003 (Dabritz et al., 2006).
† Seroprevalence (percentage of cats testing seropositive) by IgG ELISA or IFAT in each age stratum in the present survey.
‡ Age-adjusted IgG seroprevalence not accounting for the Se, Sp, and test covariances in the parallel-interpretation scheme = 2,157/7,284 (29.6%, 95% CI = 16.6–42.7%).
§ True prevalence based on the Rogan–Gladen estimator (Rogan and Gladen, 1978), adjusted for age and incorporating Se, Sp, and test covariances under a parallel-interpretation scheme. $Se = 1 - ([1 - Se_{T1}][1 - Se_{T2}]) - \gamma Se$; $Sp = Sp_{T1}Sp_{T2} + \gamma Sp$ (Gardner et al., 2000). TP = 3,275/7,284 (45.0%, 95% CI = 5.2–84.8%).

were seronegative on both tests, 2 cats were negative by ELISA and had titers of 128 in the IFAT, and the remaining 14 cats were seropositive on both tests. Of the 20 cats co-infected with FIV and *T. gondii*, only 7 cats reacted positively (1:64) in the ELISA and 1 cat reacted at 1:64 in the IFAT, which was below the cutoff selected for an IFAT-positive result. In the group of 56 cats for which toxoplasmosis was a differential diagnosis, 24 cats had ELISA titers ≥64 and 28 cats had IFAT titers ≥128.

Use of different priors for prevalence in the UCD SPF cat population, prevalence in the CSU cat population, ELISA Sp, and ELISA Se had minimal effects on the point estimates. All estimates were within the 95% probability interval for the original analysis. The most notable effect was that of the prior for ELISA Se. When ELISA Se was specified as 85%, the Se covariance in the CSU cat population increased from 12.4 to 28.1%. Because both tests demonstrated low Se (35% for the ELISA and 0% for the IFAT) in the 20 cats co-infected with FIV and *T. gondii*, the sensitivity analysis also included an assessment of test performance excluding this group. Under the aforementioned scenario, Se and Sp of the ELISA were virtually unchanged (92.3% and 96.4%, respectively), whereas Se of the IFAT increased to 94.2% from 81.0% with a trivial change in Sp (Table IV).

TABLE VI. Potential risk factors associated with *Toxoplasma gondii* seropositivity in 43 owned cats.

Risk factor	No. seropositive*	No. seronegative	*P* value	No. of cats with missing data
Hunting	3	16		
Not hunting	4	20	1.00	
Fed raw meat	1	0		
Not fed raw meat	6	36	0.16	
Female	3	20		
Male	4	15	0.68	1
Allowed outdoors	5	24		
Indoor only	2	9	1.00	3
Altered	5	18		
Not altered	0	16	0.07	4
Age >12 mo	7	16		
Age 6–12 mo	0	7		
Age <6 mo	0	11	0.04†	2
Cayucos	0	2		
Los Osos	0	14		
Morro Bay	7	16		
Neighboring community	0	4	0.08	

* IgG/IgM ELISA or IgG IFAT positive.
† Values are statistically significant by Fisher's exact test at $P < 0.05$.

Serosurvey of domestic cats

Demographics: Attributes of the 43 serologically sampled owned cats compared with population data from the telephone survey (Dabritz et al., 2006) are shown in Table II. There were statistically significant differences in the proportion of altered cats, the number of hours spent outside per day, and the median cat age. Outdoor status and age are putative risk factors for *T. gondii* seropositivity. The proportion of owned cats in the telephone survey that ate raw meat was marginally lower than the proportion in the serologically tested cat population, but the *P* value (Fisher's exact test, $P = 0.33$) was not significant. For the 80 unowned or feral cats, 44 cats were aged <6 mo, 16 cats were 6–12 mo, and 18 cats were >12 mo (age category missing for 2 animals). The unowned cats differed significantly by age strata from the owned cats sampled ($P = 0.001$) and the cat population ($P < 0.0001$). Forty-eight (60%) of the 80 unowned cats were female.

