

Prevalence and molecular characterization of zoonotic helminths in dogs

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Abstract A cross sectional study was designed to ascertain the prevalence of zoonotic helminths and protozoan parasites in dogs by employing conventional techniques and molecular characterization using polymerase chain reaction (PCR). Companion animals are reservoir hosts for helminths and protozoa species, and cohabitation with humans play a pivotal role in the transmission of zoonotic parasites to humans. A total of 510 faecal samples were collected from pet dogs belongs to various zones of Chennai city, Tamil Nadu, India and were processed by conventional techniques and molecular techniques. Out of 510 dog faeces, 121 samples were found positive for the parasitic eggs, prevalence rate was found to be 23.72%. Species-wise prevalence of the parasite was tabulated. PCR for the species-specific identification of the parasitic eggs was performed. The results revealed 38 samples were positive with product size of 540 bp specific for *Ancylostoma caninum*, 25 samples were positive which yielded a product size of 380 bp which is specific for *Toxocara canis*. None of the faecal samples tested were positive for *Echinococcus granulosus* which were positive for the presence of *Taenia* spp. eggs by microscopy. We recommend prevention and control measures focused on improving regular deworming, enhancing awareness of parasitic zoonotic diseases to minimize the transmission risk of parasitic zoonotic

diseases from companion animals to humans. PCR can be widely used for species-specific identification of the zoonotic parasites.

Keywords Parasitic zoonoses · Pet dogs · Prevalence · Molecular characterization · PCR

Introduction

Companion animals are domestic-bred or domesticated animals whose physical, emotional, behavioural and social needs can be readily met as companions in the home or in close daily relationship with humans. Among the companion animal's most popular pets are dogs and cats (Grondalen et al. 2004) which play a significant role in many households as they live in close association and contribute to the passionate development of children and the well-being of their owners in societies throughout the world. This close association tends to increase the risk of acquiring zoonotic diseases from the pets (Reynolds et al. 2016). Among all the health hazards, parasitic diseases are more common in pet animals with the potential of being transmitted to humans. Many pet owners are unaware about the zoonotic parasites that are harboured by their pets and their route of transmission. Enteric parasites harboured by pet dogs pose a potential health risk problems to humans (Schantz 1994). Transmission of these infections from pets to humans is through contact with animal hair, food and water contaminated with dog excreta or secretions (Joffe et al. 2011). In developing countries like India, the companion animal zoonotic diseases are on increasing trend which attributed due to various reasons like irregular deworming, increased population of community owned animals as pets, increase in population density of humans

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and sharing of living space between companion animals and humans in both urban and rural settings.

The common zoonotic gastrointestinal helminthic and protozoan infections are Toxocarosis, Ancylostomiasis, Echinococcosis and Giardiasis. Cutaneous contact with the infective stages of canine hookworms can lead to the development of cutaneous larva migrans (CLM) in humans. Infections with *Ancylostoma caninum* can result in eosinophilic enteritis (EE) (Landmann and Prociw 2003). Humans become infected with *Toxocara* when they accidentally ingest embryonated eggs through contamination of infected soil, food, fomites or by direct contact with dogs. Young children are at high risk of picking up the *Toxocara* spp. infection from various public places like playgrounds, parks and gardens contaminated with faeces of dogs and cats and by the habit of eating soil (Thomas and Jeyathilakan 2014) and in dogs the infection rate was much higher (62.79%) in pups as compared to (7.8%) in adult. Sangaran et al. (2014) reported that an overall incidence of hydatidosis is (6.5%) in sheep and (5.8%) in goats from slaughter house, Chennai and also found that higher number of fertile hydatid cysts was noticed in sheep which plays a major role in dissemination of infection to dogs.

Owing to difficulties with conventional protocols for diagnosis of diseases, now a day's people utilize DNA based methods because of its rapidity and reliability. Polymerase chain reaction (PCR) for identification and confirmation of the parasites were used by various researchers throughout the world targeting specific genes. Molecular tools can then provide the level of discrimination that is often not achieved by microscopy alone and which is needed to differentiate between species or intraspecific variants of the relevant parasites. Molecular characterization can provide important information about the parasite's zoonotic potential and transmission dynamics within a community.

Methodology

Collection of faecal samples

Faecal samples were collected from the dogs attending Small Animal Clinic (SAC)-Out Patient—Medicine ward, Inpatient ward, Madras Veterinary College (MVC) Teaching Hospital, community owned dogs in and around Chennai city, SPCA, Chennai corporation—Kannamapet, Private clinic-Thiruvanniyur. The samples from the subjects were collected from different zones of Chennai.

