Research Article

BASJVETRES

BASRAH JOURNAL OF VETERINARY RESEARCH, 2024, 23(2): 45-53.
https://bjvr.uobasrah.edu.iq/

Molecular Prevalence and Genotyping of *Toxoplasma gondii* from Pet Cat Using PCR-RFLP

Falah H. Ahmed[1], Suzan A. Al-Azizz[2], Muna M. Jori[2].

1-Department of Microbiology, College of Veterinary Medicine, University of Basrah, Iraq.

2-Department of Parasitology, College of Veterinary Medicine, University of Basrah, Iraq.

Corresponding Author Email Address: pgs.falah.hasan@uobasrah.edu.iq

ORCID ID: 0009-0006-0844-0344

DOI: https://doi.org/10.23975/bjvr.2024.147020.1061

Received: 10 March 2024 Accepted: 20 April 2024.

Abstract

Toxoplasma gondii is the most dangerous parasite found in humans and cats. The strong relationship between cats and their owners compelled us to explore the incidence of these parasites in companion cats and genotyping of *T.gondii* strain of the infected pets. 54 blood samples and 54 fecal samples collected from pets in order to ascertain the prevalence of *T. gondii*. The GRA6 gene was targeted in the feces and blood of examined pets and the genotypes of *T. gondii* were determined using the GRA6 gene loci by PCR-RFLP assay. The GRA6 gene amplification showed that 6% of pets were infected with *T.gondii*. PCR-RFLP assay revealed the predominance of genotype II at a rate 67% and type III at a rate 33% while type I not detected in infected pets. The incidence of toxoplasmosis in domestic felines can cause contamination of environment through excreting of oocyst in the feces which may lead to infection to cat owners specially who taking care of feeding and cleaning of pets.

Keywords: Toxoplasmosis, PCR, GRA, pet cat, genotyping

Introduction

The genus Toxoplasma only contains one species, *Toxoplasma gondii*. Most mammals, including humans, dogs, and cattle are susceptible to infection by the intracellular parasite *T. gondii*. Because of the serious illnesses it causes, it is an important issue in public health worldwide. (1). Regular interaction with felines heightens the likelihood of transmission, since domestic cats can excrete *T. gondii* oocysts for a time of up to 21 days after being initially infected. (2,3). Three stages in *T. gondii*'s life cycle have the potential to induce infection: tachyzoites, which infiltrate the host; bradyzoites, which remain inside the host; and sporozoites, which are shielded from the environment by an oocyst wall. *T. gondii* can be transmitted through vertical transmission (via the placenta) and horizontal transmission (by blood transfusions, organ transplants, oocysts in water, soil, or vegetation, and tissue cysts).(4,5). All felines are the only definitive host for the completion of the life cycle of *T. gondii* (6). Although clinical symptoms are uncommon, immunocompromised cats do occasionally have them (7). Cats commonly exhibit clinical symptoms such as both congenital and ocular toxoplasmosis, often accompanied by severe symptoms related to the respiratory and neurological systems (7). A low frequency of oocysts in fecal samples is likely attributable to the brief oocyst shedding period. Approximately one percent of a population's cats will be oocyst-shedding at any one moment.(8).

Toxoplasmosis diagnosis in the laboratory has made use of a number of techniques, including mice bioassay, microscopic detection, and polymerase chain reaction(9,10). Given the physical similarity between the oocysts of *T. gondii* and those of *Hammondia hammondi and Besnoita spp.,* microscopic identification of the oocyst is not as reliable as bioassay and PCR(7,11).

In the *T. gondii* population, three major clonal lineages are typically recognized for biological and epidemiological studies: Type I, Type II, and Type III. Type I clonal lineages include extremely dangerous strains such as RH, type II clonal lineages include non-pathogenic strains such as PLK and Beverley, and type III clonal lineages include avirulent strains such as VEG and CTG(12). These conventional genotypes have already been identified using restrictions fragment length polymorphism, (RFLP), which is frequently accustomed to identify the strain of *T. gondii* in PCR amplified SAG2 loci(13). The consequences of infection caused by Toxoplasma gondii are also influenced by the parasite's genotypes and genetic composition, which plays an important function in progression of toxoplasmosis(14–16). Comparing the molecular prevalence of *T. gondii* is the goal of current study in blood and feces of Pet cat and genotyping of local strains.