Prevalence of T. gondii in cats sampled from the Morro Bay area: Eighteen (15%) of the 123 cats tested *T. gondii* IgG seropositive, 23 cats (19%) were positive by IgM-ELISA, and 32 cats (26%) were IgG or IgM seropositive. Nine (21%) of the 43 owned cats and 23 (29%) of the 80 unowned cats were IgG or IgM seropositive. Age-adjusted seroprevalence (based on IgG results; Table V) was calculated because the proportion of cats in each age stratrum differed for the cats in the serosurvey compared with the cat population, and it was estimated to be 29.6% (95% CI = 16.6–42.7%). Using the estimates of test Se, Sp, and covariances based on the analysis without FIV-infected cats (Table IV), the age and test performance-adjusted seroprevalence was 45.0% (95% CI = 5.2–84.8%).

Factors associated with T. gondii infection for owned cats: Fisher's exact tests for the owned cats seropositive on any test indicated that age >12 mo was associated with seropositivity (Table VI). The results were the same for IgM-ELISA seropositivity, with a *P* value for age of 0.032 (data not shown). All

TABLE VII. Univariate logistic regression analysis of risk factors for *Toxoplasma gondii* seropositivity in 123 owned and feral cats from the greater Morro Bay area.*

		Seropositive on any test†			IgG positive		
Risk factor	Group	OR	95% CI	*P* value	OR	Wald 95% CI	*P* value
Ownership	Owned	1.00			1.00		
Status	Unowned	1.95	0.76–5.03	0.17	1.47	0.49–4.45	0.49
Age	<6 mo	1.00			1.00		
	6–12 mo	1.72	0.44–6.78	0.44	2.52	0.33–19.1	0.37
	>12 mo	6.39	2.24–18.2	<0.001‡	12.30	2.59–58.4	0.002‡
Gender	Male	1.00			1.00		
	Female	1.24	0.53–2.90	0.63	0.88	0.32–2.42	0.81

* IgG/IgM ELISA or IgG IFAT positive.
† IgG ELISA or IgG IFAT positive.
‡ Values are statistically significant at $P = 0.05$.

seropositive owned cats were aged >12 mo. Other characteristics with *P* values <0.20 in Fisher's exact test were neuter/spay status, a variable that is strongly associated with age ($P = 0.07$), being fed raw meat ($P = 0.16$), and city of residence ($P = 0.08$). All the owned cats testing positive lived in the city of Morro Bay. When the latter 3 variables were tested in different combinations with age in a multivariable exact logistic regression model, a model could not be fit because the data were too sparse. The median age of owned cats seropositive for any test was on average higher than uninfected cats (Mann–Whitney *U*-test, $P = 0.018$), but there was no difference between the median number of hours spent outside for infected and uninfected animals (Mann–Whitney *U*-test, $P = 0.96$). The results of the Mann–Whitney *U*-tests for IgM seropositivity were similar (data not shown).

Risk factors for T. gondii infection in owned and unowned cats: In the logistic regression model for all 123 cats, age was the only significant predictor of seropositivity (Table VII). Cats aged >12 mo were 12.3-fold more likely to be IgG seropositive than cats aged <6 mo ($P = 0.002$), and the association held for a parallel interpretation that included IgM ELISA test results with an odds ratio (OR) of 6.4. Age >12 mo compared with age <6 mo was also significantly associated with IgM ELISA seropositivity (OR = 3.7, $P = 0.017$).

DISCUSSION

Using a Bayesian approach that incorporated prior information about 1 of the tests, the IgG ELISA Se and Sp for detection of *T. gondii* seropositivity in cats was estimated to be 92.6% and 96.5%, respectively. The IgG IFAT was less sensitive (81.0%), but it had similar Sp (93.8%). Both tests performed poorly in cats co-infected with FIV and *T. gondii*. Excluding the group of 20 FIV-*T. gondii* co-infected cats, the same analytical approach yielded a much improved Se for the IFAT of 93.6%, as well as a higher value of 27.4% for Se covariance in the CSU cat population, whereas all other estimates of test performance remained similar. The ELISA and IFAT were applied to a population of owned and unowned cats from an area on California's central coast. Seroprevalence in the 123 cats was estimated to be 15% (IgG), 19% (IgM), and 26% (IgG or IgM), and it did not differ for owned versus unowned cats. The only significant predictor for seropositivity in this cat population was age >12 mo compared with age <6 mo, and this association was evident in both owned and unowned cats.

