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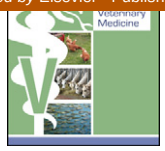

Seroprevalence and risk factors for *Toxoplasma gondii* infection in domestic cats in The Netherlands

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ABSTRACT

Cats, as definitive hosts, play an important role in the transmission of *Toxoplasma gondii*. To determine the seroprevalence and risk factors for *T. gondii* infection in Dutch domestic cats, serum samples of 450 cats were tested for *T. gondii* antibodies by indirect ELISA. Binary mixture analysis was used to estimate the seroprevalence, the optimal cut-off value and the probability of being positive for each cat. The seroprevalence was estimated at 18.2% (95% CI: 16.6–20.0%) and showed a decrease with age in very young cats, an increase up to about 4 years old and ranged between 20 and 30% thereafter. Hunting (OR 4.1), presence of a dog in the household (OR 2.1), former stray cat (OR 3.3) and feeding of raw meat (OR 2.7) were identified as risk factors by multivariable logistic regression analysis. Prevalence differences were estimated by linear regression on the probabilities of being positive and used to calculate the population attributable fractions for each risk factor. Hunting contributed most to the *T. gondii* seroprevalence in the sampled population (35%).

1. Introduction

Infection with the protozoan *Toxoplasma gondii* is one of the most common parasitic infections of man and warm-blooded animals all over the world. Nearly all species of felids, for example domestic cats, are definitive hosts. Sexual reproduction of *T. gondii* takes place in their intestines and results in shedding of environmentally resistant oocysts. In addition, *T. gondii* has an exceptionally wide range of intermediate hosts: it can reproduce asexually and form tissue cysts in all warm-blooded animals including humans. For both definitive and intermediate hosts infection occurs via one of the following routes: (1) horizontal by oral ingestion of infectious cat-shed oocysts via contaminated soil, food or water, (2) horizontal by oral ingestion of tissue cysts with bradyzoites contained in raw or undercooked meat, and (3) vertical by transplacental transmission of tachyzoites (Tenter et al., 2000).

Specific antibodies to *T. gondii* have been detected in up to 74% of adult cats in some populations (Tenter et al., 2000). Both tissue cysts and antibodies persist, while in general, a cat sheds oocysts for up to three weeks after a primary infection only (Dabritz and Conrad, 2010). The prevalence of oocyst excretion in faeces is therefore much lower than the seroprevalence: large scale screening demonstrated *Toxoplasma*-like oocysts (this includes *Hammondia hammondi* oocysts) in 0.31% of faecal samples from German and other European cats, 0.11% was confirmed as *T. gondii* (Schares et al., 2008). The quantity of oocysts produced after primary infection varies from 3 to 810 million oocysts (Dabritz and Conrad, 2010). In addition

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to the considerable amount of oocysts shed, the oocysts are very resistant and can remain viable for up to 18 months in soil depending on humidity, temperature and exposure to direct sunlight (Frenkel et al., 1975; Yilmaz and Hopkins, 1972), and for 6 up to 54 months in water (Dubey, 1998) and seawater (Lindsay and Dubey, 2009). Contamination of the environment with *T. gondii* oocysts by cats plays an important role in the epidemiology of toxoplasmosis of animals and humans, which is illustrated by the low prevalence of *T. gondii* infections in areas without cats (Dubey et al., 1997; Munday, 1972; Wallace et al., 1972).

The overall seroprevalence of *T. gondii* for the Dutch human population has recently been estimated at 26.0% (Hofhuis et al., 2011) and the incidence of congenital toxoplasmosis was estimated at 2 per 1000 live-born children leading to a disease burden similar to that of the major foodborne pathogen *Campylobacter* (Kortbeek et al., 2009). Because of the high disease burden in humans there is a need for intervention. The effectiveness of prenatal and postnatal treatment to prevent congenital toxoplasmosis is debated (Gilbert, 2009); therefore the focus is on prevention of infection. As contamination of the environment with *T. gondii* oocysts only occurs by felines and all intermediate host infections have a cat shedding oocysts as starting point of the cycle, prevention of cat infection is likely to be highly effective in reducing both oocyst- and tissue cyst-acquired infections in humans.

To prevent infection of cats and thereby reduce oocyst shedding by cats, the risk factors for infection need to be well understood. Depending on prey composition and the prevalence of *T. gondii* in these animals, prey intake from hunting is important for the prevalence of the infection (Afonso et al., 2007). Other risk factors for infection in cats identified by multivariable analyses are: age (Afonso et al., 2006; De Craeye et al., 2008; Lopes et al., 2008), outdoor access (Afonso et al., 2010; Lopes et al., 2008), raw meat in their diet (Lopes et al., 2008), number of kittens in groups of farm cats (Afonso et al., 2010), and precipitation (Afonso et al., 2006, 2010). However, in The Netherlands the risk factors for *T. gondii* infection in cats and levels of exposure of cats to these risk factors are unknown. It was our aim to determine the overall and age-related seroprevalence of *T. gondii* in cats in The Netherlands, identify the risk factors and estimate the proportion of cat infections that could be prevented if the exposure was removed from the population.

