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Environmental contamination with *Toxocara* spp. eggs in public parks and playground sandpits of Greater Lisbon, Portugal

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ABSTRACT

Toxocarosis is a zoonotic parasitic disease transmitted from companion animals to humans. Environmental contamination with *Toxocara* eggs is considered to be the main source of human infections. In Portugal, knowledge regarding the current situation, including density, distribution and environmental contamination by *Toxocara* spp., is largely unknown. The present study investigated environmental contamination with *Toxocara* spp. eggs, in soil and faecal samples collected from public parks and playground sandpits in Greater Lisbon, Portugal. A total of 151 soil samples and 135 canine faecal samples were collected from 7 public sandpits and 12 public parks, over a 4 month-period. Soil samples were tested by a modified centrifugation and sedimentation/flotation technique and faecal samples were tested by an adaptation of the Cornell-Wisconsin method. Molecular analysis and sequencing were performed to discriminate *Toxocara* species in the soil. Overall, 85.7% of the sandpits (6/7) and 50.0% of the parks (6/12) were contaminated with *Toxocara* spp. eggs. The molecular analysis of soil samples showed that, 85.5% of the sandpits and 34.4% of the parks were contaminated with *Toxocara cati* eggs. Faecal analysis showed that 12.5% of the sandpits and 3.9% of the parks contained *Toxocara canis* eggs. In total, 53.0% of soil and 5.9% of faecal samples were positive for *Toxocara* spp. Additionally, 56.0% of the eggs recovered from the samples were embryonated after 60 days of incubation, therefore considered viable and infective. The average density was 4.2 eggs per hundred grams of soil. Public parks and playground sandpits in the Lisbon area were found to be heavily contaminated with *T. cati* eggs, representing a serious menace to public health as the studied areas represent common places where people of all ages, particularly children, recreate. This study sounds an alarm bell regarding the necessity to undertake effective measures such as reduction of stray animals, active faecal collection by pet owners, awareness campaigns and control strategies to decrease the high risk to both animal and human health.

Introduction

Toxocarosis is a zoonotic infectious disease associated with parasites, transmitted from companion animals to man [1]. It can be caused either by *Toxocara canis* or *Toxocara cati*, which are both ubiquitous and prolific monoxenous nematodes. These agents are intestinal parasites of dogs and cats that are present in the majority of new-born puppies and kittens, as well as adult dogs and cats. Several species including humans may serve as paratenic hosts, where larvae migrate and encyst in tissues and organs, surviving for months or even years [2]. Infections with *Toxocara* spp. in humans may cause several clinical syndromes described as Visceral Larva Migrans (VLM), Ocular Larva Migrans (OLM), covert toxocarosis and Neural Larva Migrans (NLM) [3]. However, the vast majority of human *Toxocara* infections are asymptomatic [4].

Although humans may get infected through the ingestion of encysted larvae present in raw or undercooked meat [5,6], most infections are acquired through the ingestion of embryonated eggs by geophagia, in areas where infected cats, dogs or wildlife have defecated. This is particularly common in children [1]. In general,

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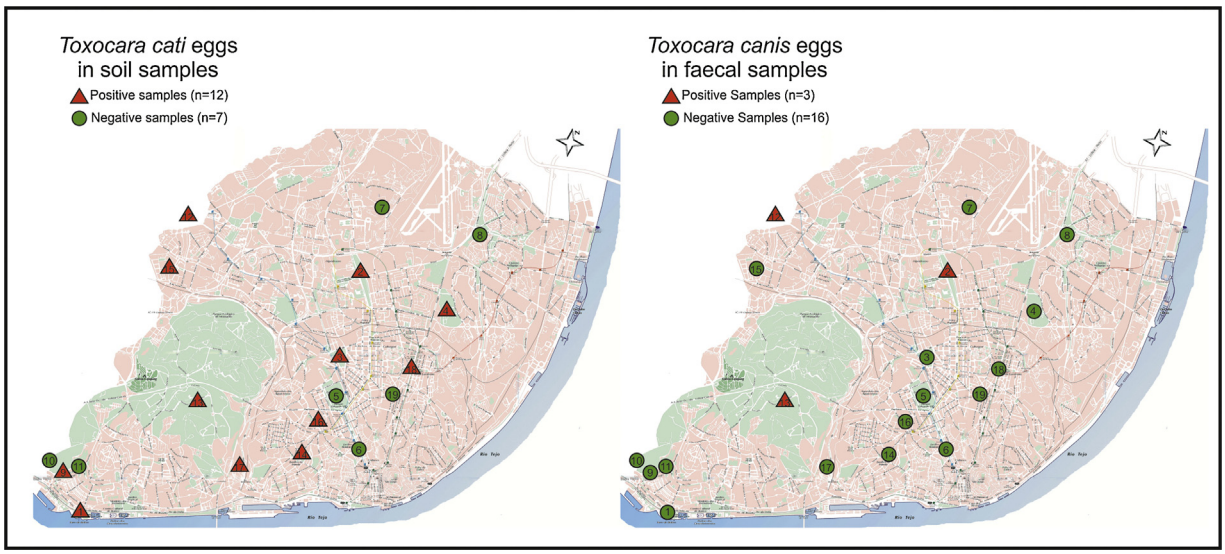