Faecal samples from rectum of dogs were collected using swabs, containers and zip lock covers. Faecal samples were kept at $-20\text{ }^{\circ}\text{C}$. Rectal swabs and faeces (510) were collected using swabs, containers and ziplock covers. After collection the faecal material was mixed with distilled water or the swab was wetted and required amount of sample was processed by sedimentation and floatation technique to identify the parasitic eggs. The remaining faecal samples were stored at $-20\text{ }^{\circ}\text{C}$ for further molecular analysis.

Processing of faecal samples

Faeces were homogenized with distilled water and strained through a sieve to remove all the coarse particles and then centrifuged at 1200 rpm for 10 min. After centrifugation supernatant was discarded. A drop of sediment was placed on glass slide and examined by placing a coverslip under $10\times$ microscope. The sediment was suspended with saturated salt solution till convex surface was formed and after 15 min coverslip was applied to the surface of the fluid and placed on clean glass slide to identify the parasitic eggs.

Polymerase chain reaction

Faecal samples which were positive by microscopy were subjected to polymerase chain reaction in order to molecular characterization of the zoonotic helminths in dogs. Stool DNA extraction kit (Qiamp[®] DNA stool mini kit, New Delhi) was used for preparation of the template DNA as per manufacturer's instructions.

Oligonucleotide primers

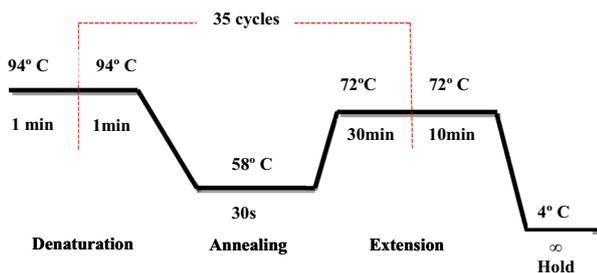
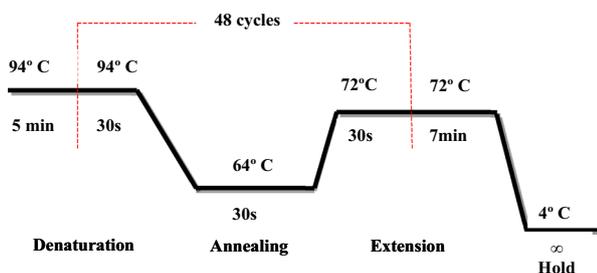
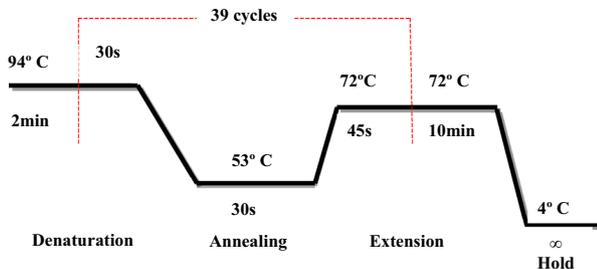
The oligonucleotide primers used in the present study were commercially synthesised from (Sigma-Aldrich, Delhi). The details of the primers and the size of the products which are given in (Table 1).

PCR cycling conditions

Amplification reaction was run with total volume of 25 μl consisting of master mix 12.5 μl , forward primer 1 μl (10 pmol), reverse primer 1 μl (10 pmol), DNA template 3 μl and nuclease free water 7.5 μl . The PCR cyclic conditions for different primers used for specific parasitic diseases as follows (Figs. 1, 2, 3):

Table 1 Details of oligonucleotide primers used in this study

S. no.	Primers	Primer sequence (5'–3')	Amplicon (bp)	References
1	Tcan1 F Nc2 R	5'-AGTATGATGGGCGCGCCAAT-3' 5'-TAGTTTCTTTTCCCTCCGT-3'	380	Khademvatan et al. (2013)
2	RTGHF1 RTABCR1	5'-CGTGCTAGTCTTCAGGACTTTG-3' 5' ATCGACGATCCAGCCAAGAC 3'	545	Traub et al. (2008)
3	Egf1 Egr1	5'-CATTAATGTATTTTGTAAGTTG-3' 5'-CACATCATCTTACAATAACACC-3'	255	Stefanic et al. (2004)

**Fig. 1** PCR cycling condition for *Toxocara canis* (Khademvatan et al. 2013)**Fig. 2** PCR cycling condition for *Ancylostoma caninum* (Traub et al. 2008)**Fig. 3** PCR cycling conditions for *Echinococcus granulosus* (Stefanic et al. 2004)