2. Materials and methods

2.1. Study population

Cats are popular pets in The Netherlands and from 1997 the population of domestic cats increased by 50% to an estimated 3.3 million in 2006 (Raad voor Dierenaangelegenheden, 2006). The number of stray cats is unknown. Based on a limited sample ($n = 150$) the seroprevalence of *T. gondii* for the Dutch cat population was estimated at 49% in 1993 (personal communication Prof. Dr. F. van Knapen, Utrecht University). With an expected seroprevalence of 49% (P), an accepted deviation of the true prevalence of 5% (d) and a confidence level of 95% ($z = 1.96$) the sample size necessary to estimate the seroprevalence was calculated at 384 (according to $n = P(1-P)\,z^2/d^2$).

Samples were collected between January and August 2010 at 26 veterinary clinics recruited by a call in the journal of the Royal Dutch Veterinary Association (Opsteegh et al., 2010a). All cats presented at the cooperating clinics were eligible to take part in the study. The cat owners, who volunteered in the project, gave their permission to collect a blood sample and filled out a questionnaire. A total of 342 cat serum samples (314 different owners) were collected. In addition, 108 cat serum samples (94 different owners) collected at 25 veterinary clinics (3 corresponding with 2010 study) between May 2005 and August 2007 as part of a vaccination status project (Dr. H. Egberink, Utrecht University, Faculty of Veterinary Medicine, Department of Virology) were tested. In this study, veterinarians were asked to include 4 types of cats based on their vaccination status (unvaccinated kittens; young cats that received the full vaccination schedule for kittens but not yet the 1-year booster; adult cats that received the full vaccination schedule for kittens and an annual or biannual booster; adult cats that had not been vaccinated in the three preceding years). Another difference was that the information on the cat was provided by the veterinarian rather than the owner. The distribution of all sampled cats over The Netherlands is shown in Fig. 1.

To enable proper fitting of the binary mixture model, 203 available domestic cat sera from Romania were tested additionally. Inclusion of these sera generates a higher proportion of positive sera (seroprevalence 50% (Györke et al., 2011)) which facilitates fitting of the positive component of the mixture. These sera were not included in the seroprevalence estimation or risk-factor analyses as risk factors may vary per country.

2.2. Serological assay

An in-house developed indirect ELISA was used to detect antibodies against *T. gondii*. The in-house ELISA and the correction of optical density (OD)-values for background absorbance and plate-to-plate variation were performed as described previously (Györke et al., 2011).

2.3. Questionnaires

To identify the risk factors for infection with *T. gondii* in cats, a self-administered questionnaire with 32 multiple-choice and 8 open-ended questions for the cat owners was formulated. Questions related to potential risk factors such as housing (type of environment, presence of a dog or other cats in the household, presence of other cats around the house), outdoor access, contact with young cats, feeding of raw meat, observed hunting behaviour, being a former stray cat, and availability and use of a litter tray were asked. Other data gathered included age, sex, and neutering and vaccination status. For the cats from the vaccination status survey ($n = 108$), only data on postal code, age, sex, neutering and vaccination status, outdoor access and the presence of a dog or more cats in the household were available.