Fig. 1. Map of the Lisbon Metropolitan area, highlighting the distribution of positive (triangle) and negative (circle) sites to *Toxocara cati* eggs in soil samples and *Toxocara canis* eggs in faecal samples. *Note*: The numbers inside the triangle or circle correspond to each of the 19 study sites referred on Table 1.

eggs require an incubation period of 3–6 weeks in the environment before becoming infective. Therefore, public parks and playground sandpits may play a crucial role in the perpetuation of this infection. Indeed, *Toxocara* spp. eggs have been recovered worldwide in sand or soil samples from playgrounds and/or public parks [7]. In Europe high levels of environmental contamination with *Toxocara* spp. have been reported, ranging from 16.4% in Spain [8], 5.0–45.0% in the Czech Republic [9] to 10.7–81.8% in Poland [10,11]. Furthermore, studies performed in humans to assess antibodies against *Toxocara* revealed high seroprevalence levels [12].

In companion animals, particularly puppies, heavy prenatal infections may cause severe disease with alternating diarrhoea and constipation, vomiting, respiratory signs due to pneumonia, reduced growth, cachexia, typical 'pot belly', poor coat and even death [13]. In older dogs less severe clinical manifestations are observed and most infections pass unnoticed. In kittens, clinical signs are similar to puppies, although generally less severe [1].

In Portugal, knowledge about the current situation, distribution and contamination by *Toxocara* spp. is largely unknown, particularly regarding environmental contamination. However, the few existent studies indicate that *Toxocara* spp. are widespread in Portugal, occurring in wild species [14], rural animals [15–17] and urban dogs and cats [18,19]. Toxocarosis was also reported in children from Portugal, causing acute pericarditis [20] and panuveitis [21]. Therefore, a large-scale study was designed to assess the environmental contamination by *Toxocara* spp. eggs in soil and faecal samples, collected from public parks and playground sandpits in Lisbon. In addition, egg density of contaminated soil was calculated and viability of the collected eggs was assessed after incubation.

Material and methods

Study design and sampling area

This survey was conducted in 7 playgrounds sandpits (PS) and in 12 main public parks (PP) of Lisbon, totalizing 19 distinct study sites (numbered from 1 to 19) (Fig. 1). The sites were chosen in order to cover all public playgrounds in Lisbon that still have sandpits, as well as the public parks located in the most populated areas of the city.

The study comprised a total of 151 soil samples and 135 canine faecal samples, undertaken from February to May 2015. Soil samples were collected at a depth of 0 cm to 15 cm and only fresh faecal samples were collected. In order to obtain a representative sample, soil and faeces were collected on three different occasions, with a minimum two months' interval, and at different periods of the day (morning, midday and afternoon). All samples were refrigerated at 4 °C until analysis (maximum of three days).

The authors were unaware of any cleaning or disinfection activities that may have been performed on the surveyed playground sandpits.