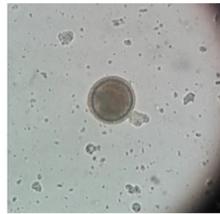
Results and discussion

Out of 510 dog faecal samples screened, 121 (23.72%) were found positive by conventional microscopic examination. The results of the present study showed that *Ancylostoma* spp. (37.19%) was the predominant species encountered followed by *Toxocara* spp. (24.79%), *Taenid* spp. (20.66%), *Diphyllobothrium latum* (1.65%), *Dipylidium caninum* (1.65%), *Isospora* spp. (1.65%), respectively. Mixed infections of *Ancylostoma* spp. and *Toxocara* spp. were found about (8.26%) and *Ancylostoma* spp. and *Taenia* spp. were found to be (4.13%), respectively (Fig. 4). The species wise distribution of the parasites encountered in this study is listed in the (Table 2). The age wise distribution of G.I parasites in dogs showed higher prevalence in 0–6 months and 6–12 months dogs, whereas dogs over 12 months of age showed a lesser prevalence percentage. The sex-wise distribution of G.I parasites in dogs showed male dogs were highly infected with zoonotic parasites of about 27.3%, while compared to that of female dogs with 18.91%.

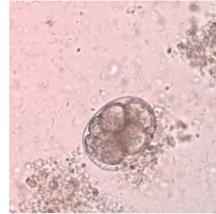
In the present study, 23.72% (121/510) of canine faecal samples were positive by conventional microscopic examination, however similar studies conducted by Palmer et al. (2008) have also recorded a similar type of overall prevalence of 23.9% in dogs and 18.4% in cats. Roger et al. (2004), Katagiri and Oliveira-Sequeira (2008) and Borkakti et al. (2013) have recorded higher prevalence as reported in the present study which might be attributed to the sample size, population targeted and species of parasite identified. The species wise distribution results of our study showed that *Ancylostoma* spp. (37.19%), *Toxocara* spp. (24.79%) and *Taenid* spp. (20.66%) were the predominant species identified in the present study. Our results are in concordance with the results of Roger et al. (2004), Katagiri and Oliveira-Sequeira (2008) and Teresa et al. (2014) who have also identified that *Ancylostoma* and *Toxocara* were the most predominant species identified. *Dipylidium*

Fig. 4 Zoonotic helminths and protozoans in dogs observed by microscopy

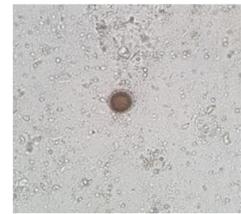
Toxocara sp. egg (40x)



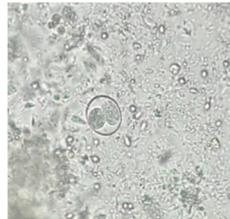
Ancylostoma sp. egg (40X)



Taenia sp. egg (40X)



Isospora sp. oocysts (40X)



Diphyllobothrium latum egg (40X) *Dipylidium caninum* egg (40X)

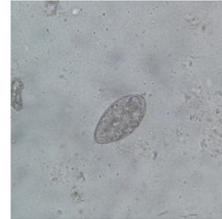


Table 2 Species wise prevalence of zoonotic helminths and protozoans in dog faeces by microscopy

Parasite	No. of positive	Percent positive (%)
<i>Ancylostoma</i> spp.	45	37.19
<i>Toxocara</i> spp.	30	24.79
<i>Taenia</i> spp.	25	20.66
<i>Ancylostoma</i> spp. + <i>Toxocara</i> spp.	10	8.26
<i>Ancylostoma</i> spp. + <i>Taenia</i> spp.	5	4.13
<i>Diphyllobothrium latum</i>	2	1.65
<i>Dipylidium caninum</i>	2	1.65
<i>Isospora</i> spp.	2	1.65

caninum and *Isopora* spp. were also identified in the present study with the prevalence rate of (1.65%). Studies conducted throughout the world to identify gastro intestinal parasites from dogs have recorded a prevalence rate of 2.4% *Dipylidium caninum* and 3.5% *Isopora* spp. (Katagiri and Oliveira-Sequeira 2008); 8.1% *Isopora* spp. (Roger et al. 2004); 10% *Isopora* spp. and 4.4% *Dipylidium caninum* (Tawin et al. 2007). Low prevalence in our study might be due to population targeted, age of the animal and few numbers of intermediate hosts to complete the life cycle. In the present study *Diphyllobothrium latum* was found in two dogs with a prevalence rate of 1.65%. Similar studies done by Pullola et al. (2006) and Reynolds et al. (2016) have showed a prevalence rate of 0.4 and 7.1% respectively. The results of the present study could be solely due to the feeding of dogs with raw or undercooked fish.