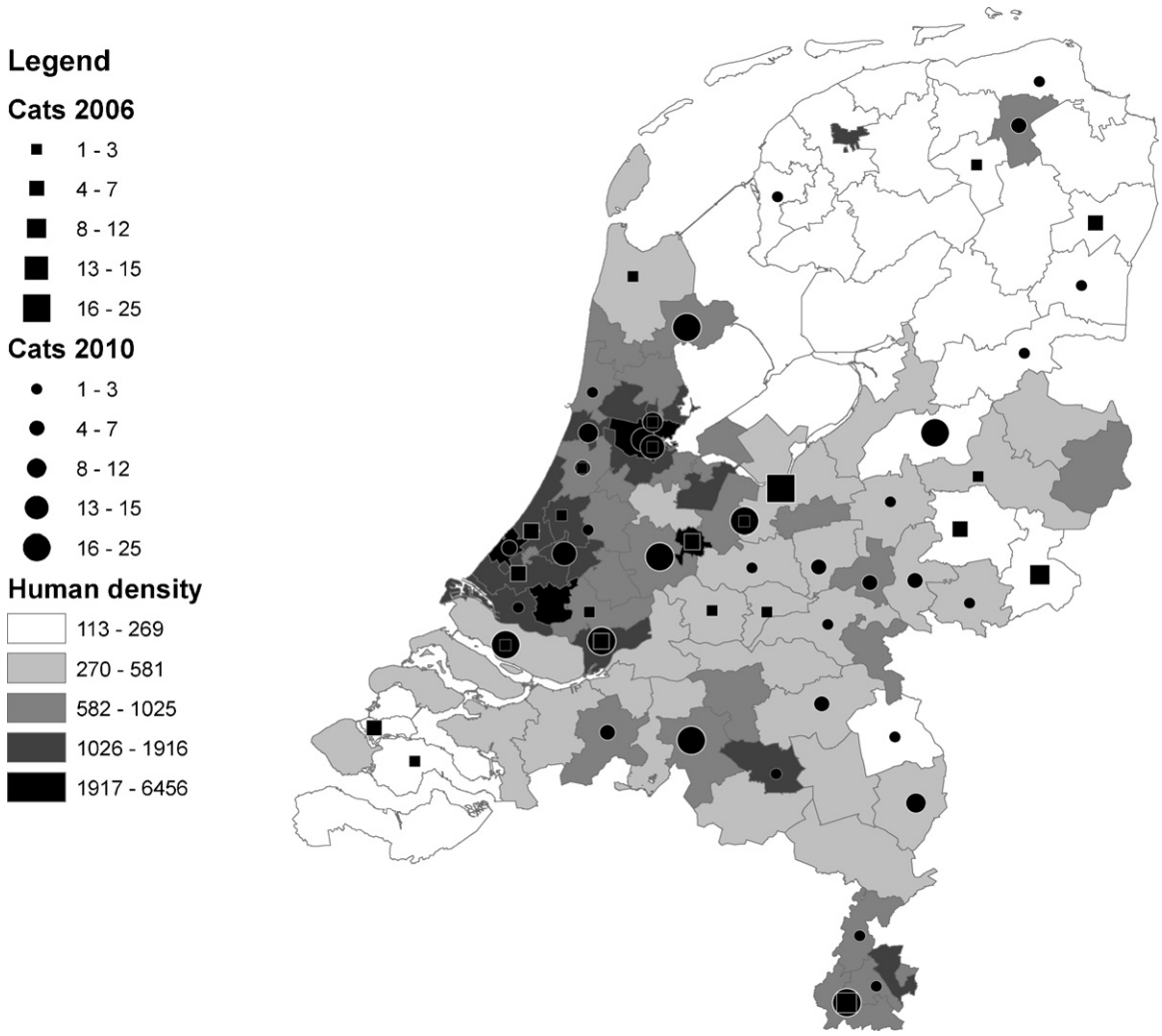

Fig. 1. Cats sampled for the vaccination status survey (2006) and the present study (2010) plotted at their 2-digit postal code. Background colour represents the population density (inhabitants per km^2).

2.4. Data analyses

2.4.1. Binary mixture model to estimate the seroprevalence, establish a cut-off value and calculate the probability of positive for each cat

The seroprevalence was estimated directly from observed corrected OD (ODc)-values using a binary mixture model (Opsteegh et al., 2010b) with the following modification: usually both OD and $\log_{10}$-transformed OD-values for positive sera show a normal distribution (Jacobson, 1998; Thrusfield, 2005). However, in this case OD-values for positive cats tended to be very high and outside of the linear relation between OD-value and antibody concentration. To account for this negative skew, a mirrored gamma distribution (having a prescribed maximum instead of a minimum of zero), rather than a normal distribution, was fitted for the positive component. The maximum for the mirrored gamma distribution was set at a log ODc of 0.7, which corresponds to the maximum OD-value measurable by the ELISA reader (5.0). Thus, a binary mixture model, in which a mirrored gamma distribution described the positive and a normal distribution the negative component, was fitted to the frequency distribution of log-transformed ODc-values. Subsequently, seroprevalence, cut-off value, and accompanying test sensitivity and specificity were estimated as described previously (Opsteegh et al., 2010b). The cut-off was used to classify all cats as positive or negative based on their log ODc-value.

In addition to scoring the animals positive and negative based on the cut-off value, the probability of being positive was calculated for each animal using:

$$P(pos|X = x) = \frac{cf_2(x)}{cf_2(x) + (1 - c)f_1(x)}$$

With X the random variable indicating the log ODc of an individual cat, $f_1(x)$ the probability density function for the negative component (in this case N(log ODc;−0.574;0.248)), $f_2(x)$ the probability density function for the positive component (in this case $Gamma$(−log ODc+0.7;20.336;0.023)), and c the mixing parameter, i.e.

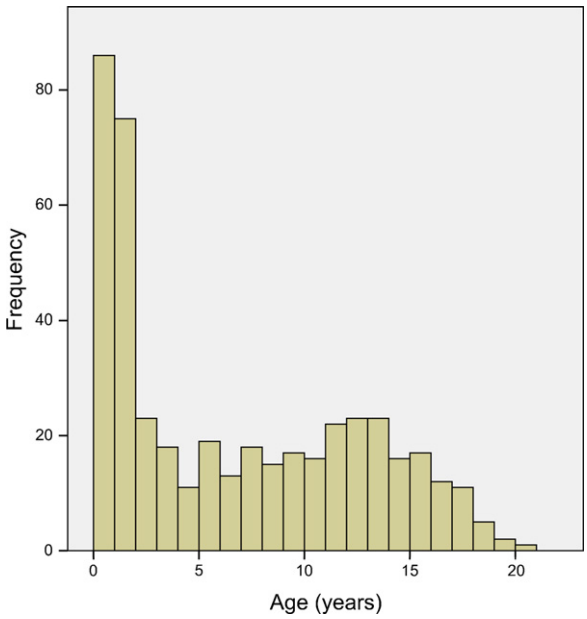

Fig. 2. Frequency distribution of age (in years) for 443 cats.

the prevalence (in this case 0.182) estimated from the binary mixture model. This is a more informative result than a simple negative or positive based on a cut-off value, because it takes into account the test characteristics.

2.4.2. Seroprevalence by age

With the parameters for the two distributions (except prevalence c) as obtained above fixed, the binary mixture model was fitted to each group of 50–100 cats categorized by age (as described previously (Opsteegh et al., 2011)). That way, for each age group, a direct estimate of the true seroprevalence c_i per age category i was obtained. These seroprevalences were subsequently plotted against the mean ages (a_i) for the groups.