Material And Methods

Collection of Blood and Feces Samples

Via vein punctures, blood samples (n=54) from pet cats were obtained in various veterinary clinics located in Kirkuk City. blood (2ml) was collected from each cat in

EDTA tube and kept in refrigerator at -20 for the DNA extraction. Then, 10 ml of Anestane® was administered (Halothan 100% BP stabilizing 0.01 thymol). Ansatane® is a product manufactured by Al-Hekma business in Jordan. Anastane is regarded as the safest form of anesthetic, as it does not cause any alterations in blood composition or its contents for a duration of 1-2 hours and a total of 54 fresh fecal samples collected from other cats during a period of February to June 2023. feces samples were collected in clean plastic cups.

Molecular Diagnosis procedure

Genomic DNA extraction

Genomic DNA was isolated from whole blood samples that were collected in EDTA tubes using a commercially available purification (Add bioPrep Genomic DNA Extraction Kit, Korea). Faecal samples were also processed to extract DNA using the QIAamp Fast DNA Stool Mini Kit from Germany according to the manufacturer's instructions. The final pellets were kept at -20.

PCR Assay

Preparation of primer: As per the guidelines provided by the synthesizer for primers. Free (ddH2O) was used to dissolve the lyophilized primers to achieve ultimately 100 µM/µl concentration. This solution was then held as a stock solution at -20°C. Stock primers (10 µM/µl) were then diluted.

Primers used in the current study: A part of the GRA6 gene was amplified to detect & genotype of *T. gondii.*

Target gene		Sequence (5'-3')	Ta (°C)	Produc t size	Accession number	Reference
GRA6	F	GTAGCGTGCTTGTTGGCGAC	56	773 bp	KX781158.1	(17)
	R	TACAAGACATAGAGTGCCCC				

PCR master mix: Reaction mixture was done according to the company's instructions. GoTaq® G2 Green Master Mix (1X ,25 µl), forward primer and reverse primer (10 µM/µl, 4 µl) for each one, ddH2O (13 µl), and DNA (40 ng, 4 µl). PCR Thermocycler T100 Thermal cycler was then applied. The nuclease-free water was used instead of DNA target as control negative in PCR Reaction. Initial denaturation (94°C, 5 min, 1X), denaturation (94°C, 30 sec, 35X), annealing (56°C, 30 sec, 1X), extension (72°C, 1 min, 35X), extension (94°C, 5 min, 1X), and final extension (72°C, 5 min, 1X) was then applied.

Restriction Fragment Length Polymorphism (RFLP): The restriction endonuclease MseI and DNA modification buffer were obtained from NEB, USA. Restriction digests were made at a final volume of 40 µl, or any larger amount that is a multiple of 40 µl, based on the specific requirements of the digests. To fulfill both preparative and analytical objectives,

samples were meticulously prepared to adhere to the specified requirements. A quantity of 1-3 µg of PCR product underwent digestion using appropriate restriction buffer (1X) and restriction enzyme (10 units). Then, the digests were incubated at 37°C (or the suitable temperature for the enzyme) for 3 h. The digests were then submitted to electrophoresis to observe and analyze the resulting products. At least three genotypes were expected to be obtained for each tested sample.

Statistical analyses

The analysis that is descriptive assessed the occurrence of parasites according to the kind of sample: Blood, feces, and sex of examined pets. Qualitative variable associations were assessed using X^2 tests in SPSS 26.0 (SPSS Inc., Chicago, IL, USA) to identify statistically significant differences (P-value <0.05).

Results

In this work, a sum of 108 cat samples were examined using molecular (PCR) test in which (6%) of the samples was positive for toxoplasmosis (Table 1). out of 54 fecal sample, 2% revealed a positive diagnosis of toxoplasmosis and out of 54 blood samples, 9% were positive for toxoplasmosis. Two types of samples (blood and feces) were taken from tested pets. The current investigation demonstrated a greater occurrence of toxoplasmosis in the blood (9%) than in the feces (2%) with highly significant differences (x2 = 2.824, P-Value = 0.053). After performing PCR-RFLP bands 623 bp, 75 bp for the type II, 544 bp and 97 bp for the type III were detected (Figure 1). While, bands of 544 bp and 168 bp type I not detected. So that the number of *Toxoplasma* infections of type II was 4/6 at a rate of 67% and type III was 2/6 at a rate of 33%. While, type I not detected (Table 2).

