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Original Article

Investigation of the role of stray cats for transmission of toxoplasmosis to humans and animals living in İzmir, Turkey

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Abstract

Introduction*: Toxoplasma gondii* is a protozoan parasite that has a widespread distribution among mammalians and birds. One of the reasons for the high prevalence may be due to ingesting oocyst disseminated by stray cats' feces. In Turkey, most of the citizens are closely associated with stray cats and they love to pet and feed them on the streets. In this study, we aimed to determine the prevalence of *T. gondii* DNA in feces of stray cats living in İzmir, Turkey in order to identify the transmission potential to humans and other animals.

Methodology: Feces and blood samples of 465 stray cats were investigated for the presence of *T. gondii* oocysts by microscopy and for the presence of *T. gondii* DNA by two real time PCR methods. Furthermore, serum samples were analyzed for anti-*T. gondii* IgG antibodies using an ELISA.

Results: Oocysts were detected in 0.43% of the stray cats by microscopy. *T. gondii* DNA was detected in 14.37% of the stray cats' feces samples. The seroprevalence rate was 37.84%. In the feces and/or blood PCR positive group, 35.89% of them were seropositive. Among the 176 seropositive cats, *T. gondii* DNA was detected in feces of 27 cats (15.34%).

Conclusions: This study first time showed the inter relation of *T. gondii* DNA in feces and blood samples and seropositivity. In sum, over 14% of the stray cats living outdoor may have an important role in transmission of toxoplasmosis to humans in İzmir as well as to other animals.

Key words: *Toxoplasma gondii*; cat; feces; PCR; Turkey.

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Introduction

Toxoplasma gondii is a protozoan parasite that has a widespread distribution worldwide among humans and warm-blooded animals [1,2]. Toxoplasmosis is the most frequent protozoan infection detected in humans [3,4]. The high prevalence is due to ease of transmission through ingesting oocysts excreted by cats and by ingestion of tissue cysts in raw or under-cooked meat. Cats shed millions of oocysts in their feces for as long as 3 weeks during *T. gondii* infection [1,4]. Worldwide, the prevalence rates in meat-producing animals such as free range chicken, free range pigs, sheep, and goats range between 0-100% and up to 92% in cattle [5-7].

Close proximity of cats as a companion species in human populations plays an important role in transmission to humans. According to the European Pet Food Industry Federation and American Veterinary Medical Association, the total number of domestic cats

in the European Union is 72 million and over 74 million in the US and toxoplasmosis seroprevalence varies between 30-40% [8-10]. *T. gondii* also causes tainted water outbreaks [11-14].

In Turkey, the seroprevalence rates among humans ranges between 30%-60%. In İzmir and Aydın (located nearby İzmir) seroprevalence rates were 30% and 49%, respectively [15,16]. In our previous studies, seroprevalence rate in stray cats of İzmir was 34.47% [17] and the prevalence of *T. gondii* infection in wild birds of İzmir was 89.6% [18]. Interestingly, there was a toxoplasmosis outbreak in Izmir that affected 171 boarding schools students which was linked to oocysts excreted by cats [11]. Stray cats are loved by most of the citizens and are under protection and free to live outdoor by law in Turkey. On the other hand, the oocysts excreted by stray cats are potential source of toxoplasmosis for humans and animals in Turkey.

Detecting oocysts in the feces sample of a cat may be an indication of *T. gondii* infection and increases the rate of toxoplasmosis in humans and animals. Diagnosing a cat that excretes *T. gondii* can be performed with mouse bioassay but it is impractical due to ethical issues and the need for an animal housing facility [17,19]. Detecting *T. gondii* DNA from the feces sample of the cat is rapid and resolves the ethical as well as animal housing issues. On the other hand, this approach can be misleading since the oocysts can be dead. Although negative feces Polymerase Chain Reaction (PCR) cannot rule out *T. gondii* infection, it may be interpreted as "this cat may not be contagious at the moment and accordingly can be released to outdoor life".