The IgG ELISA in this study had high Se and Sp that compared favorably with earlier ELISAs (Tenter et al., 1994). The latter study compared the performance of an ELISA using H4/H11 *T. gondii* polypeptides to a whole tachyzoite ELISA (termed TEA-ELISA) in a field survey of owned and stray cats in Germany. Gold standards were not available, and the authors did not consider test covariances, so Se and Sp likely would have been slightly overestimated (Gardner et al., 2000). The Se and Sp of the ELISAs in the Tenter et al. (1994) study were estimated to be 95% and 100% for the H4/H11 ELISA and 98% and 99% for the TEA-ELISA, respectively. A recent study from Japan in which gold standards were also unavailable for a field-sampled population found that a recombinant SAG2 antigen ELISA compared favorably with a latex agglutination test (LAT) for detecting *T. gondii* in feline sera (Huang et al., 2002). The same methodology used in our study could have been used to estimate Se and Sp of the SAG2 ELISA, because the 192 cats sampled came from populations in 2 different areas (Tokyo and Sapporo) in Japan (Kimbita et al., 2001), and estimates of the Se and Sp of the LAT in cats have been published previously (Tsubota et al., 1977).

Tenter et al. (1994) also estimated Se and Sp of an IgG IFAT to be 94% and 92%, respectively, using a cutoff of 1:40 for a positive test. The IgG IFAT in our study was found to have lower Se of 81.0% (94.2% if FIV-*T. gondii* co-infected cats were excluded) and comparable Sp of 93.8%. This may be attributable to the inclusion of cats co-infected with FIV and *T. gondii* in the present study and the higher cutoff selected to define a positive test. The Bayesian analysis showed that the 1:128 cutoff maximized Se and Sp for the IFAT. Only 4 (4%) of 100 cats in the group assumed to be uninfected had titers of 128. The selection of a higher cutoff may be related to the type of secondary antibody used in the test. The present study used a goat anti-cat heavy-chain IgG FITC conjugate rather than the more commonly used goat anti-cat heavy- and light-chain IgG antibody. The anti-cat heavy-chain IgG antibody produces less background reactivity, making interpretation of IFAT slides easier. Some epidemiologic studies have used much lower cutoffs, e.g., 1:16 and 1:32, to define a positive *T. gondii* IFAT, and they may be overestimating the seroprevalence of *T. gondii* in cats

(McKinney, 1973; Claus et al., 1977; Rodgers and Baldwin, 1990). Alternatively, the cats in these earlier surveys may have represented a more diverse spectrum of infection, or they may have been infected with other strains of *T. gondii* or species of protozoan parasites that were cross-reactive, giving rise to different antibody profiles. Nine of the 10 UCD SPF cats whose sera reacted at either 1:64 or 1:128 were <6 mo old. Four of these cats were shedding *G. lamblia*, *Cryptosporidium* sp., or *I. felis* in their feces, but there was no association between false-positive *T. gondii* titers in the UCD SPF cats shedding parasites in their feces compared with those that were not. However, none of the cats was tested to determine whether they had antibodies to *Cryptosporidium* sp. or *G. lamblia*. In humans, anti-*G. lamblia* antibodies may cross-react with *T. gondii* antigens (Haralabidis, 1984).

