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Spatial distribution of soil contamination by *Toxoplasma gondii* in relation to cat defecation behaviour in an urban area

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Abstract

In urban areas, there may be a high local risk of zoonosis due to high densities of stray cat populations. In this study, soil contamination by oocysts of *Toxoplasma gondii* was searched for, and its spatial distribution was analysed in relation to defecation behaviour of cats living in a high-density population present in one area of Lyon (France). Sixteen defecation sites were first identified. Cats were then repeatedly fed with marked food and the marked faeces were searched for in the defecation sites. Of 260 markers, 72 were recovered from 24 different cats. Defecation sites were frequented by up to 15 individuals. Soil samples were also examined in order to detect the presence of *T. gondii* using real-time PCR. The entire study area was then sampled according to cat density and vegetation cover type. Only three of 55 samples were positive and all came from defecation sites. In a second series of observations, 16 defecation sites were sampled. Eight of 62 samples tested positive, originating in five defecation sites. Laboratory experiments using experimental seeding of soil showed that the inoculated dose that can be detected in 50% of assays equals 100–1000 oocysts/g, depending on the strain. This study shows that high concentrations of oocysts can be detected in soil samples using molecular methods and suggests that spatial distribution of contamination areas is highly heterogeneous. Positive samples were only found in some of the defecation sites, signifying that at-risk points for human and animal infection may be very localised.

Keywords: *Toxoplasma gondii*; Soil; Oocysts; PCR; Cat defecation behaviour; Spatial heterogeneity

1. Introduction

Populations of stray cats (*Felis catus*) can reach high local densities in urban areas by making use of available resources and shelters (Macdonald, 1983; Liberg and Sandell, 1988). Areas such as parks and gardens are particularly likely to be settled by large social groups of cats (Piccoli, 1995). Because these same areas are also used for recreational activities, the proximity between humans and cats entails a significant risk of transmission of zoonoses, especially for faeces-transmitted diseases (Kumar and Smith, 2000).

Toxoplasma gondii is a protozoan parasite that infects humans, many mammals and birds. In humans, toxoplasmosis can cause abortion or severe clinical signs in foetuses, neonates and immunocompromised individuals (Dubey

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and Beattie, 1988). Felids, and particularly domestic cats (*F. catus*), are the definitive hosts of *T. gondii*. After primary infection, hosts excrete millions of environmentally resistant oocysts that may infect intermediate hosts such as small mammals and birds. The life-cycle of the parasite is completed when intermediate hosts are preyed upon by the definitive host (Dubey and Beattie, 1988; Tenter et al., 2000). Contamination of the environment by oocysts of *T. gondii* is responsible for outbreaks of acute toxoplasmosis in humans (Stagno et al., 1980; Coutinho et al., 1982; Bowie et al., 1997), and is suspected to be associated with high seroprevalence in some communities (Bahia-Oliveira et al., 2003).

Local risk of soil contamination by oocysts should depend on the initial concentration of infected cat faeces and the survival and diffusion of oocysts in the soil. The defecation behaviour of stray cats is generally structured: faeces are usually buried in the main living area but can also be exposed as scent marks around the border of a territory (Turner and Bateson, 2000). Cats often use the same place to defecate (Corbett, L. K., 1979. Feeding ecology and social organization of wildcats (*Felis silvestris*) and domestic cats (*F. catus*) in Scotland. Ph. D. thesis, University of Aberdeen, Scotland) and a single location may be used by several cats when cat density is high (Uga et al., 1996). Oocysts shed by newly infected cats can survive and remain infectious in moist soil or sand for up to 18 months (Dumètre and Dardé, 2003). Detection of oocysts in soil samples has previously been described (Ruiz et al., 1973; Coutinho et al., 1982; Dubey et al., 1995; Frenkel et al., 1995) but never related to the space used by the cats. Isolation of oocysts from naturally infected soil samples has been performed via inoculation in mice and positive results range from 10/924 samples from children's outdoor play areas in Panama City (Frenkel et al., 1995) to 4/15 samples from a yard frequented by cats (Ruiz et al., 1973). Although transport hosts have been identified (Ruiz and Frenkel, 1980), the importance of oocyst diffusion in the soil to toxoplasmosis infection is unknown.