Thus, we collected feces samples from stray cats and investigated the presence of *T. gondii* oocysts by microscopy and two PCR methods. In addition, *T. gondii* was investigated in blood samples using PCR. Seropositivity was investigated using an Enzyme-Linked ImmunoSorbent Assay (ELISA) [17].

Methodology

Cats and sample collection

Blood and feces samples were collected from 465 healthy cats, which were brought to Veterinary Clinics for sterilization purposes from Konak (n = 183), Narlıdere (n = 145), Çiğli (n = 74), Karşıyaka (n = 38), and Karabağlar (n = 25) (Figure 1) districts of İzmir between November 2017-January 2019. Feces and blood samples were collected from the anesthetized cats, inserted into 50 mL tubes and kept at +4°C until use. The protocol for collecting cat samples from stray cats was approved according to the instructions of the Institutional Animal Care and Use Committee (IACUC) of Ege University.

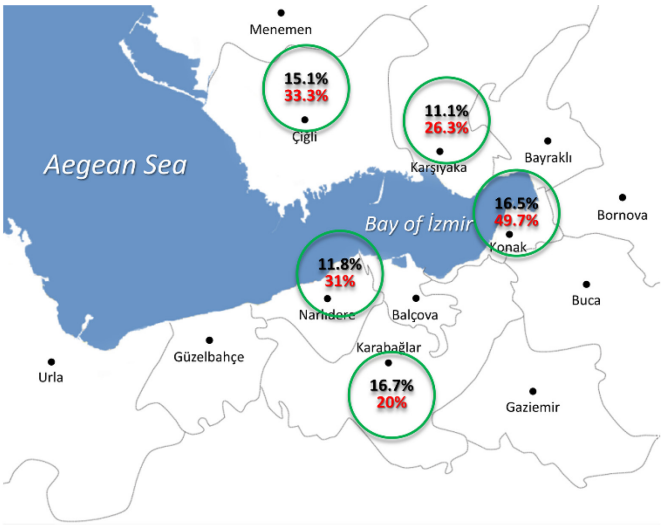
Microscopy

Briefly, after taking a sample from the feces for DNA extraction, tap water was added to remaining feces (5-10 g) in 50 mL tube and incubated for 2 hours at room temperature. Thereafter, tap water was discarded without loss of feces and sucrose solution (53 g sucrose, 100 mL water) was added up to the top level of feces and emulsified by sterile tongue depressor. Next, the mixture was filtered through 2 layers of gauze and centrifuged at 400×g for 10 minutes [20-22]. Thereafter, supernatant was collected from the top of each tube using a sterile Pasteur pipette and the presence of oocysts was investigated using a phase contrast microscope (Nikon, Melville, USA).

Real Time Polymerase Chain Reaction

T. gondii repeat region (RE) gene (GenBank accession no: AF146527) was investigated by 2 different Real Time PCR approaches using hydrolysis and hybridization probes, respectively. Briefly, isolation of DNA from the cat blood and feces was performed by blood and stool kits (RTA DNA Isolation Kit from Stool and Blood, RTA Labs (İstanbul, Turkey), according to the manufacturer's protocol. During DNA isolation, 100 mg feces and 500 µL buffy coat samples were used and they were eluted with 100 µL and 200 µL elution buffer, respectively. For each sample, 2.5 µL of internal control provided by the

Figure 1. Prevalence of *T. gondii* DNA and seropositivity in Konak, Narlıdere, Çiğli, Karşıyaka, and Karabağlar districts of İzmir. *T. gondii* DNA as detected from feces of stray cats by PCR are shown with black letters. Seropositivity rates of stray cats detected in each district are written with red letters.



