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Prevalence of *Toxoplasma gondii* in pet and stray cats in Klang Valley, Malaysia

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Abstract. *Toxoplasma gondii*, a zoonotic protozoan that has a worldwide distribution, is known to infect many warm-blooded vertebrates. The feline species including domestic cats are the definitive hosts for *Toxoplama gondii* and shed the infective oocyst. There is lack of information on the prevalence of *Toxoplasma gondii* in cats in Malaysia. The objective of this study was to determine both the seroprevalence of *T. gondii* and the prevalence of *T. gondii* DNA in cats' feces in Klang Valley, Malaysia. 198 blood and 201 fecal samples were collected from pet and stray cats from the local council, Dewan Bandaraya Kuala Lumpur (DBKL) and University Veterinary Hospital, Universiti Putra Malaysia respectively. The overall seroprevalence of *Toxoplasma gondii* in cats in the Klang Valley was found to be 5.5%. There was a high prevalence (10.5%) of *T. gondii* DNA detected in the cat fecal samples in both pet and stray cats suggestive of *T. gondii* oocyst shedding. Stray cats showed a higher seroprevalence and molecular prevalence of *T. gondii* than the pet cats. However, comparative analysis using *Chi*-square test showed no significant difference between both groups (P>0.05). Higher prevalence (10.5%) of cats shedding *T. gondii* DNA as compared to the seroprevalence (5.5%) was found in the cat population in the Klang Valley. The high prevalence of cats shedding *T. gondii* DNA is alarming as this may directly reflect the number of oocysts excreted into the environment posing a significant public health hazard.

INTRODUCTION

Toxoplasma gondii is the only species that belongs in the genus *Toxoplasma*. *T. gondii* is a protozoan parasite that infects most mammalian species including cats, dogs, cattle as well as humans. It is a major human health concern in many countries as it causes clinically important diseases (Robert-Gangneux and Dardé, 2012). Seroprevalence of toxoplasmosis in humans has been reported between 3%–70% in Southeast Asian (SEA) countries (Nissapatorn, 2007). In Malaysia, prevalence of *T. gondii* in healthy humans can be up to 30.2% and 33% in children (Nissapatorn, 2007). Pregnant women as well as immunocompromised patients have been reported to have higher seroprevalence rates of *T. gondii* of up to 49% (Nissapatorn *et al.*, 2003; Nissapatorn, 2007; Andiappan *et al.*, 2014).

The main cause of toxoplasmosis in humans was found to be due to consumption of raw meat as well as frequent contact with cats (Nissapatorn, 2007; Andiappan *et al.*, 2014; Brandon-Mong *et al.*, 2015). A study in Klang Valley, Malaysia in 2015 showed that pet owners and veterinary technicians had a seroprevalence between 30–33% (Brandon-Mong *et al.*, 2015). Frequent contact with cats increases the risk of zoonotic transmission as domestic cats will shed *T. gondii* oocyst

up to 21 days upon initial infection (Lilly and Wortham, 2013; Lappin *et al.*, 2015). Reshedding of *T. gondii* in cats has also been reported (Dubey *et al.*, 2009; Elmore *et al.*, 2010).

Toxoplasma gondii completes its life cycle in the domestic cats (Cerro *et al.*, 2014). It rarely causes clinical manifestations however, clinical cases are seen in immunocompromised cats (Dubey *et al.*, 2009; Dabritz and Conrad, 2010). Clinical manifestations frequently observed in cats include ocular toxoplasmosis and congenital toxoplasmosis, some with severe respiratory and neurological signs (Dubey *et al.*, 2009). Severe clinical cases are usually seen in kittens infected transplacentally (Dubey *et al.*, 2009).