Soil samples analysis

Soil samples were analysed using a modified centrifugation and sedimentation/flotation technique [22–24]. For each soil sample, one hundred grams were weighed and mixed with 100 mL of 5% Tween20 solution, homogenized for 10 min and allowed to stand for 12 h. The contents were then sieved (diameters 1.000 mm; 0.300 mm; 0.150 mm and 0.063 mm) and samples washed in running water for 30 min. The sediment present in the sieve of 0.063 mm was transferred to a sedimentation cup, to which was added distilled water up to 2/3 of the top and leaved to rest for 12 h. The supernatant was then discarded and the superficial layer of the sediment was transferred to the centrifuge tubes with a Pasteur pipette until 1/4 of them were filled. Distilled water was added to half of each tube, vortexed, centrifuged at $200 \times g$ for 10 min, after which the supernatant was discarded. Sucrose solution ($\rho \sim 1.3\ g/cm^3$) was added to half of each tube, vortexed and centrifuged at $200 \times g$ for 10 min, after which each of them was filled with the same saturated solution to form a positive meniscus. A cover slip was added to the top of each tube and after 30 min was observed with an optical microscope at a magnification of 100×.

Faecal samples analysis

An adaptation of the Cornell-Wisconsin method was used [25,26], in which 10 g of faeces of each sample were homogenised in 100 mL of distilled water. The suspension was transferred into 4 centrifuge tubes which were vortexed, centrifuged at $200 \times g$ for 10 min, and the supernatant discarded. Sucrose solution with a density of approximately $1.3\ g/cm^3$ was added until half of each tube, vortexed, centrifuged at $200 \times g$ for 10 min, and later each tube was filled with the same saturated solution to form a positive meniscus.

Table 1
Soil and faecal contamination by *Toxocara* spp. eggs in public parks and playground sandpits of Lisbon.

	Park reference number	Name of study sites	Positive for *Toxocara* spp. eggs (n)	No. of soil samples	Positive soil samples (n)	No. of faecal samples	Positive faecal samples (n)
Public Parks (PP)	1	Torre de Belém	+	10	8	8	0
	2	Campo Grande	+	9	7	8	3
	3	Gulbenkian	+	9	7	8	0
	4	Parque da Bela Vista	+	7	5	8	0
	5	Parque Eduardo VII	–	4	0	8	0
	6	Avenida da Liberdade	–	3	0	8	0
	7	Quinta das Conchas	–	3	0	6	0
	8	Vale do Silêncio	–	3	0	7	0
	9	Quinta de Santo António	+	8	1	4	0
	10	Miraflores (urban park)	–	10	0	8	0
	11	Miraflores (dog park)	–	10	0	10	0
	12	Queluz (urban park)	+	20	5	20	1
PP Total		12 sites	50.0% (6/12)	96	34.4% (33/96)	103	3.9% (4/103)
Playgroud Sandpits (PS)	13	Alameda Keil do Amaral	+	8	8	8	4
	14	Guerra Junqueiro	+	10	9	4	0
	15	Silva Porto	+	10	9	4	0
	16	Marcelino Mesquita	+	8	7	4	0
	17	Quinta da Cabrinha	+	8	7	4	0
	18	D. Afonso Henriques	+	8	7	4	0
	19	Jardim Constantino	–	3	0	4	0
PS Total		7 sites	85.7% (6/7)	55	85.5% (47/55)	32	12.5% (4/32)
Total		19 sites	63.2% (12/19)	151	53.0% (80/151)	135	5.9% (8/135)

After 30 min, the cover slip was placed on a slide for observation under an optical microscope at a 100× magnification.

Assessment of Toxocara spp. egg infection ability

In order to evaluate egg's ability to become infective, a modification of the *T. canis* egg incubation technique was used on the eggs recovered from the soil samples [27]. Collected eggs were incubated in 0.05 M H_2SO_4 solution and stored in the dark at room temperature for 60 days. For each study site a different flask was used. The content of each flask was then transferred to tubes and centrifuged at 200 × *g* for 10 min, after which the superficial 2 mL were pipetted and transferred to 6 microscope slides. After the incubation period, eggs were observed under an optical microscope at 100× magnification. The number of embryonated eggs was counted and registered, and the percentage of embryonated eggs was calculated by dividing the number of embryonated eggs by the total number of eggs observed.