manufacturer was added to lysis solution of the Stool and Blood DNA Isolation Kits. Initially, a commercial multiplex Real Time PCR (RTA *T. gondii* Real-Time PCR, RTA Labs (İstanbul, Turkey) was used to screen all the feces and blood samples for the presence of *T. gondii* RE gene (n=465) according to the manufacturer's protocol. Briefly, reaction mix was prepared by adding 11.5 µL of Mix A, 3.5 µL of Mix B, and 5 µL DNA sample or controls. The PCR amplification reactions were performed by the following calculated protocol: 10 minutes initial denaturation step at 95ºC, followed by 45 cycles of 30 seconds at 95ºC, 30 seconds at 60ºC, and 30 seconds at 72ºC. Fluorescence intensity is measured at 72°C; using FAM and HEX channels of a Real Time PCR instrument (CFX96-IVD, Bio-Rad, Hercules, CA, USA). As positive control, plasmid containing *T. gondii* RE gene diluted serially 10-fold ranging from 10^6 to 10^1 copy plasmids/µL and one negative control prepared by replacing template DNA with distilled water were used.

After screening the samples by commercial Real Time PCR, another Real Time PCR method [23], using hybridization probe was performed to confirm the presence of *T. gondii* RE gene by quantification and melting curve analyses as previously described [17,18,23,24]. During the *in house* Real Time PCR, the primers targeting the 134 bp region of *T. gondii* RE gene were 5'-AGGCGAGGGTGAGGATGA-3' (18nt, TOX-SE forward primer; final concentration: 0.5 µM) and 5'-TCGTCTCGTCTGGATCGCAT-3' (20nt, TOX-AS reverse primer; final concentration: 0.5 µM) and the hybridization probes were 5'-GCCGGAAACATCTTCTCCCTCTCC-3'-FL (24nt, TOX FLU, labeled at the 3' end with fluorescein; final concentration: 0.1 µM) and 5'-640-CTCTCGTCGCTTCCCAACCACG-3' (22nt, TOX LCR labeled at the 5' end with LC-Red 640; final concentration: 0.5 µM) (IDT). Quantification and melting curve analysis were performed by Real Time instrument (1.5 LightCycler, LightCycler software, Version 3.5, Roche, Mannheim, Germany). The PCR reaction with a 20 µL final volume included primers and probes, 1× mix with 5 mM $MgCl_2$ (LightCycler Fast Start DNA Master HybProbe, Roche, Mannheim, Germany), 5 µL purified DNA template or controls. The amplification reaction was performed as follows: 10 minutes initial denaturation incubation at 95ºC, followed by 50 cycles of 5 seconds at 95ºC, 10 seconds at 60ºC, and 15 seconds at 72ºC. As positive control, *T. gondii* genomic DNA serially 10-fold diluted ranging from 10^6 to 10^1 parasites per µL was used. One negative control prepared by replacing template DNA with distilled water was used in each experiment to assess contamination and standard measures were taken to prevent contamination. Melting curve analysis was performed using the following calculated protocol: 20 s denaturation at 95ºC with temperature transition rate 20ºC/s followed by 20 seconds annealing at 40ºC with temperature transition rate 20ºC/s and extension incubation gradually increasing temperature to 85ºC with temperature transition rate 0.2ºC/s.

IgG Enzyme-Linked ImmunoSorbent Assay

ELISA detecting anti-*T. gondii* IgG antibodies of cats was developed in a previous study of our study group and performed as previously described [17]. Briefly, each well of microtiter plate (MaxiSorp, Nunc, Roskilde, Denmark) was coated with 100 µL antigen suspension containing 6×10^5/mL *T. gondii* Ankara strain tachyzoites [25], and incubated for one hour. Next, the plates were washed thrice with 200 µl PBS-T [PBS containing 0.05% (v/v) Tween 20 (pH: 7.4)]. Then, the plates were blocked (5% nonfat dry milk containing 0.05% PBS-T) for 30 minutes at room temperature and washed thrice with PBS-T. Thereafter, the plates were probed with sera at a dilution of 1/64 in blocking buffer for one hour and washed thrice with PBS-T. Next, the plates were probed with peroxidase conjugated *anti*-cat IgG (1:5000) (Goat anti-cat IgG-HRP, Santa Cruz, Dallas, Texas, USA) diluted in PBS-T for 1 hour and washed thrice with PBS-T. Bound antibodies were visualized after adding 3, 3', 5, 5' tetramethylbenzidine (TMB) substrate. Reaction was stopped by adding 75 µL of 2 N sulfuric acid and the results were evaluated in a micro titer plate reader (ELx808, Bio-Tek, Vinooski, VT, USA) at 450 nm. Serum was considered positive if the absorbance value (AV) exceeded the mean AV+2SD of the negative control serum samples. Negative and positive control serum samples were obtained from our previous study in which a commercial multispecies ELISA kit (ID Screen, Toxoplasmosis Indirect Multi-species ELISA test, ID.VET, Montpellier, France) was used to determine the negative and positive controls [17].