Due to the short oocyst shedding period, the prevalence of oocysts found in fecal samples tends to be low. Approximately, only about 1% of cats in a population are found to be shedding oocysts at any given time (Hill and Dubey, 2002; Elmore *et al.*, 2010). A high prevalence of 6% of cats shedding *T. gondii* oocyst was found in a study in the United States (Lilly and Wortham, 2013). Various laboratory methods have been used to diagnose toxoplasmosis such as microscopic detection, bioassay in mice and PCR (Hooshyar *et al.*, 2007; Salant *et al.*, 2007; Lilly and Wortham, 2013; Jung *et al.*, 2015). Microscopic detection of oocyst is less reliable than bioassay and PCR as *T. gondii* oocyst is morphologically similar to *Hammondia hammondi* and *Besnoita* spp. (Dubey *et al.*, 2009; Györke *et al.*, 2011). On the other hand, bioassay in mice is a more time consuming diagnostic method as compared to PCR (Salant *et al.*, 2010; Saki and Khademvatan, 2014). Polymerase chain reaction is deemed to be the most sensitive, specific and convenient test for *T. gondii* oocyst detection (Salant *et al.*, 2010; Zewdu *et al.*, 2015). Serological tests of *T. gondii* are more commonly used as the diagnostic method for infection (Dubey *et al.*, 2009). Studies across the world estimated seroprevalence of *T. gondii* in domestic cats with a wide range, between 11–91% (Jittapalapong *et al.*, 2007; Lee *et al.*, 2011; Castillo-Morales *et al.*, 2012; Opsteegh *et al.*, 2012; Garcia *et al.*, 2014; Liu *et al.*, 2014). The huge range of seroprevalence of *Toxoplasma gondii* may be due to different serological assays used in each individual study (Vollaire *et al.*, 2005; Millán *et al.*, 2009; Castillo-Morales *et al.*, 2012; Oi *et al.*, 2015). Enzyme-linked immunosorbent assays were shown to have a higher sensitivity and specificity in comparison with other serological assays such as indirect immunofluorescent antibody test (IFAT) and modified agglutination test (MAT) in recent studies (Györke *et al.*, 2011; Paştiu *et al.*, 2015). There is scant data regarding the seroprevalence of *T. gondii* in cats in Malaysia. Only one study has been conducted in the pet cat population in Ipoh, Malaysia using IFAT which detected a seroprevalence of 14.5% (Chandrawathani *et al.*, 2008). Furthermore, there is no study on the prevalence of *T. gondii* oocyst in the cat fecal samples using molecular detection techniques. Thus, the objectives of the present study were to determine the presence of *T. gondii* in pet and stray cats in the Klang Valley, Malaysia and to determine the risk factors that may be involved.

METHOD

Study design

This cross-sectional study involved 101 pet cats and 100 stray cats. Cats that were owned and kept indoors or allowed to roam outdoors were categorized as pet cats, whereas cats without owners were considered as stray cats. Pet cat samples included those presented to University Veterinary Hospital, Universiti Putra Malaysia. Stray cat samples were obtained from the local council (Dewan Bandaraya Kuala Lumpur). The age, sex and management of both the pet and stray cats were noted upon collection. Management was only noted for the pet cat population if they were kept totally indoors or allowed to roam outdoors as well as their household management of either kept in a single or multi-cat household. This study was approved by the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (AUP-R088/2016).

Sample collection

Blood from 198 cats were collected via jugular venipuncture in plain tubes and were centrifuged at 5000rpm for 5 minutes to separate the serum. Serum samples were kept in -20°C until further use. Fecal samples were collected from 101 pet cats and 100 stray cats using a fecal swab. Fecal samples collected were then placed in 100ul lysis buffer and kept at -20°C for storage until use.

Serological analysis

A total of 198 (100 pet cats and 98 stray cats) serum samples collected were screened for IgG antibodies against *Toxoplasma gondii* using indirect-ELISA ID Screen®Toxoplasmosis Indirect Multi-species (ID.vet Innovative Diagnostics, Grabels, France). The optical density of each sample was read under 450nm using a microplate reader TECAN Infinite® 200M Pro (Tecan Group Ltd, Mannedorf, Switzerland). The sample/positive ratio (S/P %) were calculated using the following formula

$$S/P\ \% = \frac{OD_{sample} - OD_{NC}}{OD_{PC} - OD_{NC}} \times 100$$

According to the manufacturer's guidelines S/P values that were greater than 50% were considered positive.