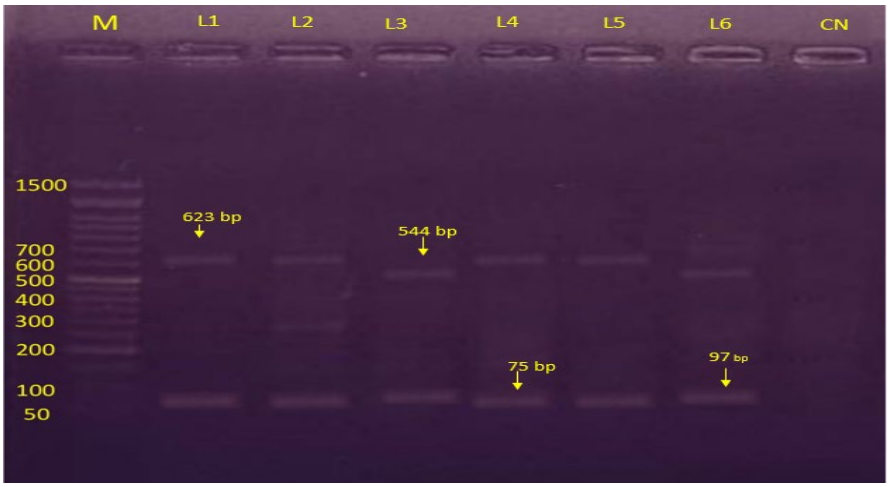

Figure.1: PCR-RFLP genotyping for *T. gondii* based on amplification of a partial portion of the parasite's GRA6 gene. Each genotype's fragments showing the following size: 544 bp and 168 bp for the Type I, 623 bp and 75 bp for the Type II, 544 bp, and 97 bp for the type III RedSafe (Intron, Korea) DNA dye was used on a 1.5% gel. Time: 45 min, V: 90. M: A ladder DNA

Table 1: Overall infection rate of toxoplasmosis in pet cats

Sample type	No. of Samples	No. of Positive	positive %
Feces	54	1	2%
Blood	54	5	9%
Total	108	6	6%

Pearson Chi-Square = 2.824 P-Value = 0.053

***Association is significant at the 0.05 level.**

Table 2: Number of samples for I, II, and III types, and the number of unknown samples

Samples	Genotype I	Genotype II	Genotype III	Unknown
6	0	4	2	0
Percentage	0	67%	33%	0

Discussion

In this research, PCR assay was carried out to estimate the prevalence of toxoplasmosis in Households cat and PCR-RFLP was used to identify the genotypes of positive samples. The environment is widely contaminated by oocysts, which are expelled by domestic cats as well as other felidae family members (18). Most oocysts are generated soon after the parasite is first acquired, reaching their highest level within a month after the first infection (19). The existence of diseased pets means that there is a risk to human population due to contaminated environment and small ruminants (20). Shedding of oocysts usually lasts no more than 21 days (21). Infected cats typically excrete oocysts only once during their lifetime and for a brief duration (22). Cats that are infected carry the bradyzoite stage of the parasite within their cells throughout long durations, and the latent infection can remain. The parasite's biological behavior can account for the greater incidence of its DNA in the blood of infected cats compared to their feces(23) . Research from all across the globe shows that the prevalence of cat toxoplasmosis ranges from 2.7% to 90%, depending on factors such as geographic location, climate, socioeconomic status, historical context, and testing methods used (24) The toxoplasmosis prevalence overall in this study was 6%. Present study documents higher occurrence of toxoplasmosis in the blood samples (9%) compared to fecal samples (2%). There is not enough data about distribution of toxoplasmosis among pet cats in Iraq. However, this study documents lower prevalence of toxoplasmosis in household cats than those conducted in Mosul city (22%) by (25) and (26) . The documented occurrence of *T. gondii* in cats across different regions of globe varies depending on the diagnostic techniques employed and the diversity in geographical, environmental, and dietary factors of the cats under investigation. The result of current study revealed greater than the prevalence reported in Switzerland

compared with previous molecular studies worldwide (0.58%) (27), Poland (2.4%) (28), Malaysia (4%) (29), Thailand (4.7%) (30), considerably lower than that reported in Italy (20.5%) (31).