The aim of this study was to estimate the level of contamination of *T. gondii* oocysts in the grounds of the Croix-Rousse Hospital in Lyon (France) and to relate oocyst presence to cat defecation behaviour. At this site, a population of domestic cats lives at high-density (Devillard et al., 2003) and infection by *T. gondii* has been serologically measured at 18.6% (Afonso et al., 2006). This paper first presents results from laboratory experiments elaborated to estimate the sensitivity of a new molecular method for oocyst detection in soil (Romand S., Fromont, E., Pontier, D., Thulliez, P. 2003. Détection par PCR en temps réel de *Toxoplasma gondii* dans les prélèvements de sol. Congrès de la Société Française de Parasitologie, 16–18 December 2003, AFSSA, Maisons-Alfort). Then, results from a description of both the presence of oocysts in soil samples and cat defecation behaviour in the Croix-Rousse site are presented. We predicted that soil contamination should be higher in defecation sites than in the rest of the study area and should be highest in sites most frequently used by cats.

2. Materials and methods

2.1. Detection of oocysts

Detection of oocysts was carried out according to a molecular method modified from Dubey et al. (1972; Romand S., Fromont, E., Pontier, D., Thulliez, P. 2003. Détection par PCR en temps réel de *Toxoplasma gondii* dans les prélèvements de sol. Congrès de la Société Française de Parasitologie, 16–18 December 2003, AFSSA, Maisons-Alfort). First, 10 g of soil were mixed with 5 mL of 2% sulphuric acid. After 1 h, mixtures were filtered through four layers of gauze and centrifuged at 500*g* for 5 min. The sediment was suspended in a sucrose solution (specific density of 1.15). After centrifugation (400*g* for 5 min), the supernatant (1.5 mL) was collected and submitted to three cycles of freezing/thawing for at least 4 h at +20 °C and −80 °C. DNA extraction was made with the High Pure PCR Template Preparation kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions, including a treatment with proteinase K. DNA was subjected to real-time quantitative PCR targeting the 529 bp repeated element on a LightCycler instrument (Roche Diagnostics), according to the method previously described (Reischl et al., 2003) and using 50 cycles.

Laboratory experiments were performed to estimate the sensitivity of *T. gondii* oocyst detection in soil samples. Three strains of sporulated oocysts that had been obtained from experimentally infected cats were used. Five-year-old oocysts of strain GUI (RMS/99/GUI; type II) were obtained from the Laboratoire de Parasitologie – Mycologie, EA 3800 at Reims (France), and produced as described by Villena et al. (2004). Oocysts of strain VEG (type III, 13-months-old) and strain PIG3 (type II, 5-months-old) were provided by J.P. Dubey of Beltsville, Maryland (Dubey et al., 1996). All oocysts were kept in 2% H_2SO_4 and stored at 4 °C until used in inoculation experiments.

First, 1-mL samples of distilled water were inoculated with 10,000, 1000, 100, or 10 oocysts. Each inoculation was repeated four times (4 concentrations × 3 strains × 4 repetitions = 48 inoculations). Next, oocysts were inoculated in soil samples. The soil came from an area that was chosen to be representative of defecation sites in the study area but was negative for oocysts during a field study (site M; see below), it was not tested again at the time of the experiments. We inoculated 10 g of soil with 10,000, 1000, 100 or 10 oocysts. For both water and soil experiments, the proportion of positive samples for each strain was compared using Cochran–Mantel–Haenszel tests (Agresti, 1990). We calculated $D_{0.5}$ as the inoculation dose that was detected in 50% of assays.