2.4.3. Logistic regression analysis to identify predictors of seropositivity

For most variables the original answers from the questionnaires were categorized or combined into fewer categories because seroprevalences were similar or numbers per category were low. Age was divided into three categories consisting of equal numbers of cats (≤1.5 years, 1.5–10 years, >10 years). Outdoor access was aggregated into two categories with *no* including indoor cats as well as cats with restricted outdoor access (e.g. balcony, area without contact with other cats) and *yes* only those with free outdoor access. Vaccination status categories were limited to *yes* (including *annually* and *once*) and *no*. The use of the litter tray was re-categorized by combining *seldom* and *never* into *no* and *always* and *often* into *yes*. The number of cats around the house was re-categorized from *none*, *one or two*, *three to five*, and *more than five* into *no* (none) and *yes* (one or more). Although owners that fed their cat raw meat were asked to answer whether this was done a *few times a year* (22 cats), *monthly* (9 cats) or *weekly* (5 cats) these categories were combined into *yes*. The two categories for hunting are *no* and *yes* with *yes* including only cats that bring home prey or have been observed eating prey animals. Residential environment was divided into *urban* and *not urban* (including *rural*, *nature*, *industrial area* and *other*). Missing values in the analysis were coded with an indicator variable.

To identify predictors of seropositivity for *T. gondii* infection in cats, first bivariable logistic regression analyses were performed to test which of the variables were

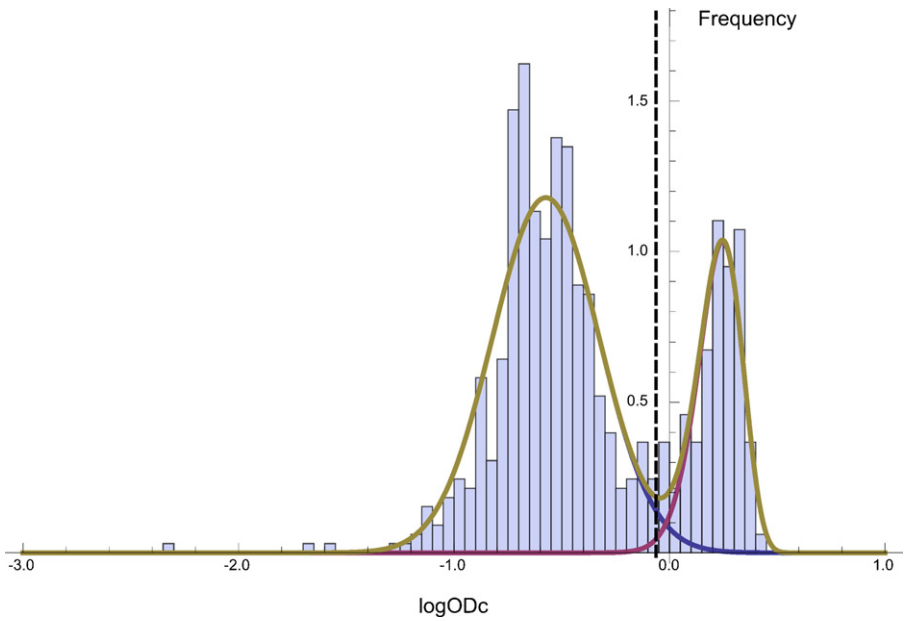

Fig. 3. Frequency distribution of $\log_{10}$-transformed ODc values for 653 cats in *Toxoplasma gondii* ELISA (bars), fitted mixure of normal (left) and gamma (right) distribution (lines), and cut-off value (log ODc = −0.058) (vertical dashed line).

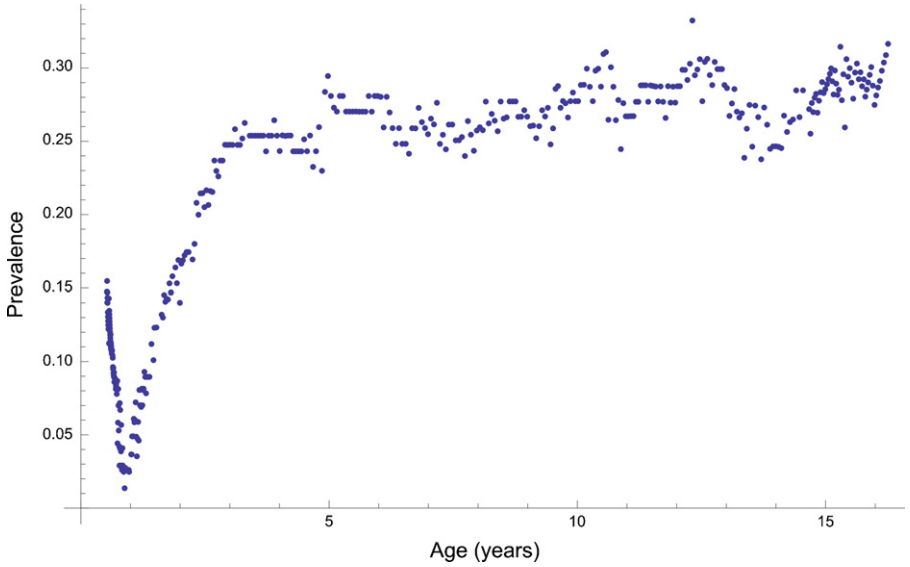

Fig. 4. *Toxoplasma gondii* seroprevalence by age group in cats. Seroprevalence was estimated by fitting the binary mixture model to age groups including 50–100 cats and plotted at the mean age of the group in years.

age-adjusted predictors of seropositivity. Age was always included because with a persistent infection such as *T. gondii*, the prevalence of antibodies is known to accumulate with age. Next, all variables which reached a significance level of $P \leq 0.20$ (by likelihood ratio test) in bivariable analysis were included in a multivariable logistic regression model (full model). The model was reduced by backwards elimination of non-significant variables ($P > 0.05$ based on likelihood ratio test). Model fit was examined using the Hosmer and Lemeshow goodness of fit test. All logistic regression analyses were performed in SPSS 18.0 (PASW Statistics, Chicago, IL, USA).