The parasite genotyped using in this work was GRA6 because of the significant polymorphism in the coding region of this gene. Compared to other investigated, *Toxoplasma* coding genes such as SAG1, SAG2, and GRA4, GRA6 exhibits greater variability (32). The rate of amino acid substitutions, namely non-synonymous to synonymous modifications, is significantly high. This observation indicates that the diversity in GRA6 genes among various isolates of *T. gondii* might have a significant impact on the parasite's survival, particularly within the parasitophorous vacuole (33). The result of this study showed that 67% of isolates was type II and 33% was type III. While, type I not detected after performing PCR-RFLP. The frequency of type II strain found in (4/6) 67% which is nearest to the result of study conducted by (34) in Wasit who recorded 80% of type II strain and agree with the research carried out by (35) who determined that, of all the genotypes, type II strain was the most prevalent. Many previous studies like (36) and (37) concluded that type II is the most widespread, 76.7% of cases in France and 85.7% in Iran. Type III found in 33% (2/6) of isolates in contrast to another global research was greater than (38) . It has been found that type III strain was in 9% of the isolates (revise)and (39) who recorded 4% type III. sample size, pet nutrition, and geographic location Factors might provide an explanation for why this study's findings differ from those of previous investigations.

Conclusion

Owning pet cats has recently become a lifestyle for some Iraqis, especially women and cats are only final host that can excrete durable oocysts in environment. This poses a risk to those taking care of pet feeding and cleaning cat cages. Our results recorded the prevalence of toxoplasmosis in 6% of examined pets even though all pets were commercially feed also the study's findings supported the prevalence of the *T. gondii* type II strains, highlighting its higher occurrence compared to both type I and III.

Financial support and sponsorship

Self-funding

Conflicts of interest

All of the writers have declared no conflicts of interest with the studies presented in this research study.

Ethical Clearance

This work is approved by The Research Ethical Committee.

References

1.Robert-Gangneux F, Dardé ML. (2012). Epidemiology of and diagnostic strategies for toxoplasmosis. *Clin Microbiol Rev. 25*(2):264–96.

2.Lilly EL, Wortham CD. (2013). High prevalence of Toxoplasma gondii oocyst shedding in stray and pet cats (Felis catus) in

Virginia, United States. *Parasit Vectors. 6*(1):1–4.
3.Scorza V, Lappin MR. (2021). Giardiasis. In: Greene's Infectious Diseases of the Dog and Cat. Elsevier; p. 1263–77.
4.Jg M. Liesenfeld O. (2004). Toxoplasmosis. *The lancet. 363*(9425):1965–76.
5.Al-Qassab S, Reichel MP, Su C, Jenkins D, Hall C, Windsor PA, et al. (2009). Isolation of Toxoplasma gondii from the brain of a dog in Australia and its biological and molecular characterization. *Vet Parasitol. 164*(2–4):335–9.
6.Cerro L, Rubio A, Pinedo R, Mendes-de-Almeida F, Brener B, Labarthe N. (2014). Seroprevalence of Toxoplasma gondii in cats (Felis catus, Linnaeus 1758) living in Lima, Peru. *Revista Brasileira de Parasitologia Veterinária. 23*:90–3.
7.Dubey JP, Lindsay DS, Lappin MR. (2009). Toxoplasmosis and other intestinal coccidial infections in cats and dogs. Veterinary Clinics: *Small Animal Practice. 39*(6):1009–34.
8.L.P. T, P.A. MAbdR, R.S.K. S, S.S. SH, M. W. (2020). Prevalence of Toxoplasma gondii in pet and stray cats in Klang Valley, *Malaysia. Trop Biomed. 1*;542–50.
9.Houshyar H, Rostamkhani P, Talari SA, Arbabi M. (2007). Toxoplasma gondii infection in stray cats.
10.Salant H, Markovics A, Spira DT, Hamburger J. (2007). The development of a molecular approach for coprodiagnosis of Toxoplasma gondii. *Vet Parasitol. 146*(3–4):214–20.
11. Györke A, Opsteegh M, Mircean V, Iovu A, Cozma V. (2011). Toxoplasma gondii in Romanian household cats: evaluation of serological tests, epidemiology and risk factors. *Prev Vet Med. 102*(4):321–8.
12.Howe DK, Sibley LD. (1995). Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease. *Journal of infectious diseases. 172(*6):1561–6.
13.Khan A, Taylor S, Su C, Mackey AJ, Boyle J, Cole R, et al. (2005). Composite genome map and recombination parameters derived from three archetypal lineages of Toxoplasma gondii. *Nucleic Acids Res. 33*(9):2980–92.
14.Suzuki Y, Conley FK, Remington JS. (1989). Differences in virulence and development of encephalitis during chronic infection vary with the strain of Toxoplasma gondii. *J Infect Dis. 159*(4):790–4.
15.Roberts F, Kirisits MJ, Estes R, Johnson EJJ, Roberts CW, Pope C. (2002). In Vitro Correlates of Ld-Restricted. *J Immunol.169*:966–73.
16.Jungersen G, Jensen L, Riber U, Heegaard PMH, Petersen E, Poulsen JSD, et al. (1999). Pathogenicity of selected Toxoplasma gondii isolates in young pigs. *Int J Parasitol. 29*(8):1307–1319.
17.Norouzi M, Tabaei SJS, Niyyati M, Saber V, Behniafar H. (2016). Genotyping of Toxoplasma gondii strains isolated from patients with ocular toxoplasmosis in Iran. *Iran J Parasitol. 11*(3):316.
18.Dubey JP, Beattie CP. (1988). Toxoplasmosis of animals and man. CRC Press, Inc.
19.Elmore SA, Jones JL, Conrad PA, Patton S, Lindsay DS, Dubey JP. (2010). Toxoplasma gondii: epidemiology, feline