2.2. Study area and cat population

Cats live in gardens and underground areas of the Croix–Rousse Hospital in Lyon (France). Underground areas are basement levels under the hospital buildings. They are used for technical materials and have mud floors. A large group of cats lives at high-density (9.7 ± 0.5 cats/ha; Devillard et al., 2003) in the 7.2-ha site, including 3 ha not covered by roads or buildings. The population ranged between 37 and 94 cats, depending on the time of year (Devillard et al., 2003), with four to eight feeding points maintained throughout the grounds (Fig. 1). Feeding points consisted of canteen waste and canned food that was regularly deposited by volunteers from the hospital.

The cat population had been monitored visually between 1993 and 2004. We also recorded information and took blood samples from individuals during trapping sessions twice a year (Say et al., 1999; Courchamp et al., 2000; Devillard et al., 2003; Afonso et al., 2006). Cats were identified by coloured collars and coat colour combinations. In a previous study (Afonso et al., 2006), antibodies for *T. gondii* were detected in 56/301 trapped cats (18.6%), so the number of susceptible cats in the population was estimated between 30 and 76 ($(1 - 0.186) \times 37$ and $(1 - 0.186) \times 94$). Incidence was also estimated as 17 for 100 cat-years (cat-year is defined by the cumulative follow-up period for all the cats), so 5 to 13 cats on average could have been infected in any one year and excreted oocysts in the study area.

The area was first searched systematically to locate sites used by cats for defecation, which were systematically cleared before the experiment. Pre-defined walking surveys were carried out in February 2005 to identify all cats that were present. Next, individual cats were fed with bait containing eight small coloured plastic markers on a daily basis, according to Ikeda et al. (1979). Faeces were then collected daily from each defecation site during February 2005 (1 month), March and April 2005 (6 weeks) and August 2005 (1 month). Plastic strips were removed from faeces by dilution in water and filtering the mixture. The location of faecal deposits was thus recorded, as well as the identity of cats producing them. The experimental protocol was approved by the Director of Veterinary Services, Rhone Department, France.

2.3. Soil contamination

The first soil sampling session assessed contamination across the entire study site. This was done in April 2004 by sampling two square metres of quadrats in a regular grid pattern. Each quadrat (87 m^2) was described for the presence of cats (number of cats observed in the quadrat during the previous year) and vegetation cover. Three categories of cat density were defined: (i) more than five cats observed in the quadrat, (ii) one to five cats observed and (iii) no cat observed. Four categories of vegetation cover were also defined: (i) grass, (ii) bushes, shrubs and trees, (iii) flowerbeds and (iv) gravel and sand. Five quadrats were randomly sampled for each combination of cat density and cover type.

For each sample, 20 sub-samples were taken from all over the surface of the sampling unit (to a depth of 2 cm), in order to be representative of the whole quadrat. From each quadrat, 200–300 g of soil were thus collected and kept in plastic boxes at ambient temperature (22 °C). All samples were collected in the field on the same day and laboratory analysis was carried out over the next few days.

Because few samples were positive for *T. gondii* during the first sampling (see Section 3), a second sampling session focused on the identified defecation sites. In November 2005, four randomly selected square metres were thus sampled from each defecation site. For the two areas less than 4 m^2 in size, the number of squares sampled was equal to the sampled surface area (3 m^2). Soil samples were collected and kept as described above.

Each soil sample was thoroughly mixed in the laboratory and then we removed 100 g of soil for detection of DNA of *T. gondii* (see above), adjusting the detection method to the soil quantity.