Molecular analysis

DNA was extracted from the fecal samples using QIAamp stool mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Extracted DNA was subjected to nested-PCR to amplify the *T. gondii* B1 gene with two sets of primers that produces a product size of 90bp (Jones *et al.*, 2000). First amplification reaction mixture consisted of 5µL of Promega 5x Green GoTaq®Flexi Buffer (Promega Corporation, Madison,USA). 1mM $MgCl_2$, 0.1mM dNTP mix, 1.25U of Taq polymerase with 0.1pmoL of both first set of primers and 5µL of sample DNA with a total volume of 25µL. 1µL of the first PCR product was then used in the second PCR amplification mix which included 5µL of Promega 5x Green GoTaq®Flexi Buffer, 1.5mM, 0.1mM of dNTP mix, 1U of Taq polymerase and 0.5pmoL of second set of primers with a total volume of 25µL. Both amplification conditions were as used by Jones *et al.*, 2000. Products were then loaded on 2% agarose gel for gel electrophoresis. Gel was stained with ethidium bromide and viewed under Biorad Gel Doc™ XR (Biorad, Hercules, CA, United States). Positive samples were then purified using Qiagen PCR purification kit (QIAGEN, Hilden, Germany) and were sent for sequencing.

Statistical analysis

Samples were grouped into different risk factors which included age, sex, and management of the cats. Comparative analysis of each group was performed using *Chi*-square test and Fisher exact test. Statistical analysis was performed using IBM SPSS Statistics 22 (IBM, Greenville, United States). The differences were considered statistically significant when $P<0.05$.

RESULTS

Seroprevalence of *T. gondii* in pet and stray cats

The overall seroprevalence in the cat population was 5.6% (11/198). Among serum samples from the stray cats, 7 out of 98 were positive for IgG antibodies with a seroprevalence of 7.1%. In the pet cat population, 4 samples were positive with a seroprevalence of 4%, calculated at a 95% confidence interval. Stray cats showed a higher seroprevalence as compared to the pet cats however, *Chi*-square test demonstrated no significant difference between the two groups. All 4 positive samples in the pet cat population were from cats 1 year old and above, and there were no positives among the kitten and junior cats less than 1 year old. Despite the difference, Fisher exact test showed no significant difference between these two groups (OR = 0.177; 95% = 0.020, 1.570; P> 0.05). Age of the stray cats also showed no

significant association with seropositivity (OR = 2.200; 95% = 0.388, 12.483; P>0.05). Comparative analysis using Fisher exact test showed no significant difference between the sex of the cats in both the pet and stray cat population (P>0.05). Management of the pet cat population also showed no significant association with the seropositivity of toxoplasmosis (P>0.05). Results are listed as in Table 1 and Table 2.

Molecular prevalence of *Toxoplasma gondii* in feces of pet and stray cats

Fecal samples of 201 cats (100 stray cats and 101 pet cats) were collected to detect *Toxoplasma gondii* DNA. A total prevalence of 10.5% (21/200) for both pet and stray cats positive for *T. gondii* was obtained. A high prevalence of 15% (15/100) was found in stray cats and 6% (6/100) in pet cats. Positive samples are as shown in Figure 1. All samples

Table 1. P value, odd ratio, confidence interval for sex, age, management of the pet cat sampled for seroprevalence *T. gondii*

	P value	Odd ratio	Confidence interval	
			Lower	Upper
Sex	0.097	0.162	0.018	1.442
Age	0.135	0.177	0.018	1.774
Indoor/Outdoor	0.282	2.667	0.286	24.896
Single/Multi cat household	0.363	3.318	0.277	39.732