Statistical analysis

Data obtained during the study were processed by a statistical program (Prism 3.03, GraphPad). A two-tailed unpaired t test or one way-ANOVA with 95% confidence interval was used to determine the significance between the PCR results and seropositivity rates.

Results

Prevalence of Toxoplasma gondii infection in stray cats using direct microscopy

According to the results of microscopy, oocysts of the size of *T. gondii* were detected in feces sample of 2 cats (0.43%; 2/465). Specifically, these cats were from Konak district where the oocyst prevalence was 1.09% (2/183). Under light microscopy, the unsporulated oocysts were morphologically like spherical and approximately 10-12 µm in diameter and the sporont almost filled the oocyst as described [26]. Regarding these 2 cats (named #K7 and #K147), RT-PCR targeting *T. gondii* RE gene was negative in both feces and blood samples and only K7 was seropositive for anti-*T. gondii* IgG antibodies.

Prevalence of Toxoplasma gondii infection in stray cats using PCR

According to the results of commercial PCR screening, *T. gondii* RE gene was detected in 68 feces samples. After excluding 4 samples (2 samples from Karşıyaka, one sample from Çiğli and one sample from Karabağlar) that inhibited the PCR from the total number of samples, the samples used to determine prevalence for PCR decreased to 461 and the prevalence rate of *T. gondii* RE gene in feces samples of all cats was 14.75% (68/461).

We assayed the PCR positive 68 feces samples by Real Time PCR using hybridization probe method to confirm the presence of *T. gondii* RE gene as well as to eliminate cross positivity due to DNA homology between *Hammondia hammondi* or *Besnoitia besnoiti* using melting curve analyses. Using NCBI Blast tool, blasting *T. gondii* repeat region (GenBank No: AF146527) resulted with similarities to *H. hammondi* repetitive sequences (GenBank Nos: EU493279.1; EU493280.1; EU493281.1; EU493282.1; EU493283.1; EU493284.1; EU493285.1; JX477424.1; KC223619.1) and there was 3 to 5 disassociation (mismatches) in the hybridization probe regions of *in house* real Time PCR target sequence which is 48bp in size. *T. gondii* repeat region (GenBank No: AF146527) also showed similarity to *B. besnoiti* (an Apicomplexan parasite from Sarcocystidae family) WD domain, G-beta repeat-containing protein (GenBank No: XM_029363716.1) in a 40 bp region in which the identity rate was 85% (34/40) and *B.besnoiti* had 3 mismatches and 3 deletions. During the blast analyses of top 100 hits, there were some similarities to *Parastagonospora nodorum* (fungal pathogen of wheat), *Lutra lutra* (Eurasian otter), *Linum usitatissimum* (Flax), *Oryzias latipes*, (Japanese rice fish), *Cucumis sativus* (Cucumber), *Gadus morhua* (Atlantic cod), *Kaloula latidisca* (a species of frog), *Rhodotorula toruloides* (oleaginous red yeast), *Scleropages formosus* (Asian arowana), *Glycine soja* (wild soybean), *Oryza sativa* (Asian rice), *Spirodela polyrhiza* (species of duckweed) but not at the same level as *H. hammondi*.