Molecular analysis and sequencing

Eggs collected from the soil were identified through molecular techniques. For molecular detection, DNA was extracted using a commercial kit (Qiagen DNA mini-kit), according to the manufacturer protocol. PCR was performed in a 20 μL reaction using SensiFast No-Rox kit from Bioline. In each reaction, 10 pmol of forward and reverse primers were used and 2 pmol of probe. Two distinct PCRs (called Nemo and Nautilus) targeting the 18S rRNA gene of the Ascaridoidea superfamily were used. Primers and probe used were: Nemo18Sf, 5′-ggctaagccatgcatgtc-3′, Nemo18Sr, 5′-acttgatagacacgtcgcc-3′ and probe Fam-5′-aaaccgcgaacggctcatv3′-BHQ1 for the first PCR and Nautilus18Sf, 5′-agaggttcgaaggcgatca-3′, Nautilus18Sr, 5′-gtcaatcctcacggtgtcc-3′ and probe Vic-5′-aacgataccaactagcgttccgt-3′-BHQ1 for the second PCR. The amplification reactions were performed in a Roche Lightcycler. PCR products were sequenced using standard Sanger sequencing by Baseclear, Leiden.

Data analysis

Statistical analysis was performed using SPSS Statistics 20 software (SPSS Inc. Chicago, Illinois, USA). Since all frequencies were small, Fisher's exact test was used to check for associations between the variables related to the prevalence of *Toxocara* spp. eggs in parks. Pearson correlation coefficient was used to analyse correlations between the variables related to the viability of the recovered eggs and the type of park. A $p < 0.05$ was considered significant for both tests.

Results

In total, 85.7% of the playground sandpits (6/7) and 50.0% of the public parks (6/12) were contaminated with *Toxocara* spp. eggs (Fig. 1), with an overall prevalence of contamination of 63.2% (12/19) (Table 1).

Soil analysis showed that, 85.5% of the sandpits and 34.4% of the parks were contaminated, giving a total of 53.0% (80/151) positive soil samples, all containing only *T. cati* eggs (Figs. 2 and 3). Faecal analysis showed that, 12.5% of the faeces from sandpits and 3.9% from the parks contained *T. canis* eggs, giving a total of 5.9% (8/135) positive faecal samples (Table 1).

Overall, *T. cati* was detected in the soil samples obtained from 12 out of the 19 study sites and *T. canis* in the faecal samples obtained from 3 out of the 19 study areas (Fig. 1). Only *T. canis* eggs were found in faecal samples, and only *T. cati* eggs were found in soil samples. A significant difference was found between the positive soil samples collected from the PS and PP ($p < 0.001$), although this was not observed in faecal samples between PS and PP.

A total of 570 *Toxocara* spp. eggs were recovered from soil samples, with an average of 4.2 eggs per hundred grams of collected soil. Of these, 11% were already embryonated (L1 and L2 stages). After 60 days of incubation, 319 eggs had developed into a third stage larvae (L3) resulting in an overall viability rate of 56.0%. The individual viability rates were 53.1% for PS (180/339) and 60.2% for PP (139/231). A positive correlation was detected between the percentage of embryonated eggs after incubation and the number of retrieved eggs ($p = 0.647$). The same correlation was not found with the type of examined park (PS or PP).

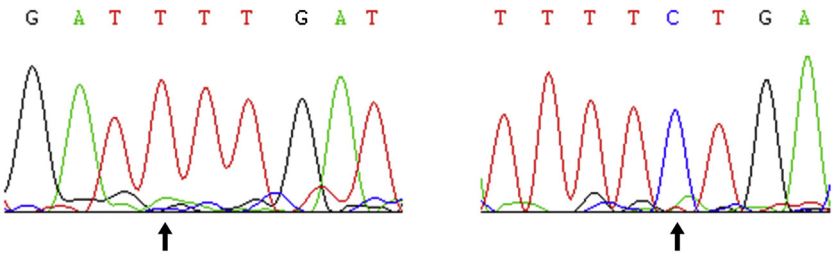

Fig. 2. Sequencing chromatograms from PCR Nemo products. The T and the C letters highlighted by the arrows are indicative of a *Toxocara cati* sequence.

```
T.cati  ctcattataacagctattatatacttgattttgatgtcctacgtggataact
   Nemo ..................................................
T.canis ............................c.....................

T.cati  gtggtaattctagagctaatacatgcaccaaagctccgattttctgacgagc
   Nemo ..................................................
T.canis ............................................g........
```

Fig. 3. Alignment of *Toxocara cati* EF180059 and *Toxocara canis* AF036608 with PCR Nemo products.