Table 2. P value, odd ratio, confidence interval for sex and age of the stray cat sampled for seroprevalence *T. gondii*

	P value	Odd ratio	Confidence interval	
			Lower	Upper
Sex	0.439	2.446	0.451	13.261
Age	1.000	1.289	0.271	6.129

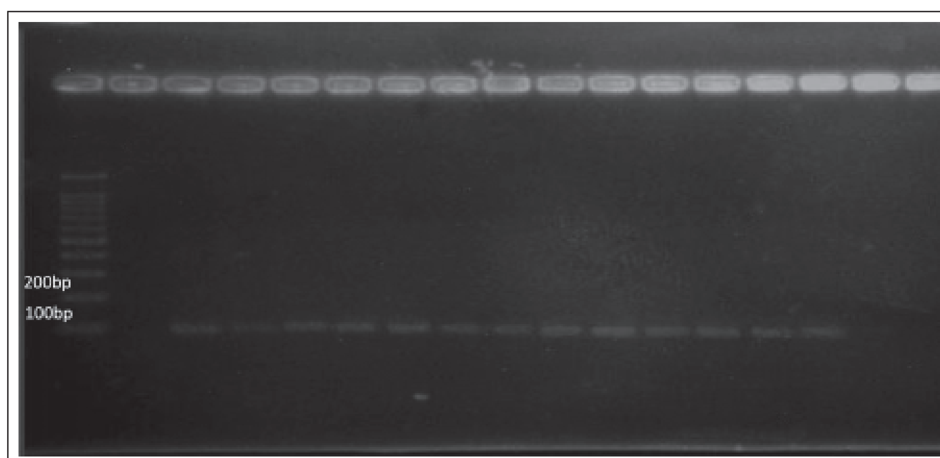

Figure 1. PCR amplification of B1 gene from the cat fecal samples. This figure illustrates positive samples at 96bp size.

Table 3. P value, odd ratio, confidence interval for sex, age, management of the pet cat sampled for shedding *T. gondii* oocyst

	P value	Odd ratio	Confidence interval	
			Lower	Upper
Sex	1.000	0. 958	0.184	4.993
Age	1.000	0.811	0.129	5.103
Indoor/Outdoor	0.207	0.300	0.052	1.929
Single/Multi cat household	1.000	1.167	0.133	10.221

Table 4. P value, odd ratio, confidence interval for sex and age of the stray cat sampled for shedding *T. gondii* oocyst

	P value	Odd ratio	Confidence interval	
			Lower	Upper
Sex	0.585	1.348	0.460	3.956
Age	0.280	0.513	0.151	1.749

sequenced were compared with those in the GenBank database using BLAST (Basic Local Alignment Search Tool). All positive samples were matched and confirmed to be *Toxoplasma gondii*. *Toxoplasma gondii* isolates that were sequenced showed 98-100% similarity to the isolates from small ruminants in Mexico, KX2770388 and Bangladesh KU900748. Statistical analysis using *Chi*-square test showed no significant difference between sex, age of the cats as well as management of the cats for both pet and stray cats. The results are as in Table 3 and Table 4.

DISCUSSION

The overall seroprevalence for *Toxoplasma gondii* in cats in the Klang Valley was found to be 5.6%. This seroprevalence is comparatively lower compared to several other studies using Indirect-ELISA (Györke *et al.*, 2011; Castillo-Morales *et al.*, 2012; Opsteegh *et al.*, 2012; Deksne *et al.*, 2013; Rengifo-herrera *et al.*, 2017). Previously a study in Ipoh, Perak Malaysia showed a higher seroprevalence of 14.5% (Chandrawathani *et al.*, 2008) and in neighboring Thailand 11.7% (Jittapalapong *et al.*, 2007) and Philippines 22% (Garcia *et al.*, 2014). The difference in seroprevalence in different countries has been suggested due to different climate and geographical location of each study conducted as well as serological tools used in the different studies. However the ELISA used in the current study has high sensitivity (96.8%) and specificity (96.1%) for the detection of antibodies against *T. gondii* in the feline species (Györke *et al.*, 2011) therefore the low seroprevalence obtained in this study may be an indication of either actual decrease in number of infected cats in the region or recent infections where the cats have yet to seroconvert.