According to the melting curve analysis, a peak at 68 ± 0.5°C (*T. gondii* specific melting point) was detected in all positive samples except 2 samples (cat #K82 and cat #N96) that shifted. We excluded these 2 PCR positive samples from *T. gondii* infection cases due to possible cross reactivity with *H. hammondi* and the samples used to determine prevalence for PCR decreased to 459. Thereafter, the prevalence of *T. gondii* infection in stray cats of İzmir was updated as 14.37% (66/459). The prevalence rates were 16.48% (30/182) for Konak, 11.80% (17/144) for Narlıdere, 15.06% (11/73) for Çiğli, 11.10% (4/36) for Karşıyaka, and 16.66% (4/24) for Karabağlar. The difference between them was not statistically significant (Figure 1, Table 1).

According to the results of PCR screening of blood samples, the prevalence rate of *T. gondii* RE gene was 8.81% (41/465). Prevalence rates of Konak (8.74%; 16/183), Narlıdere (7.58%; 11/145), Çiğli (10.81%; 8/74), Karşıyaka (10.52%; 4/38), and Karabağlar (8%; 2/25) were similar to each other and the difference between them was not statistically significant (Table 1). Overall, the prevalence rates in feces and blood samples were 14.37% and 8.81%, respectively. On the other hand, feces and blood sample PCR results of the same cat were not always positive. In 6.31% of the cats (29/459), feces and blood PCR positivity were both positive. The specific rates for each district are shown in Table 1.

Table 1. Stray cats with acute toxoplasmosis in Konak, Narlıdere, Çiğli, Karşıyaka, and Karabağlar districts of İzmir.

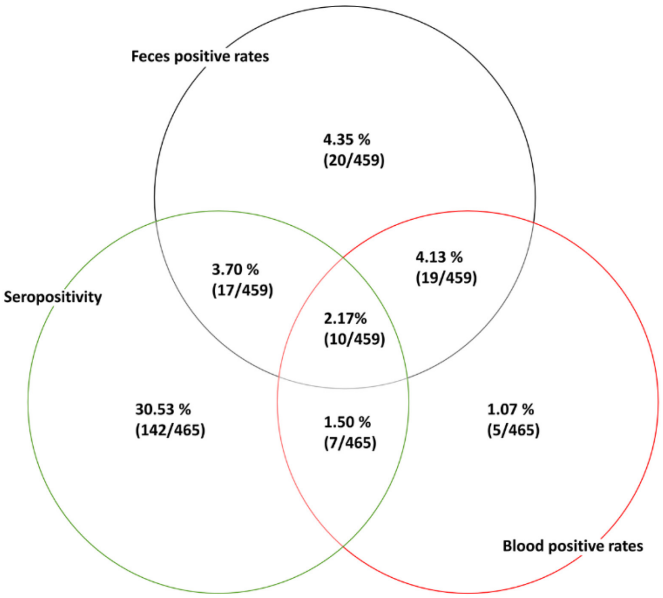
	Konak	Narlıdere	Çiğli	Karşıyaka	Karabağlar	Totally İzmir
Prevalence of *T. gondii* DNA in feces by PCR	17.03% (31/182)	11.11% (16/144)	15.06% (11/73)	11.10% (4/36)	16.66% (4/24)	14.37% (66/459)
Prevalence of *T. gondii* DNA in blood by PCR	8.74% (16/183)	7.58% (11/145)	10.81% (8/74)	10.52% (4/38)	8% (2/25)	8.81% (41/465)
Feces and blood PCR positive cats	4.94% (9/182)	6.25% (9/144)	9.58% (7/73)	8.33% (3/36)	4.16% (1/24)	6.31% (29/459)

Seroprevalence rates

The results of IgG ELISA showed that the seroprevalence was 37.84% (176/465) in İzmir. The seropositivity rates were 49.72% (91/183) in Konak, 31.03%; (45/145) in Narlıdere, 33.33% (25/74) in Çiğli, 26.31% (10/38) in Karşıyaka, and 20% (5/25) in Karabağlar (Figure 1) (Table 2).