clinical aspects, and prevention. *Trends Parasitol. 26*(4):190–6.
20.Santos TR, Costa AJ, Toniollo GH, Luvizotto MCR, Benetti AH, Santos RR, et al. (2009) Prevalence of anti-Toxoplasma gondii antibodies in dairy cattle, dogs, and humans from the Jauru micro-region, Mato Grosso state, Brazil. *Vet Parasitol. 161*(3–4):324–6.
21.Lappin MR. (2015). Clinical Feline Toxoplasmosis. Parasitoses & Vector Borne Diseases Of Cats. 87.
22.Dubey JP, Jones JL. (2008). Toxoplasma gondii infection in humans and animals in the United States. *Int J Parasitol. 38*(11):1257–78.
23.Karimi P, Shafaghi-Sisi S, Meamar AR, Nasiri G, Razmjou E. (2022). Prevalence and Molecular Characterization of Toxoplasma gondii and Toxocara cati Among Stray and Household Cats and Cat Owners in Tehran, Iran. *Front Vet Sci. 22*;9.
24.Dubey JP, Cerqueira-Cézar CK, Murata FHA, Kwok OCH, Yang YR, Su C. (2020). All about toxoplasmosis in cats: the last decade. *Vet Parasitol. 283:1*09145.
25.Alzori SK, Hamoo reedh. (2023). Molecular Detection of Toxoplasma Gondii in Naturally Infected Cats In Mosul City. *Egyptian Journal of Veterinary Sciences. 54*(5):971–82. Available from: https://ejvs.journals.ekb.eg/article_308281.html
26.Al-Ani ANA, Al-Badrawi TYG, Hussein ZS. (2020). Toxoplasmosis in cats: Serological and molecular study in Baghdad Province. *Ann Trop Med and Public Health. 23*: S445.
27.Berger-Schoch AE, Herrmann DC, Schares G, Müller N, Bernet D, Gottstein B, et al. (2011). Prevalence and genotypes of Toxoplasma gondii in feline faeces (oocysts) and meat from sheep, cattle and pigs in Switzerland. *Vet Parasitol. 177*(3–4):290–7.
28.Sroka J, Karamon J, Dutkiewicz J, Wójcik-Fatla A, Zając V, Cencek T. (2018). Prevalence of Toxoplasma gondii infection in cats in southwestern Poland. *Annals of Agricultural and Environmental Medicine. 25*(3):1–5.
29.Nasiru Wana M, Mohd Moklas MA, Watanabe M, Zasmy Unyah N, Alhassan Abdullahi S, Ahmad Issa Alapid A, *et al.* (2020). Molecular detection and genetic diversity of Toxoplasma gondii oocysts in cat faeces from Klang Valley, Malaysia, using B1 and REP genes in 2018. *Pathogens. 9*(7):576.
30.Chemoh W, Sawangjaroen N, Nissapatorn V, Sermwittayawong N. (2016). Molecular investigation on the occurrence of Toxoplasma gondii oocysts in cat feces using TOX-element and ITS-1 region targets. *The Veterinary Journal. 215*:118–22.
31.Veronesi F, Santoro A, Milardi GL, Diaferia M, Morganti G, Ranucci D, et al. (2017). Detection of Toxoplasma gondii in faeces of privately owned cats using two PCR assays targeting the B1 gene and the 529-bp repetitive element. *Parasitol Res. 116*:1063–9.
32.Mawlood HH, Mero WMS, Bapeer YAA, Su C. (2019) Genotyping of Toxoplasma gondii from Stray Cats by Multilocus PCR-RFLP Techniques in Erbil city -Kurdistan Region/Iraq. In: Journal of Physics: Conference Series. Institute of Physics Publishing.