2.4.4. Linear regression on probability of positivity to determine population attributable fractions

Seroprevalence was additionally checked for differences between groups using linear regression on the probabilities of being positive $P(pos|X = x)$. Although these probabilities are continuous data, they are limited between 0 and 1, and often close to 0 or 1. This leads to violation of the assumption of normally distributed residuals (Dohoo et al., 2003). In this case, it cannot be solved by data transformation, because only linear regression on untransformed data provides estimates of risk differences. Fortunately, the effects of this violation may be limited, as the average probability of being positive for cats grouped by exposure to the predictors might still be estimated correctly (as has been shown for binary dependent variables in a simulation experiment by Hellevik, 2009). This was checked by Hosmer and Lemeshow test, thus comparing the mean observed $P(pos|X = x)$, with the mean predicted $P(pos|X = x)$ per decile group of cats based on the predicted $P(pos|X = x)$. As mentioned, the coefficients in the linear regression model for the probability of being positive represent prevalence differences (RD) between cats exposed and not exposed to a certain risk factor. By multiplication of these coefficients with the prevalence of exposure ($p(E+)$) the population attributable risk for each factor is calculated ($PAR = RD * p(E+)$) (Dohoo et al., 2003). Next the population attributable fraction is calculated by dividing the PAR by the prevalence of disease ($AF_p = PAR/p(D+)$) to get an indication of the proportion of disease in the population that would be avoided if the exposure was removed from the population (Dohoo et al., 2003). All factors included in the final logistic regression model were included in the linear regression model. Risk differences, population attributable risks, and population attributable fractions were calculated for all (borderline) statistically significant factors in the model except age because this is a confounder and not a causal factor.

3. Results

3.1. Descriptive statistics

Of the 450 cats tested, 91 (20.2%, 95% CI: 16.5–23.9%) tested positive for *T. gondii* antibodies. The apparent seroprevalences did not differ significantly for the newly collected samples (68/341) and those from the vaccination status study (23/109) (Pearson's $\chi^2(df = 1)$, $P = 0.79$). For 443 cats (98.4%) age was reported and it varied between 0.25 and 20.0 years. Many young cats were sampled (Fig. 2).

3.2. Seroprevalence and cut-off value by binary mixture model

The frequency distribution of the 653 measured log ODc-values showed two components (Fig. 3). Using the parameters of the distributions fitted to all cats including those from Romania (N(log ODc; −0.574;0.248) and $Gamma$(−log ODc+0.7;20.336;0.023)), the seroprevalence for the Dutch cats ($n = 450$) was estimated at 18.2% (95% CI: 16.6–20.0% based on 500 bootstrap samples). The ROC-curve analysis showed that the test had a good

Table 1
Response rate per variable, percentage of cat serum samples positive for antibodies against *Toxoplasma gondii* (Prevalence) by variable category, and age-adjusted odds-ratios (OR) with 95% confidence interval and *P*-values (based on likelihood ratio test) for those variables in bivariable logistic regression analysis.

Variable	*N*	Response	Prevalence (95 CI%)	OR (95% CI)	*P*-value
Sex					
Male	236		24.6 (19.1–30.1)	Reference	0.129
Female	201		15.9 (10.9–21.0)	0.63 (0.38–1.04)	
Missing	13	97.2%	7.7 (0–22.2)	0.36 (0.04–3.02)	
Former stray					
No	261		18.0 (13.3–22.7)	Reference	0.043
Yes	44		36.4 (22.1–50.6)	2.58 (1.24–5.37)	
Missing	145	89.2%[a]	19.3 (12.9–25.7)	1.05 (0.61–1.81)	
Dog in the household					
No	334		17.7 (13.6–21.8)	Reference	0.059
Yes	109		28.4 (20.0–36.9)	1.92 (1.13–3.26)	
Missing	7	98.5%	14.3 (0–40.2)	1.20 (0.12–12.16)	
More cats in the household					
No	152		26.3 (19.3–33.3)	Reference	0.057
1 or 2	222		19.4 (14.2–24.6)	0.74 (0.44–1.25)	
3 or more	62		9.7 (2.3–17.0)	0.30 (0.12–0.77)	
Missing	14	96.9%	14.3 (0–32.6)	0.86 (0.16–4.53)	
Raw meat					
No	297		17.2 (12.9–21.5)	Reference	0.046
Yes	36		38.9 (23.0–54.8)	2.65 (1.22–5.73)	
Missing	117	97.4%[a]	22.2 (14.7–29.7)	1.36 (0.78–2.38)	
Cats around the house					
No	30		10.0 (0.0–20.7)	Reference	0.272
Yes	306		21.2 (16.7–25.8)	2.54 (0.73–8.86)	
Missing	114	98.3%[a]	20.2 (12.8–27.6)	2.43 (0.66–8.98)	
Residential environment					
Urban	284		18.0 (13.5–22.4)	Reference	0.042
Not urban	51		31.4 (18.6–44.1)	2.55 (1.24–5.21)	
Missing	115	98.0%[a]	20.9 (13.5–28.3)	1.28 (0.73–2.27)	
Outdoor access					
No	176		9.7 (5.3–14.0)	Reference	<0.0005
Yes	188		28.7 (22.3–35.2)	3.47 (1.89–6.39)	
Missing	86	80.9%	23.3 (14.4–32.2)	2.93 (1.40–6.14)	
Hours outside per day					
Not outside	104		9.6 (3.9–15.3)	Reference	0.007
<1 h	69		15.9 (7.3–24.6)	1.41 (0.54–3.68)	
1–5 h	127		22.0 (14.8–29.3)	2.17 (0.98–4.84)	
>5 h	64		34.4 (22.7–46.0)	4.29 (1.81–10.16)	
Missing	86	80.9%	23.3 (14.4–32.2)	2.66 (1.13–6.30)	
Hunting					
No	196		11.2 (6.8–15.6)	Reference	<0.0005
Yes	104		36.5 (27.3–45.8)	4.49 (2.39–8.42)	
Missing	150	87.8%[a]	20.7 (14.2–27.2)	2.07 (1.12–3.83)	
Neutered					
No	86		10.5 (4.0–16.9)	Reference	0.935
Yes	337		23.4 (18.9–28.0)	1.10 (0.48–2.52)	
Missing	27	94.0%	11.1 (0–22.9)	0.89 (0.20–3.92)	
Vaccinated					
No	70		25.7 (15.5–36.0)	Reference	0.208
Yes	344		19.5 (15.3–23.7)	0.56 (0.30–1.07)	
Missing	36	92.0%	16.7 (4.5–28.9)	0.50 (0.17–1.50)	
Contact with young cats or kittens					
No	194		16.5 (11.3–21.7)	Reference	0.176
Yes	56		23.2 (12.2–34.3)	1.62 (0.76–3.47)	
Missing	200	73.1%[a]	23.0 (17.2–28.8)	1.59 (0.94–2.68)	
Use of litter tray					
No	20		30.0 (9.9–50.1)	Reference	0.040
Yes	280		15.7 (11.5–20.0)	0.54 (0.19–1.53)	
Missing	150	87.7%[a]	27.3 (20.2–34.4)	1.01 (0.35–2.93)	