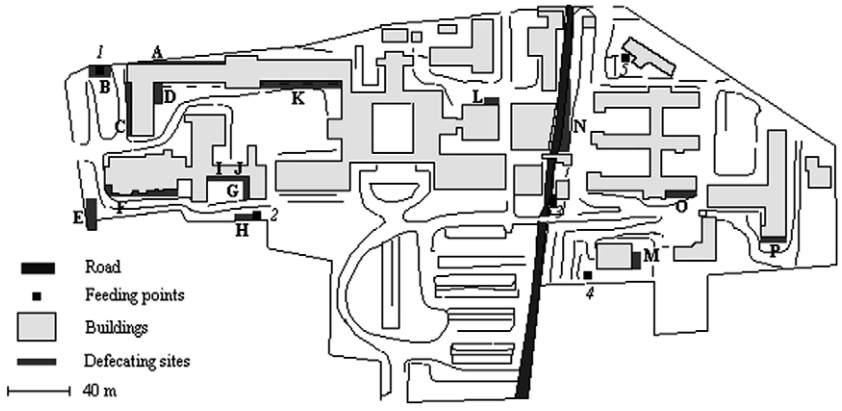

Fig. 1. Location of feeding (1–5) and defecation (A–P) sites recorded at the Croix-Rousse Hospital in November 2005. Defecation sites I and J were located underground.

3. Results

3.1. Sensitivity of the detection method

Oocysts of the oldest strain (GUI) were only detected in water inoculated with 10,000 oocysts, whilst more recently produced oocysts were detected at lower concentrations (Table 1). $D_{0.5}$ values were 10,000, 100 and 10,000 oocysts in 1 mL of distilled water for strains GUI, VEG and PIG3, respectively.

Results of soil seedings are shown in Table 1. Although *T. gondii* DNA was not detected in the soil used for experiments, we cannot preclude the hypothesis that a small quantity of DNA was present in this soil and that it explained part of the variability in further tests. Results obtained for the three strains in soil samples tended to be different. Results for GUI and VEG strains did not differ significantly (Mantel–Haenszel test, $P = 0.580$), whilst results differed significantly between GUI and PIG3 strains, or VEG and PIG3 strains (Mantel–Haenszel test, $P = 0.025$ and $P = 0.014$, respectively). Oocysts that were produced earliest (strain GUI) gave inconsistent results and the $D_{0.5}$ was not estimated in this case. *T. gondii* DNA was detected repeatedly when soil samples were inoculated with 10,000 oocysts for strains VEG and PIG3 (Table 1). $D_{0.5}$ value equalled 10,000 oocysts per 10 g of soil for strains VEG (shed 13 months before the experiments) and 1000 oocysts per 10 g of soil for PIG3 (shed 5 months before the experiments).

3.2. Defecation behaviour of cats

During preliminary observations, 16 defecation sites were identified in the study area. Fourteen sites were located in areas with soil and two sites were underground (Fig. 1). Total surface area of defecation sites was 189 m^2 (between 3 and 60 m^2 each), representing less than 1% of the total surface area of the hospital grounds (Table 2).

A total of 51 cats were observed, of which 45 individuals (88.2%) could be fed with marked bait. Marked cats were representative of the population in terms of sex and age. Of the 260 markers used in baits, 72 were found in the faeces of 24 different cats (Table 2). Cats that were identified as producing faeces used one to four different defecation sites. The 16 defecation sites were not used equally. No markers were found in five sites (B, D, E, K and L), nine sites were used by five cats or less (A, C, F, I, J, M, N, O and P), whilst sites G and H were frequented by 15 and seven cats, respectively (Fig. 1).

3.3. Contamination of soil

A total of 354 quadrats of 87 m^2 were described in the study area, amongst which 55 were sampled. No analysis was performed for the combination of high cat density and flowerbed vegetation because this combination did not occur. *T. gondii* DNA was detected in three samples. The first was located in a lawn where cat density was high, the second was located in a flowerbed where cat density was intermediate, and the third was under a tree where no cat had been observed. Taking into account the frequency of these three types of quadrats across the study area, the estimated prevalence of positive samples in the entire site was 4.8%. All three positive samples were located in defecation sites. *T. gondii* DNA was thus found significantly more often in defecation sites (3/15) than in other parts of the study area (0/40; Fisher's exact test, $P = 0.017$).