Feces and blood sample PCR results as well as serology result of the same cat were not always positive. The *T. Gondii* DNA positivity rates in feces and blood as well as seropositivity rates were detailed given in Figure 2.

Figure 2. Positivity rates compared to tests.



Discussion

It may be misleading to determine the transmission potential of *T. gondii* infection from cats to humans and animals using only serology or microscopy due to two major reasons; 1) In cats infected by *T. gondii*, antibody response against *T. gondii* usually occurs after the cat stops shedding oocysts 2) During the microscopic examination of feces, *H. hammondi* and *Besnoitia* spp. oocyst can't be discriminated from *T. gondii* oocysts because they are very similar in size [27,28]. Microscopy and/or PCR have been used commonly, to determine the presence of oocysts in cat feces [19,28-35]. Although mouse bioassay confirms the definite diagnosis it is time consuming and cannot be performed easily due to ethical issues and cost effectiveness.

Microscopy has been used to determine the prevalence of *T. gondii* oocysts. In 2008, 24,106 cats from Germany and other European countries were examined by microcopy and *T. gondii*-like oocysts were detected in 74 samples (0.31%) [28]. In another study, among the 18,259 cat feces examined by microscopy, *T. gondii*-like oocysts were detected in 105 samples (0.57%) [32]. In the United States, among the 326 feces samples examined by microscopy, *T. gondii*-like oocysts were detected in only three samples (0.9%) [30]. According to the results of microscopy in this study, *T. gondii* like oocysts were detected in two feces sample of 465 cats (0.43%). These two cats were seropositive but *T. gondii* RE gene was not detected in feces sample indicating a source of oocysts other than *T. gondii*. According to the results of these studies and our study, *T. gondii* like oocyst shedding rates are pretty low using microscopy. This can be due to the low sensitivity of microscopy in minimally oocyst shedding cats [19,31,34]. In a study conducted in Virginia, United States, among the 60 fecal samples, 11 inhibited PCR and of the 49 cats tested, three samples were true positive (6%) [34]. In Switzerland, out of 252 feces samples, *T. gondii* like oocysts were detected in two samples by microcopy and one was confirmed by PCR (0.4%) [29]. In Finland, *T. gondii* like oocysts were detected in two feces samples obtained from 131 shelters cats and one was confirmed by PCR (0.7%) [33]. In Italy, *T. gondii* like oocysts were not detected in feces samples obtained from an urban population of colony cats (n =50) by microscopy however *T. gondii* DNA was detected in 8 of them (16%) [35].

In this study, according to the results of PCR screening, *T. gondii* RE gene was detected in 66 feces samples of 459 cats and the prevalence rate of *T. gondii* RE gene in feces samples was 14.37%. The commercial RT-PCR using hydrolysis probe couldn't eliminate the 2 samples with shifting TM and *in house* RT-PCR using hybridization probe resolved this issue. Future studies targeting *T. gondii* RE gene are suggested to use RT-PCR using hybridization probe to resolve such problems.

According to the results of PCR screening of blood samples, *T. gondii* RE gene was detected in 41 samples (8.81%; 41/465). Overall, the prevalence rates in feces

Table 2. Stray cats with chronic toxoplasmosis by ELISA in Konak, Narlıdere, Çiğli, Karşıyaka, and Karabağlar districts of İzmir.

	Konak	Narlıdere	Çiğli	Karşıyaka	Karabağlar	Totally İzmir
Seropositivity rate	49.72% (91/183)	31.03% (45/145)	33.33% (25/74)	26.31% (10/38)	20% (5/25)	37.84% (176/465)

and blood samples were 14.37%and 8.81%, respectively. Feces and blood sample PCR results of the same cat were not always positive. In 6.31% of the cats, feces and blood PCR were both positive. This difference in prevalence rates can be linked to some cats that may be at the very first hours of infection and tachyzoites did not appear in blood yet. This is supported by a study in a murine model in which after the ingestion of oocysts or tissue cysts, sporozoites and bradyzoites are released, invade the intestinal cells and turn in to tachyzoites in 12 and 18 hours, respectively [36,37]. Another possibility can be that the feline immune deficiency virus (FIV) may affect *T. gondii* oocyst shedding [38], and cats with FIV may excrete oocyst but tachyzoites may not present in blood samples or vice versa. *H. hammondi* remains confined to the enterocytes and do not penetrate extra-intestinal tissues of cat [39], and because of this *H. hammondi* cannot be kept responsible for the prevalence difference between feces and blood.