Faecal sample analysis by PCR in dogs

Ancylostoma caninum

The faecal samples which were positive for *Ancylostoma* spp. by microscopy were subjected to PCR for detection of *A. caninum* by targeting RTGHF1 and RTABCR1 gene. PCR results showed of the 45 samples subjected to PCR, 38 samples were found positive, which yielded *A. caninum* specific 540 bp product size (Fig. 5). In our study only 84.44% of the faecal samples were found positive which might be due to PCR inhibitory factors of faecal origin, inadequate amount of DNA in the sample and presence of other *Ancylostoma* spp. other than *A. caninum*. In a similar manner a study was conducted in Australia in which dog and cat faecal samples which were positive by microscopy were checked by PCR. Results of the study showed, out of 96 microscopy positive dog hookworm samples, 92 were amplified successfully by PCR and out of 14 microscopy positive cat hookworm samples 10 were amplified successfully. The researchers have also documented that inability to amplify all of the samples was thought to be associated with inhibitory factors within the PCR reaction or an inadequate amount of DNA in the sample (Palmer et al. 2007). In another study by Traub et al. (2005), ninety-three dog’s faecal samples which were microscopically positive for hookworm eggs were subjected to *Ancylostoma*-specific PCR–RFLP showed ninety one faecal samples were found positive by PCR and they also reasoned that the above mentioned factors were responsible for the lower detection limits.

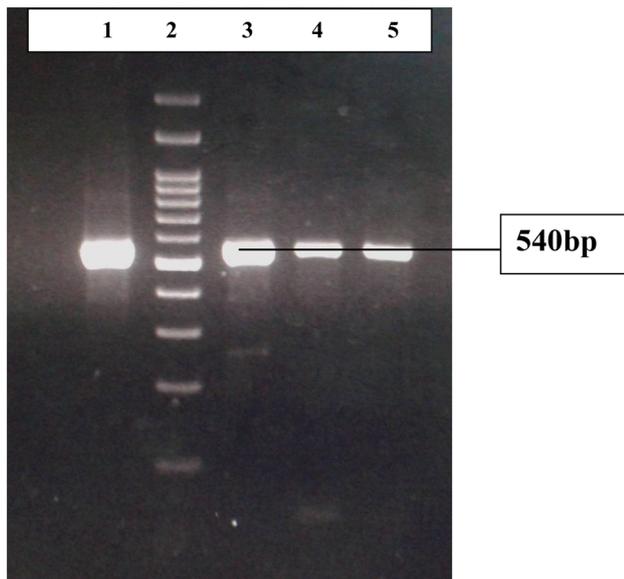


Fig. 5 Agarose gel showing amplicons specific for *RTGHF1* and *RTABCR1* gene of *Ancylostoma caninum* from canine faeces. Lane 3,4,5: amplicon showing positive. Lane 2: 100 bp DNA ladder. Lane 1: +ve CONTROL (540 bp)

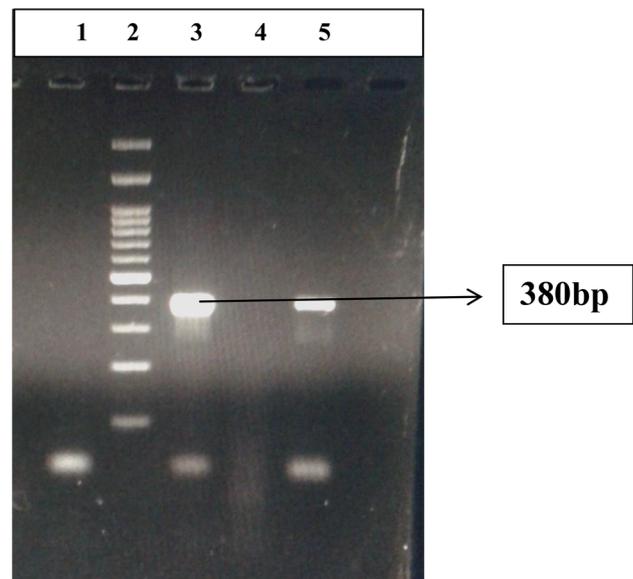


Fig. 6 Agarose gel showing amplicons specific for *Tcan1 F* and *Nc2 R* gene of *Toxocara canis* from canine faeces. Lane 5: sample showing positive, Lane 1 and 4: sample showing negative. Lane 2:100 bp DNA ladder, Lane 3: +ve CONTROL (380 bp)