Table 2
Characteristics of cat defecation sites and number soil samples positive for *Toxoplasma gondii*

Defecation site	Surface area (m^2)	Number of faecal markers found	Number of different cats found defecating	Positive samples
A	20	1	1	1/4
B	4	0	–	0/4
C	6	1	1	0/4
D	5	0	–	0/4
E	17	0	–	0/4
F	8	9	5	0/4
G	10	24	15	0/4
H	5	9	7	0/4
I[a]	3	6	3	2/3
J[a]	3	2	2	3/3
K	20	0	–	1/4
L	5	0	–	1/4
M	4	4	3	0/4
N	60	1	1	0/4
O	4	6	3	0/4
P	15	9	3	0/4
Total	189	72	24	8/62

[a] Located underground.

Table 1
Number of distilled water (1 mL) or soil (10 g) samples found to be positive for three strains of *Toxoplasma gondii* using PCR, following experimental inoculation

Strain (time since oocyst production)	Number of oocysts seeded (mean cycle threshold for positive results)							
	10,000		1000		100		10	
	Water	Soil	Water	Soil	Water	Soil	Water	Soil
GUI (5 years)	3/4 (33.7)	0/4	0/4	0/4	0/4	0/1	0/4	0/2
VEG (13 months)	4/4 (27.2)	3/4 (41.9)	3/4 (36.1)	1/4 (40.8)	2/4 (37.8)	1/4 (41.6)	0/4	–
PIG3 (5 months)	3/4 (32.1)	4/4 (36.5)	1/4 (37.8)	3/4 (37.5)	0/4	0/4	0/4	–

During the second soil sampling session, DNA of *T. gondii* was detected in 8/62 samples. The positive samples were collected from five different defecation sites, either on the ground surface (3/14 sites) or underground (2/2; Table 2). Underground sites were located in shaded, warm and moist zones and were often infected (5/6 positive results). Contrary to expectations, infected areas were not located in sites used most frequently by cats. The five infected sites were observed to be used by only zero to three cats.

The frequency of positive samples was twice as high in defecation sites (8/62) than across the study area in general (3/55; McNemar chi-squared test, $P < 0.001$). In addition, two of the three areas where DNA was found in 2004 were also positive in 2005 (A and K). The third area was not sampled in 2005 because it was no longer accessible.

4. Discussion

Here, we present the use of a newly described molecular method to estimate soil contamination by *T. gondii*. Laboratory experiments showed that three different concentrations of oocysts were not detected with equal likelihoods. Concentrations of 100 oocysts/mL of distilled water and 100–1000 oocysts/g of soil resulted in positive identification for recently shed oocysts, whilst 5-year-old oocysts were not detected, except at very high concentration in water (10,000 oocysts/mL). The older oocysts had previously been detected at a dose of 100 oocysts in 100 L of deionised water when 2-years-old (Villena et al., 2004). We assume that the decrease in sensitivity of the detection method may have resulted from a breakdown of the oocysts. The sensitivity that was measured is comparable to other techniques proposed to detect oocysts in cat faeces (Salant et al., 2007), however our method has the advantage of being more feasible since there is no bioassay. Because a newly infected cat may excrete millions of oocysts per day (Dubey and Beattie, 1988), local soil concentrations should be at least in the order of magnitude of hundreds/g, thus this method is sensitive enough to detect recent contamination. Improving the sensitivity of the detection method through more efficient DNA extraction would allow us to detect lower levels of concentration due to spatial diffusion. Finally, although oocysts are known to remain infectious for long periods, only a proportion of detected oocysts is likely to be infectious and biological assays are necessary to determine this proportion.

Although the seroprevalence of *T. gondii* was low in the studied cat population, the high-density of cats living in the study area was expected to result in high levels of contamination. In the field, we first found that the overall proportion of soil that was recently contaminated was relatively low (4.8%). This result was related to the low proportion of the study area that was actually used by cats for defecation (<1%). The spatial distribution of soil contamination thus appears to be highly heterogeneous, with a limited role for diffusion of oocysts in the soil.