In order to correlate the PCR results with antibody response against *T. gondii*, an ELISA developed in our previous study [17], was used to determine the seroprevalence in this cat population. The results of IgG ELISA showed that the seroprevalence was 37.84% (176/465) in İzmir which increased compared to our previous study (34.47%) conducted between 2012 and 2014.

Among the feces and/or blood PCR positive cats 35.89% of them were seropositive. In the feces PCR positive group, seropositivity was 40.90% and 41.46% in blood PCR positive group (Figure 2). These results are expectable since an antibody positive cat is very unlikely to shed oocysts and antibodies against *T. gondii* need 2 to 3 weeks to develop [31]. Moreover, at the beginning of *T. gondii* infection in cats in which the entero-epithelial stage occurs, oocyst propagation in intestinal epithelium may not induce strong specific anti-*T. gondii* IgG response. This is supported by the result in which *T. gondii* oocyst was detected by PCR in 15.34% seropositive cats. Thus, seronegative cats with PCR positive results may seroconvert soon also.

Conclusions

Overall, this study evaluated the prevalence of *T. gondii* DNA in feces samples of the stray cats of İzmir which is the third biggest city located on Western coast of Turkey with a human population of more than 4.2 million. The prevalence of *T. gondii* DNA in feces samples of the stray cats living in İzmir is over 14%. Detecting *T. gondii* DNA in feces sample is an indication of *T. gondii* infection and we can assume that these cats can be contagious for other animals, humans, and birds. Similarly, depending on our previous study showing the high *T. gondii* prevalence in birds in our study area, chronically infected birds can also be a toxoplasmosis source for non-infected cats [18]. Taking into consideration that the seroprevalence rate in humans and wild birds in İzmir which is around 30% and 90%, respectively [16,18]; avoiding feeding of stray cats with raw meat may help decrease the disease burden in cats, other animals, and humans. But we must keep in mind that we cannot prevent a stray cat from hunting birds, rodents and eating insects (e.g. cockroaches harbor *T. gondii* in high quantity) [39,40]. Thus, monitoring *T. gondii* DNA in the feces of stray cats by municipality veterinary clinics, collecting feces of stray cat and treatment of positive cat cases has utmost importance and may lower the prevalence of the *T. gondii* infection in cats, other animals, humans, and birds in İzmir.

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Authors' contributions

Conceptualization: MK, HC, NS, BY, EA, FD, AG, HGÖ, NA, MY,OÖ,MA, TÇ, ŞC, ADD, AYG, MD; Data curation: MK, HC, EAŞ, HGÖ, ŞC, ADD, AYG, MD; Formal analysis: MK, HC, NS, BY, EA, EAŞ, FD, AG, HGÖ, NA, MY, OÖ, MA, TÇ, ŞC, ADD, AYG, MD; Funding acquisition: MD Investigation: MK, HC, NS, BY, EA, EAŞ, FD, AG, HGÖ, NA, MY, OÖ, MA, TÇ, OÖ, MA, TÇ, ADD, MD; Methodology: MK, HC, HGÖ, ŞC, ADD, AYG, MD; Project administration: name; AYG, MD; Resources: MK, ADD, AYG, MD; Supervision: AYG, MD; Validation: ŞC, MD ; Visualization: MD; Writing - original draft: MK, MD; Writing - review & editing: MK, HC, NS, BY, EA, EAŞ, FD, AG, HGÖ, NA, MY, OÖ, MA, TÇ, ŞC, ADD, AYG.

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