The poorer performance of the IFAT in the present study also may be attributable to the inclusion of 20 cats co-infected with *T. gondii* and FIV. Surveys of FIV in cats from various countries since 1995 estimate seroprevalence ranged between 2.3 and 42.8% (D'Amore et al., 1997; Malik et al., 1997; Hill et al., 2000; Dorny et al., 2002; Lee et al., 2002; Maruyama et al., 2003; Luria et al., 2004; Nutter et al., 2004; Clifford et al., 2006). In a study where cats were infected with FIV 18 wk before inoculation with *T. gondii*, none of the 4 cats surviving to day 14 developed detectable IgG ELISA titers (Davidson et al., 1993). In the present study, none of the 20 cats experimentally co-infected with FIV and *T. gondii* was seropositive in the IFAT, whereas the ELISA yielded positive results for only 7 cats. Previous studies of cats co-infected with *T. gondii* and FIV have shown that they were more likely to produce IgM antibodies in response to *T. gondii* infection than IgG antibodies (O'Neil et al., 1991; Lappin et al., 1993) and that they were more likely to have lower *T. gondii* IgG ELISA or IFAT titers than cats without FIV (Svobodova et al., 1986; Lappin et al., 1993). Contrary evidence regarding IgG antibody detection in FIV-*T. gondii* co-infected cats also has been reported, possibly because natural co-infection differs from experimental co-infection (Witt et al., 1989). Elevated *T. gondii* IgG IFAT titers in 8 naturally FIV-*T. gondii* co-infected cats were detected in a survey of 585 stray, relinquished, and veterinary client cats from Baltimore, Maryland. Possible explanations for this phenomenon include the magnitude of exposure to *T. gondii* in the natural environment, nonspecific polyclonal B-cell activation as a result of FIV infection, and recrudescence of *T. gondii* due to FIV-induced immunosuppression (Witt et al., 1989).

The immune response to *T. gondii* also may be affected by whether FIV infection occurs before or after *T. gondii* infection, and the length of time that the cat was infected before superinfection. Cats infected with FIV 12 mo before *T. gondii* inoculation had lower IgM ELISA titers, and they developed IgG antibody later in the course of infection than FIV-seronegative cats, probably due to delayed IgM-to-IgG class switching and/or poorer immune response as a result of $CD4^+$ T-lymphocyte depletion (Lappin et al., 1996). *Toxoplasma gondii*-infected cats seropositive for at least 6 mo before FIV infection maintained stable *T. gondii* IgG titers, but they had transiently elevated *T. gondii* IgM titers 4-6 wk post-FIV infection along with immunosuppression (Lappin et al., 1992). In the United States, the largest, most recent FIV serosurveys estimated prevalence to be 4-5% (Lee et al., 2002; Luria et al., 2004; Nutter et al., 2004). If the Se of the IFAT and ELISA are 0% and 35%, respectively, for detection of *T. gondii* antibodies in cats co-infected with FIV, then *T. gondii* serosurveys of U.S. cat populations may slightly underestimate the *T. gondii* prevalence. Concurrent testing for FIV may be helpful. If FIV infection is uncommon (<5%), then it would be reasonable to use estimates of Se and Sp for the IFAT and ELISA that were calculated for cats without concurrent FIV infection.

Agreement between readers and for the same reader on different days for the IFAT was high (weighted kappa 0.90 and 0.98, respectively). Most disagreements occurred at low dilutions, where there is more difficulty in interpreting IFA slides because of background fluorescence. More than half of the disagreements were below the 1:128 cutoff for a positive test, and they would not impact the interpretation (negative or positive) of the test result.

Older age was positively associated with *T. gondii* infection in this study, as has been reported in many other *T. gondii* cat serosurveys (Ruiz and Frenkel, 1980; Witt et al., 1989; O'Neil et al., 1991; Lappin et al., 1993; Maruyama et al., 2003; Miro et al., 2004; Salant and Spira, 2004; Pena et al., 2005; Vollaire et al., 2005). Based on the IgG results for the present study, age >12 mo was associated with 12.3-fold greater odds of *T. gondii* infection compared with age <6 mo. Two (4%) of 55 cats <6 mo old, 4 (17%) of 23 cats aged 6–12 mo, and 13 (32%) of 41 cats aged >12 mo tested IgG seropositive. The crude IgG prevalence of 15% in the present study was related to the high proportion (55% of unowned and 26% of owned) cats that were <6 mo old at the time of sampling. Eighteen (42%) of 43 owned cats sampled in the serosurvey were <12 mo old compared to only 9% in the general population (Dabritz et al., 2006).