During feeding experiments, 28% of the markers distributed to cats were subsequently found in faeces, which is a high proportion compared with previous studies (Ishida and Shimizu, 1998). The Croix-Rousse site is surrounded by walls and busy roads and the rates of dispersal to/from the site are low (Devillard et al., 2003), thus the use of defecation areas outside the grounds is probably uncommon and we assume that we identified most defecation sites used by the studied population. A few cats were too shy to be fed with marked food. This may partly explain why no cats using defecation sites B, D, E, K and L were identified.

Defecation sites were the places most often found to be positive for *T. gondii* DNA. The proportion of samples from defecation sites testing positive was relatively high and similar for the two sampling sessions: 3/15 versus 8/62. The five positive defecation sites were located either underground or in the margin of the distribution range of the cats. Contrary to expectations, positive sites were not those most frequented. The two areas that were positive in April 2004 were also contaminated in November 2005, however, suggesting that they were regularly used by newly infected cats. Because toxoplasmosis can induce severe clinical signs in cats, such as diarrhoea or vomiting (Dubey and Beattie, 1988), we cannot preclude the hypothesis that primary infected cats adapt their defecating behaviour to defecate in unusual areas, particularly in areas away from humans (underground) or other cats. Another possible explanation is that the soil showed favourable conditions for the survival of oocysts, such as consistent shade and moisture underground. Oocyst survival is known to be affected by moisture (Frenkel et al., 1975), temperature (Dumètre and Dardé, 2003) and U.V. radiation (Wainwright et al., 2007). Here, we have no information on the physical characteristics of the sampled sites, thus we can only hypothesise that the relationship between cat behaviour and soil contamination is modulated by local conditions that may influence the survival of oocysts.

Beside these high-risk sites are caused by dense cats populations using common defecation sites, other places contaminated at an intermediate level are expected to occur. Such areas were not detected here because of the sensitivity of the detection method, so we have no information on their spatial distribution. Specifically, intermediate levels of contamination are expected to be frequent in rural environments where cat density is intermediate and territoriality is more pronounced (Turner and Bateson, 2000). Human contact with soil is more frequent in rural than urban environments so even low contamination in the landscape may become risky if exposure is high. Our detection method should now be improved to characterise the risk of toxoplasmosis in less-infected areas, particularly the rural environment.

Studying the spatial distribution of soil contamination by *T. gondii* will be useful in understanding the risk of toxoplasmosis in both intermediate hosts and humans. Gardening and consumption of raw vegetables have been demonstrated to be significant risk factors for toxoplasmosis in humans (Bobic et al., 1998; Cook et al., 2000; Kort-

beek et al., 2004). Here, we showed that only a few areas were highly contaminated by *T. gondii* in a dense cat population. No rodent has been observed on the studied site (Afonso et al., 2006), however rodents are often abundant in urban areas, so the defecation sites may play a significant role in the perpetuation of the cycle of *T. gondii*, by creating a risk for rodents and/or cats. These highly contaminated points may be viewed as "hot spots" of risk, where the level of contamination was at least 100 oocysts/g of soil. Contact with these areas is expected to result in a high-risk of infection but because contaminated sites represent a low proportion of the area, only a few humans are likely to be directly exposed. These persons include those feeding the cats, gardeners, maintenance workers in these sites and also dog owners who allow pets to roam in these sites and become indirectly exposed through contact with dogs (Frenkel et al., 1995).

The management of zoonotic risk in urban areas often relies on control of the demography or sanitary status of host populations: for example, reducing fox and/or rodent density, or treating foxes have been proposed as methods to control the urban cycle of *Echinococcus multilocularis* (Deplazes et al., 2004). However, this approach often presents practical difficulties and/or raises ethical concerns. Direct interventions in the environment, either to decrease contamination or to limit contact between high-risk areas and hosts or people, may constitute a valuable alternative when parasites are localised, for example in sandpits contaminated by eggs of *Toxocara* sp. (Uga et al., 1996). In the case of toxoplasmosis, characterising the determinants of environmental contamination opens the possibility of targeted interventions towards the contaminated areas. Such measures would be facilitated if high-risk areas are localised and can be practically detected.

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