33.Fazaeli A, Carter PE, Darde ML, Pennington TH. (2000). Molecular typing of Toxoplasma gondii strains by GRA6 gene sequence analysis. *Int J Parasitol. 30*(5):637–42.
34.A'aiz NN. (2016). Genotyping of Toxoplasma gondii isolates from human being in Wasit province/Iraq. *Al-Qadisiyah Medical Journal. 12*(22):26–32.
35.Mohammed NS, Al-A'ssie AHA, Al-saqur IM. (2015). Genotyping of Toxoplasma gondii isolated from aborted Iraqi women. *Diyala Journal of Medicine. 9*(1):44–52.
36.Honore S, Couvelard A, Garin YJ, Bedel C, Henin D, Darde ML, *et al.* (2000). Genotyping of Toxoplasma gondii strains from immunocompromised patients. *Pathol Biol (Paris). 48*(6):541–547.
37.Behzadi R, Razavi MR, Hovanessian A, Asmar M, Rouhvand F. (2003). Genotyping of Toxoplasma gondii strains isolated from patients and mice by PCR-RFLP assay.
38.Howe DK, Honoré S, Derouin F, Sibley LD. (1997). Determination of genotypes of Toxoplasma gondii strains isolated from patients with toxoplasmosis. *J Clin Microbiol. 35*(6):1411–4.
39.Ferreira IMR, Vidal JE, Costa-Silva TA, Meira CS, Hiramoto RM, de Oliveira ACP, et al. (2008). Toxoplasma gondii: Genotyping of strains from Brazilian AIDS patients with cerebral toxoplasmosis by multilocus PCR–RFLP markers. *Exp Parasitol. 118*(2):221–7.

الانتشار الجزيئي والنمط الجيني لطفيلي داء المقوسة الغوندي (*Toxoplasma gondii*) في قطط الزينة باستخدام اختبار PCR-RFLP

فلاح حسن احمد[1] , سوزان عبدالجبارعبدالعزيز[2] , منى محمد جوري[2].

1-فرع الاحياء المجهرية – كلية الطب البيطري – جامعة البصرة، العراق.

2-فرع الطفيليات– كلية الطب البيطري – جامعة البصرة، العراق.

الخلاصة

داء المقوسة الغوندية (*T.gondii*) هو اخطر طفيلي يصيب القطط وبقية الحيوانات بما فيها الانسان . نظرا للعلاقة القوية بين القطط وأصحابها، دفعنا لاستكشاف انتشار هذه الطفيليات في القطط المنزلية وتحديد النمط الجيني لها في القطط المصابة. تم جمع 54 عينة دم و54 عينة براز من القطط المنزلية لتحديد انتشار والنمط الجيني لطفيلي *T.gondii* عن طريق استهداف جين GRA6 باستخدام تقنية PCR-RFLP . اظهر تضاعف جين GRA6 ان 6% من القطط التي تم فحصها كانت مصابة ب *T.gondii* واظهرت نتائج اختبار PCR-RFLP عن تفوق نمط II بنسبة 67% والنمط III بنسبة 33% في حين لم يتم اكتشاف النمط I في القطط المصابة. يمكن ان يؤدي انتشار طفيلي *T.gondii* في قطط الزينة إلى تلوث البيئة من خلال إفراز البويضات في البراز، مما قد يؤدي إلى عدوى أصحاب القطط خاصة الذين يهتمون بتغذية وتنظيف حيواناتهم الأليفة.

الكلمات المفتاحية: قطط الزينة , داء المقوسة الغوندية , تفاعل البلمرة المتسلسل, النمط الجيني, جين GRA6.