[a] Exposure to these factors was not recorded in the vaccination status survey, therefore these variables are missing for all 108 cats from this survey and response rates were calculated for 342 rather than 450 cats.

discriminatory power with an area under the curve (AUC) of 0.998. The optimum cut-off was estimated at a log ODc-value of −0.058, with sensitivity (*Se*) at 99.0% and specificity (*Sp*) at 98.1%.

3.3. Seroprevalence by age

The seroprevalence by mean age per age group showed a decrease from 6 months up to 1 year (Fig. 4). After the

Table 2
Odds-ratios (OR) with 95% confidence interval and *P*-values based on likelihood ratio test for variables associated with *Toxoplasma gondii* seropositivity in cats in multivariable logistic regression analysis.

Variable	*N*	OR (95% CI)	*P*-value
Age (years)			
<1.5	158	Reference	<0.0005
1.5–10	152	5.20 (2.35–11.51)	
>10	133	7.49 (3.37–16.66)	
Former stray			
No	261	Reference	0.012
Yes	44	3.26 (1.46–7.30)	
Dog in the household			
No	334	Reference	0.043
Yes	109	2.09 (1.18–3.72)	
Raw meat			
No	297	Reference	0.056
Yes	36	2.67 (1.13–6.25)	
Hunting			
No	196	Reference	<0.0005
Yes	104	4.13 (2.14–7.97)	

decline during the first year of age, the seroprevalence rose up to 25% around 4 years and ranged between approximately 20 and 30% in cats over 4 years old.

3.4. Predictors of seropositivity identified by logistic regression analysis

The bivariable analyses showed significant differences ($P < 0.05$, based on likelihood ratio test) in seroprevalence for former stray cats, feeding of raw meat, residential environment, outdoor access, hours outside per day, hunting, and the use of a litter tray; and additional differences at the $P < 0.20$ level for sex, presence of a dog in the household, more cats in the household, and contact with young cats/kittens (Table 1). The final model for the multivariable logistic regression analysis (Hosmer and Lemeshow goodness of fit test $P = 0.674$) is presented in Table 2. Age, former stray cat, presence of a dog in the household, and hunting behaviour remained significant ($P < 0.05$) predictors of seropositivity (Table 2). Feeding of raw meat was borderline significant ($P = 0.056$) and was retained because it is well known risk factor that can act as a confounder.

3.5. Population attributable fractions estimated using linear regression

The Hosmer and Lemeshow test did not indicate a lack of fit ($P = 0.173$) for the linear regression model on $P(pos|X = x)$. The model showed the highest risk differences for feeding of raw meat (0.150) and hunting (0.202). Taking the prevalence of exposure into account, it was shown that the largest fraction of *T. gondii* infections in the whole population (AF_p) could be attributed to hunting (35%, Table 3).

4. Discussion

To determine the seroprevalence of *T. gondii* in cats in The Netherlands and investigate the risk factors for infection, we used an in-house ELISA to test cat serum samples and determined a cut-off value with corresponding test characteristics by binary mixture analysis.