Toxocara canis

A total of 30 faecal samples which were positive by microscopy were subjected to PCR for detection of *Tox Can1* and *Nc2* gene which are specific for *Toxocara canis*. Results of our study showed of the 30 samples tested by PCR only 25 samples were positive with 83.33% positivity (Fig. 6). Similarly, Fahrion et al. (2011) isolated *Toxocara* eggs from faecal samples of 35 dogs from Switzerland and identified the *Toxocara* spp. by PCR. Amongst the isolates originating from dogs, 24 (68.5%) were determined as *T. canis* and 11 (31.5%) as *T. cati*. Another study by Khademvatan et al. (2013) in which 63 cat faeces which were positive by microscopy were subjected to PCR targeting *T. cati* primers of the 63 samples tested by PCR only 59 samples were positive for *T. cati* and the remaining four samples turned out to be *T. canis*. Based on the results of the present study and the experiments conducted by various researchers throughout the world for detection of *Toxocara* spp. by PCR, we can conclude that there is a possibility of infection with both *T. canis* and *T. cati* which needs to be ruled out by using species specific primers and this might an important reason for the lower detection limit with our microscopy positive samples. Apart from identification of *Toxocara* parasites, various researchers have exploited PCR assay for differentiation of *Toxocara* spp. which helps in molecular epidemiological investigations and to better understand the species wise distribution of the parasite (Jacobs et al. 1997; Fogt-Wyrwas et al. 2007; Borecka 2008).

Echinococcus granulosus

Microscopical detection of eggs by routine coprological floatation technique in faecal samples is not suitable in case of the family *Taenidae* which has indistinguishable morphology from other; hence molecular analysis is needed to differentiate species of *Taenidae* (Casaravilla et al. 2005). In the present study (30) faecal samples which were positive by conventional method for the presence of *Taenid* eggs were analysed by PCR. Out of 30 samples screened by PCR none of them were positive for *Echinococcus* (Fig. 7). This is in accordance with the study conducted in Portugal by Teresa et al. (2014) in which faecal samples which were positive for *Taenid* spp. eggs by microscopy were found negative for PCR assay targeting *Echinococcus*. Numerous studies reported that indoor dogs are having less chance or risk of being copro antigen positive when compared to free roam dogs (Mastin et al. 2011). Negative PCR results for *Echinococcus* in the present study might be due to majority of samples were collected from pet dogs, the lower sensitivity limit of PCR which needs at least 100 eggs per gram of faeces, besides extraction of DNA can be often hampered by the presence of inhibitory substances (Abbasi et al. 2003).

Conclusion

Prevalence of zoonotic helminths and protozoan parasites in dogs and cats indicates that dogs may serves as a reservoir of infections to human beings. *Ancylostoma* spp.

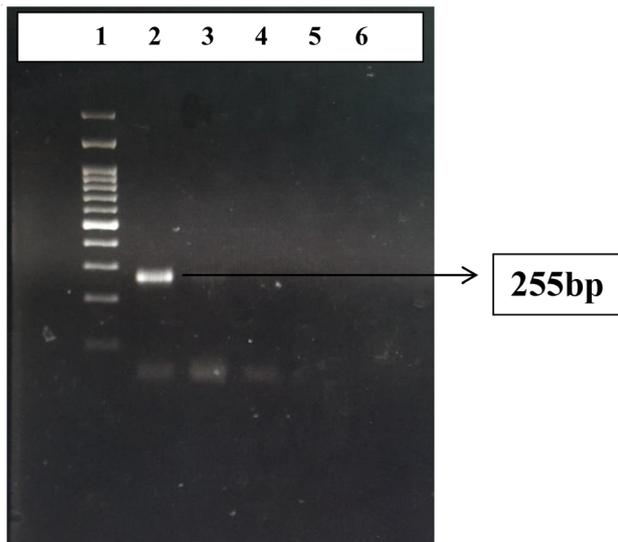


Fig. 7 Agarose gel showing amplicons specific for *Egfl* and *Egfl2* gene of *Echinococcus granulosus* from canine faeces. Lane 3, 4, 5, 6: samples negative by PCR. Lane 1: 100 bp DNA ladder. Lane 2: +ve CONTROL (255 bp)

and *Toxocara* spp. was found to be predominant zoonotic helminths which is associated with cutaneous larval migrans and visceral larval migrans in human beings. PCR-based approaches were employed in several studies to differentiate among the eggs of parasitic spp. from adult helminths, which are closely related and/or morphologically similar. It can be widely used for species-specific identification of the zoonotic parasites.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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