This study demonstrated a high prevalence of 10.5% *T. gondii* detected in the cat fecal samples in the Klang Valley. Detection of *T. gondii* in cat feces using PCR is usually assumed to be difficult due to the short oocyst shedding period, and a few studies have demonstrated lower prevalence rates between 0–6% (Mancianti *et al.*, 2010; Lilly and Wortham, 2013; Jung *et al.*, 2015). However, there is one study that showed a high prevalence of 19.4% in cat fecal samples in Ethiopia (Dubey *et al.*, 2013). Therefore again the prevalence rates

appear to differ based on geographical location and the degree of exposure of cats to the protozoa.

Stray cats in the Klang Valley demonstrated a molecular prevalence of 15% which was much higher than that in pet cats (6%). Similarly, seroprevalence of *T. gondii* was higher in stray cats compared to the pet cats in the Klang Valley. This finding is similar to other studies where feral or stray cats generally showed a higher seroprevalence than pet cats (Gauss *et al.*, 2003; Nutter *et al.*, 2004; Vollaire *et al.*, 2005; Hooshyar *et al.*, 2007; Liu *et al.*, 2014). Higher seroprevalence in stray cats may be due to constant exposure to the outdoor environment with higher chances of coming in contact with soil contaminated with *T. gondii* oocysts. Stray cats also have higher risk of ingesting tissue cysts from infected rodents, small mammals as well as wild birds *(Gauss et al.*, 2003; Jung *et al.*, 2015). Thus, pet cats that are allowed to roam outdoors have higher chances of getting *T. gondii*, as seen in this study where 4 out of 6 roaming cats positive for *T. gondii* in their feces. However, statistical analysis showed no significant difference (P>0.05) between stray and pet cats. This could be due to the sampled pet population in this study as 60% of the sampled cats were managed indoor.

The present study showed a low seroprevalence of IgG antibodies against *T. gondii* in the cat population of Klang Valley but in contrast, a high molecular prevalence of *T. gondii* detected from the feces of the cats. Seropositivity of *T. gondii* has been shown to increase with the age of cats in several studies (Must. K *et al.*, 2017; Wang *et al.*, 2017). The low seroprevalence in the present study may be due to the age of the sampled cats. 84% of the samples were collected from cats aged 6 years and below. Cats aged above 10 years old was found to be a significant risk factor for *T. gondii* seropositivity (Györke *et al.*, 2011). Eventhough, comparative analysis using *Chi*-square test showed no significant difference (P>0.05) in this study, older cats showed higher seropositivity as all seropositive samples were cats 1 year old and above. IgG antibodies against *T. gondii* in cats has been reported to appear around 1 to 3 weeks after the initial infection usually regarded as chronic infections. (Robert-Gangneux and Dardé, 2012). All cats that were shedding *T. gondii* DNA in the fecal samples were negative for IgG antibodies when tested in this study, which suggests that those cats that were PCR positive had not yet seroconverted. Thus, this could suggest that these cats have been recently infected and might be shedding *T. gondii* oocyst at that point of time (Robert-Gangneux and Dardé, 2012; Lilly and Wortham, 2013; Lappin, 2015).

In conclusion, the present study demonstrated a high *T. gondii* molecular prevalence of 10.5% among cats in the Klang Valley, Malaysia. 5.5% of the cat population in the Klang Valley were seropositive for IgG antibodies against *T. gondii*. The high prevalence of cats possibly shedding *T. gondii* DNA is alarming as this may indicate the number of oocysts directly excreted into the environment increasing the risk of zoonotic disease transmission.

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