The test characteristics of the ELISA were estimated at 99.0% sensitivity and 98.1% specificity using the binary mixture model. These estimates are higher than those previously determined for the same ELISA by Bayesian analysis (sensitivity 88.3% and specificity 95.8%) (Györke et al., 2011). It is unclear what is causing the discrepancy. In our opinion, both methods provide a more realistic estimation of test characteristics than when estimated based on reference sera, because it is likely that the separation between OD-values for positive and negative sera is greater for reference than for field sera. However, both evaluation methods still have disadvantages: in Bayesian analysis the agreement between tests is used to evaluate the tests, and there is a risk that a good assay is compared to several assays that are misclassifying in the same direction. This will lead to an overestimation of the specificity of the assays for comparison and an underestimation of the sensitivity of the assay of interest, if the assays for comparison have a cut-off value too low for use with field sera. In the binary mixture analysis, test characteristics are based only on the results with the assay of interest. Therefore a low analytic sensitivity (i.e. a low concentration of antibodies is not detected) or cross-reactivity might not be identified. If the test characteristics determined by Bayesian analysis are correct, the overall prevalence estimated by binary mixture analysis (18.2%) is a slight underestimation of the true prevalence (the apparent prevalence of 91 test-positives out of 450 cats would result in an estimated true prevalence of 19.0% using the Rogan–Gladen estimator).

Using the binary mixture model the seroprevalence of *T. gondii* was estimated at 18.2% (95% CI: 16.6–20.0%). This prevalence is significantly lower than the prevalence of 49% (95% CI: 41.3–57.3%) in 1993 (personal communication, Prof. Dr. F. van Knapen, Utrecht University), but because no details are available for the population sampled in the nineties (this population may for example have been older on average) and the use of a different test, this difference does not necessarily represent an actual decrease in prevalence. The estimated seroprevalence is comparable to seroprevalences in other European countries, for example 18.6% in an urban cat population in France (Afonso et al.,

Table 3
Age-adjusted risk difference (*RD*), prevalence of exposure ($p(E+)$), population attributable risk (PAR) and population attributable fraction (AF_p) for identified risk factors for *Toxoplasma gondii* infection in Dutch domestic cats.

Variable	*RD* (95% CI)	$p(E+)$	*PAR*	AF_p
Former stray	0.144 (0.032; 0.255)	0.144	0.021	10%
Dog in the household	0.086 (0.011; 0.162)	0.246	0.021	10%
Raw meat	0.150 (0.029; 0.270)	0.108	0.016	8%
Hunting	0.202 (0.118; 0.286)	0.347	0.070	35%

2006) and 25.0% in house cats in Belgium (De Craeye et al., 2008).

The seroprevalence increased with age for cats from 1 year up to almost 4 years of age. After this rise, the seroprevalence up to 12 years old ranged between 20 and 30%. In correspondence with our observations in wild boar (Opsteegh et al., 2011), this relatively stable seroprevalence could be explained by a decline of IgG titre to a level below cut-off (i.e. reversion to seronegative). However, this type of pattern can also be expected if 60–70% of the cats is either not exposed or resistant to infection. Review of the literature on persistence of antibodies in cats does not clarify much: although IgG titres are known to stay high for months up to 6 years in cats (Dubey, 1995; Dubey and Thulliez, 1989), it has been described that 4 out of 9 cats that had shed oocysts earlier, re-excreted oocysts after infection with tissue cysts six years after primary infection (Dubey, 1995) which may indicate a loss of immunity, even though all nine cats were still seropositive at re-infection (titre $\geq$1:10,000 in modified agglutination test). Whether antibodies decay to a level that would be scored negative in our ELISA needs further investigation.

In addition, we observed a decreasing seroprevalence by age in cats less than one year old. Kittens can develop detectable antibody titres upon infection at a very young age, but can also obtain high IgG antibody titres via colostrum. Maternal antibodies will decrease to non-detectable levels at 8–12 weeks after birth (Omata et al., 1994), and a decrease in seroprevalence for very young cats has been observed previously (Afonso et al., 2006). Although the youngest cats in our study were over 3 months old, part of the observed decrease in prevalence might still be due to declining maternal antibody titres if natural variation in decline is larger than was observed experimentally (Omata et al., 1994). However, because misclassification of age for these young cats is likely (owners were asked to report age in years and, whereas many reported age in months, others may have rounded the age to one year) the observed decrease may also be an artefact.

Next, risk factors for *T. gondii* infection were identified by logistic regression analysis. Before interpreting the identified risk factors, it is important to realize that there is potential participation bias. For the current study clinics were recruited through a call in the journal of the Royal Dutch Veterinary Association without any further inclusion criteria, and the owners visited those veterinarians with their cat and volunteered in the study. In the vaccination status survey, cats were selected based on their vaccination history. Both selection methods may have resulted in a sampled population that is not representative of the total Dutch cat population. This can have various effects. Firstly, although sampling seems to have followed population density (which is likely correlated to cat density) reasonably well, not all areas in The Netherlands were sampled (Fig. 1). In The Netherlands, regional differences in *T. gondii* seroprevalence have been shown for humans (Hofhuis et al., 2011) and sheep (Opsteegh et al., 2010b). If these differences also exist for cats sampling may have influenced the overall estimation of the seroprevalence. Secondly, the cats are relatively young (mean age 6.5 years) compared to the age distribution reported by the Council for Animal Affairs (Raad voor Dierenaangelegenheden, 2006). From the seroprevalence by age curve, it can be understood that an overrepresentation of young cats means that the overall prevalence for the Dutch cat population is probably somewhat higher than 18.2%. Selection based on vaccination history is unlikely to affect the seroprevalence directly because it was found uncorrelated to seroprevalence. Lastly, sampling at veterinary clinics may, for example, have limited the number of farm cats kept for rodent control included in our study, therefore the prevalence of outdoor access and hunting behaviour are likely underestimated. The effects on the frequency of other characteristics or possible predictors (e.g. feeding of raw meat) are less obvious, but can be present. In general, this means that the calculated population attributable fractions (which are calculated based on the observed prevalences of exposure) apply to the sampled population and would not necessarily be the same for the total population.