Contrary to the findings of the present study, in Costa Rica exposure to *T. gondii* occurred at an early age, and oocyst shedding was detected most frequently in cats <600 g or 2 mo old (Ruiz and Frenkel, 1980). Of the 55 cats <6 mo old in the present survey, only 7 (14%) cats were seropositive for *T. gondii* IgM or IgG antibodies, suggesting that exposure to *T. gondii* is infrequent in the first months of life in this area. Five of the 7 cats were positive only for IgM antibody, and all 7 cats were the offspring of feral cats. *Toxoplasma gondii*-specific IgM antibodies are most commonly detected in recently infected cats (Lappin, 1996), including some kittens that are infected transplacentally or lactationally (Dubey, Lappin et al., 1995; Cannizzo et al., 1996; Powell and Lappin, 2001). Thus, we think it is most likely that the 5 feral kittens with IgM antibodies alone were *T. gondii* infected. However, antigen recognition patterns consistent with lactational transfer of IgM antibodies also have been detected in some kittens (Cannizzo et al., 1996).

Toxoplasma gondii seroprevalence in cats from the United States has been recently reviewed (Conrad et al., 2005). Applying the age stratum-specific IgG prevalence estimates to the cat population structure derived for the Morro Bay area from an earlier survey (Dabritz et al., 2006), the age-adjusted prevalence was estimated to be 29.6%. The latter estimate is consistent with the 26.3% reported for clinically ill cats from the California–Nevada region for which toxoplasmosis was a differential diagnosis (Vollaire et al., 2005) and with the 33.7% seroprevalence for feral cats on 3 islands off California's central coast (Clifford et al., 2006). The 29.6% seroprevalence in the current

study is much lower that the prevalence of 60% (by serology or detection of oocysts in feces) in Costa Rica, where 80% of cats were reported to be free-roaming strays (Ruiz and Frenkel, 1980). Seroprevalence ranges from 0 to 81% in cats from Brazil where cats are similarly managed, but it is commonly ≥26% (Silva et al., 2002).

There was no significant difference in *T. gondii* seroprevalence for owned cats observed hunting compared with those that were not. This may be related to the fact that cat owners were unaware of their cat's predatory abilities, because they did not look for evidence of their cat's hunting activities or they did not find it because cats often consume small prey items, e.g., mice, whole. Thus, hunting behavior may be underreported for owned cats. Owners also may have been reluctant to admit their cat was killing small rodents and birds. Although unowned or feral cats are assumed to hunt more avidly to survive, there was no significant difference in IgG/IgM seroprevalence for owned cats (21%) compared with unowned cats (29%) in the present study. Similar results were reported in serosurveys of cats from Rhode Island and Florida (U.S.A.) and Poland (DeFeo et al., 2002; Smielewska-Los and Pacon, 2002; Luria et al., 2004). One possible explanation for not detecting a difference in seroprevalence for owned cats compared with unowned animals may be because the desired sample size of 82 cats per group was not achieved, reducing the power for detecting a statistically significant difference from 80 to 53%. This association also may be masked by confounding factors such as access to the outdoors and a diet that includes raw meat for owned cats, or supplemental feeding of feral cats that reduces their need for predation. Outdoor access and feeding raw meat were not associated with *T. gondii* infection of owned cats in the present study. However, the power to detect significant differences was low because we did not achieve the desired sample size for owned cats. We nonetheless determined that owned cats recruited for the study differed in important characteristics from the general population (Table II). They spent about half as much time outside, had a median age 5 yr younger, and fewer were spayed or neutered (an attribute correlated with age). A greater amount of time spent outside may increase the opportunity for cats to hunt and eat infected rodents, whereas older age is related to increasing risk for *T. gondii* exposure over the cat's lifetime. Other surveys have detected significantly higher proportions of *T. gondii* seropositivity in feral or unowned cats than in pet cats, and in outdoor cats compared with indoor cats (Wallace, 1971; Knaus and Fehler, 1989; Tenter et al., 1994; Nogami et al., 1998; Lucas et al., 1999; Dubey et al., 2002; Maruyama et al., 2003; Nutter et al., 2004). However, comparison across studies is complicated by the use of different serologic tests and cutoffs, collection of varying types of demographic data, and differences in ownership or management of the cat populations studied.