Discussion

We described a high level of environmental contamination with *T. cati* eggs in soil from public parks and sandpits in Lisbon. This is seriously concerning as the studied areas represent common places where people of all ages, including children, recreate. The overall prevalence of 63.2% for positive sampled sites, as well as the 53.0% for positive soil samples are higher than those previously reported in other European cities, such as 50% in the Marche region of Italy [28], 5–45% in Prague, Czech Republic [9], 16.4% in Madrid, Spain [8] and 15.7–7.7% in Lodz, Poland [11]. The sampling period occurred in an especially hot and dry year, with two heat waves [29]. Accounting that *Toxocara* spp. eggs need moisture and pO_2 from the soil, the observed prevalence may be significantly different if those environmental conditions are present, namely in cooler and moisty months from Autumn till Spring, for the Lisbon area.

A higher prevalence of *T. cati* was observed in PS soil in comparison to PP. This suggests that the hygiene and disinfection maintenance measures currently applied to the playground sandpits are insufficient to keep the parasitic load at a low level. According to [30], children who live and play in areas surrounding contaminated parks or sandpits, show higher seropositivity values for *Toxocara*. Considering that all the surveyed PS were surrounded by high fences and have an access door that is closed and locked at night, dog access is difficult and unlikely. Therefore, and according to the molecular results, stray cats are the main disseminators of this parasite, as previously suggested in other European countries [31–33]. Precedent data regarding the prevalence of parasitic diseases in stray cats from Lisbon ranged from 10.8 to 38.3%, corroborating our findings [34,35].

The prevalence of *Toxocara* spp. eggs in faecal samples from PP was much lower than the prevalence registered for soil samples, with only one PS and two PP positive for *Toxocara* spp. Indeed, in faecal analysis, 15.8% of the studied parks were positive for faecal samples *versus* 63.2% that were positive for the soil samples. This explains why some study sites without positive faecal samples were reported as positive. Besides, all the parks that reported positive faecal samples also reported positive soil samples. Our results are in accordance with the literature, where the reported prevalence for faecal samples is often lower than the soil sample prevalence [36,37,8]. These values also warn of a potential undervaluation of the prevalence reported in studies that only take faecal samples into account. A possible explanation for this difference might be the fact that faecal samples commonly tested are almost exclusively from dogs, as cats tend to bury their faeces precluding their collection. Similarly, if cats are primarily responsible for elevated environmental contamination, then the eggs they shed will be more readily found when soil samples are analysed.

The viability rate of the eggs recovered was 56.0%, meaning that more than half of the eggs found in the soil had the capacity to develop a larva and become infective. This represents an average of 4.2 eggs per hundred grams of contaminated soil sites or 2.4 eggs per hundred grams of all studied soil samples. These results are in line with the previous data reported in Japan [38] and the Czech Republic [9]. The number of eggs per hundred grams of soil should be taken into account thoughtfully, considering the distinct immunological competence of the population. Although a healthy individual may need to ingest a large amount of soil to become infected, a single egg might be enough to cause infection, especially in individuals with a compromised immune system. Furthermore, it is known that infections caused by a few or even by just one larva of *Toxocara* spp. might cause even more damage in humans than a larger amount of larvae, with consequent increases in the probability of developing OLM and VLM infection [39–41].

Conclusions

Public parks and playground sandpits in Lisbon are heavily contaminated with *T. cati* eggs, presenting a serious menace to the public health, especially if the longevity of these infective eggs is considered in the environment. Preventing environment contamination through rigorous faecal removal practices should be encouraged, combined with regular or targeted anthelmintic treatment of companion animals. The high contamination levels detected in PS emphasize the need of adopting effective strategies to prevent human infections, such as precluding pets and especially stray animals from accessing children's play areas, regularly replacing sand or sterilizing it, covering sandpits, fencing the whole playground or even eliminating sandpits altogether from public areas. Furthermore, health education and discouraging geophagia in children are fundamental. An integrated multidisciplinary 'One Health' approach involving the collaboration between local authorities, veterinarians, physicians, and policy makers, is needed to achieve a better control of this zoonotic disease.

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Conflict of interest

The authors declare that they have no conflict of interest.

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