The population attributable fractions are based on risk differences estimated by linear regression on the probability of being positive for each cat. The residuals from this model are not normally distributed (data not shown) but the average probability of being positive for decile groups of cats was predicted accurately. An accurate prediction for groups of cats is more important, because the model is used to estimate the population effect of exposure to risk factors rather than to predict the probability for individual cats. In our opinion, this method is useful because the coefficients from this linear model provide direct estimates of risk differences (which are easier to interpret than odds-ratios, and can be used directly to calculate population attributable fractions) that have been controlled for confounding factors (multivariable analysis) while taking test sensitivity and specificity into account.

The risk factors identified were: age, former stray cat, presence of a dog in the household, hunting behaviour and (at $P = 0.056$) feeding of raw meat. Feeding of raw meat was borderline statistically significant but this is probably due to the small number of cat owners that feed their cat raw meat present in the study. Eating raw meat was previously identified as a risk factor for *T. gondii* seropositivity in cats by others (Lopes et al., 2008). The small fraction of owners feeding their cat raw meat also means that a total ban would reduce the seroprevalence by 8% only. Nevertheless, it would still be useful to advise cat owners against feeding of raw meat as this is a simple measure that can be expected to be effective because, from the life cycle of *T. gondii*, it can be understood that this risk factor is directly causal.

Hunting is another risk factor with a direct mechanism: eating infected prey animals will cause infection. It is also one of the strongest risk factors identified, and because many cats hunt (34.7% of the cats in our study), prevention could reduce the seroprevalence by 35%. However, preventing cats from hunting may be more complicated than banning feeding of raw meat. As 93% of the cats showing hunting behaviour had free outdoor access, keeping the cat inside will be very effective. However, keeping the cat indoors may not be preferred by the owner. Because outdoor access itself is not a significant predictor in multivariable analysis, measures aimed at preventing hunting without completely limiting outdoor access may be just as

effective in reducing the risk of *T. gondii* infection. Possible measures to reduce hunting behaviour include equipping the cat with a bell (Ruxton et al., 2002; Woods et al., 2003) or a bib (Calver et al., 2007), keeping the cat indoors at night (Robertson, 1998; Woods et al., 2003) and including raw meat in the diet (Robertson, 1998). Inclusion of raw meat is contradictory: Although it can decrease hunting behaviour, the cat may ingest *T. gondii* tissue cysts with the meat. For that reason, this is not recommended as a measure to reduce hunting behaviour, unless the meat is frozen first. Freezing of meat at −12 °C for two days effectively kills the *T. gondii* tissue cysts that can be present in meat (Kotula et al., 1991).

Besides feeding of raw meat and hunting, being a former stray cat and presence of a dog in the household were identified as independent predictors for *T. gondii* infection. These factors are unlikely to have a direct effect. Probably the effect of formerly being a stray cat is due to high levels of hunting in the past. Because the fraction of former stray cats (14.4%) may not represent the fraction of stray cats in The Netherlands (this fraction is unknown), the population attributable risk for being a former stray cat in this study does not give an indication of potential reduction in seroprevalence that can be achieved by preventing cats from straying. Nevertheless, preventing cats from straying is expected to give a reduction of the overall seroprevalence.

The presence of a dog in the household has also been identified as a risk factor for *T. gondii* infection in humans, and especially children (Etheredge et al., 2004; Frenkel et al., 1995; Sroka et al., 2010). In that case dogs may facilitate human exposure to environmental oocysts by rolling in contaminated cat faeces or soil, or passing oocysts in faeces after eating cat faeces (Frenkel et al., 1995, 2003; Lindsay et al., 1997). Whether dogs fulfill the same role for their feline housemates which, in contrast to humans, are not very susceptible to oocyst-infection (Dubey, 1996) is questionable. Possibly the presence of a dog in the household indicates the presence of an unidentified risk factor. For that reason, it cannot be assumed that removal of dogs from the household will have the effect as estimated by the population attributable fraction.

We were interested in the risk factors for *T. gondii* infection in cats because these risk factors indicate potential targets for measures to reduce infections and thereby oocyst shedding into the environment. However, the amount of oocysts shed into the environment also depends on the cat population size (including stray cats), the fraction of cats defecating outside, and the amount of oocysts shed by an infected cat (Dabritz and Conrad, 2010). Therefore reduction of the cat population (for example by controlling stray cat populations and advocating timely neutering of domestic cats) and increasing the use of the litter tray in combination with proper disposal of the filling (i.e. with household waste and not in organic waste, toilet or compost) could be effective intervention measures too. From the questionnaire, it was clear that almost 90% of the cat owners have a litter tray for their cat, but less than 40% of the cats use it often (data not shown). That the total amount of oocysts shed by an individual cat influences the environmental contamination level is an important consideration in vaccine development: a cat vaccine that does not induce sterile immunity but reduces the number of oocysts shed could still be a useful part of a strategy to reduce the exposure of humans and animals to *T. gondii*. 85% of the owners participating in this study were willing to pay some amount for *T. gondii* vaccination of their cat (data not shown).

In conclusion, the overall prevalence in domestic cats in The Netherlands was estimated at 18.2% (95% CI: 16.6–20.0%) and hunting behaviour and feeding of raw meat were identified as risk factors that could be potential targets for intervention measures in order to prevent future human infections.

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