The crude IgG seroprevalence of 15% in cats in the current study reflected the study population's younger age and shorter number of hours spent outside per day. Application of age-specific prevalences from the sampled cats to population-based age strata derived from a random sample enabled us to adjust for the age sampling bias. Given an age-adjusted seroprevalence of 29.6%, 2,156 *T. gondii* infections in total would be expected to occur in the estimated 7,284 cats in the population over 50,988 cat-yr at risk (median survival time of 7 yr × number of cats in the population at risk) for an average incidence of 0.042 *T. gondii* infections/cat-yr. This represents about 4.2% of owned cats in the population (308 cats) becoming infected each yr. If each cat shed 10 million *T. gondii* oocysts/infection (Davis and Dubey, 1995; Dubey, 2001, 2005), and 36% of them always defecated outside (Dabritz et al., 2006), then owned cats would defecate about 1.1 billion oocysts into this 3,104-ha coastal ecosystem each year. An environmental burden of 36 oocysts/m^2 over the region (being most likely more intense in areas with large cat populations) suggests that oocysts are present in sufficient numbers to reach marine waters, where they could be ingested by sea otters. *Toxoplasma gondii* oocysts in soil and nearshore marine waters also may pose a health risk for humans. Reducing the number of *T. gondii* oocysts in the environment could mitigate this wildlife and human health hazard. Measures to diminish oocyst contamination of the environment could include placing unowned cats in rescue facilities at an early age, relocating feral cat colonies, and encouraging cat owners to confine their cats inside, where opportunities for predation on infected rodents and birds are low.

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APPENDIX I

```
model
{
## Likelihood ##
  y1[1:Q, 1:Q]~dmulti(p1[1:Q, 1:Q],n1)
  y2[1:Q, 1:Q]~dmulti(p2[1:Q, 1:Q],n2)
  p1[1,1]<-pi1*Se11 + (1-pi1)*Sp11 ## pi=Prevalence ##
  p1[1,2]<-pi1*Se12 + (1-pi1)*Sp12
```

```
   p1[2,1]<-pi1*Se21 + (1-pi1)*Sp21
   p1[2,2]<-pi1*Se22 + (1-pi1)*Sp22
   p2[1,1]<-pi2*Se11 + (1-pi2)*Sp11
   p2[1,2]<-pi2*Se12 + (1-pi2)*Sp12
   p2[2,1]<-pi2*Se21 + (1-pi2)*Sp21
   p2[2,2]<-pi2*Se22 + (1-pi2)*Sp22

## Re-Parameterization ##
   Se11 <-lambdaD*Se1
   Se12 <-Se1 - Se11
   Se21 <-gammaD*(1-Se1)
   Se22 <- 1 - Se11 - Se12 - Se21
   Sp11 <- 1- Sp12 - Sp21 - Sp22
   Sp12 <- gammaDc*(1-Sp1)
   Sp21<-Sp1-Sp22
   Sp22 <- lambdaDc*Sp1
   Se2 <- Se11 + Se21
   Sp2 <- Sp22 + Sp12
   rhoD <- (Se11 - Se1*Se2)/sqrt(Se1*(1-Se1)*Se2*(1-Se2))
      ## correlation in diseased ##
   rhoDc <- (Sp22 - Sp1*Sp2)/sqrt(Sp1*(1-Sp1)*Sp2*(1-Sp2))
      ## correlation in disease free ##

## Priors ##
   pi1 ~ dbeta(1.8816,88.28)
   pi2 ~ dbeta(27.0502,7.5125)
   Se1 ~ dbeta(99.6983,6.1946)
   Sp1 ~ dbeta(99.6983,6.1946)
   lambdaD ~ dbeta(1,1)
   gammaD ~ dbeta(1,1)
   plambdaDc ~ dbeta(1,1)
   gammaDc ~ dbeta(1,1)
   }

## Data ELISA 64, IFAT 128 ##
   list(n1=101,n2=94,Q=2,y1=structure(.Data=c(0,2,4,94),
      .Dim=c(2,2)),
   y2=structure(.Data=c(37,8,7,42),.Dim=c(2,2)))

## Initial Values ##
   list(pi1=0.05,pi2=0.60,Se1=.95,Sp1=.95,lambdaD=0.80,
      lambdaDc=0.50,gammaD=0.70,gammaDc=0.80))
```