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DETECTION AND IDENTIFICATION OF TOXOCARA CANIS IN DOGS IN TABUK REGION, SAUDI ARABIA BY PCR AND DNA SEQUENCING

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Abstract

Dog feces are important for spreading and transmitting parasite eggs, larvae, and oocysts by contaminating soil, food, or water. Dog feces can be a source of intestinal parasites. This study investigated the prevalence of *Toxocara canis* infection in stray dogs in the Tabuk Region in northwest Saudi Arabia. Eggs were discovered using the sedimentation flotation method in the feces of stray dogs, and they were recognized using DNA sequencing and polymerase chain reaction (PCR). Out of the 200 fecal samples that were randomly obtained from public places, the Tabuk region's west (an agricultural area) showed the highest prevalence of eggs (7.5%). The molecular approaches for identification of this parasite are sensitive sufficient to distinguish between the various *T. canis* in feces and to detect low parasite levels. Due to Saudi Arabia's ineffective environmental hygiene controls, the comparatively high prevalence of this parasite may continue to rise. This study accurately identified *T. canis* in dogs in Tabuk Region by a new technique, eliminating the possibility of misdiagnosis by traditional fecal microscopy.

Keywords: Saudi Arabia, Tabuk, Dog, *Toxocara canis*, PCR, Sequencing.

Introduction

The most common canid that has adapted to living alongside humans is dog, which serves as the main reservoirs for many zoonotic parasites (Sabry *et al,* 2012). The most common canid that has adapted to living alongside humans is dog. The direct ownership of a cherished pet does, have certain possible health risks, as well as the potential for ecto-parasites, bites, scratches, and allergies (Diba and Garoma, 2021). The commonest enteric nematode parasites of dogs were *Toxocara* canis (Khante *et al*, 2009). Its name is derived from the Greek word "*toxon*," meaning bow or quiver, and the Latin word "caro," meaning flesh (Bassert and Thomas, 2014). Toxocariasis is a neglected parasitic zoonosis that afflicts millions of the pediatric and adolescent populations worldwide, especially in impoverished communities (Chen *et al,* 2018). *Toxocara canis* was described as a parasitic roundworm of dogs with a high zoonotic potential inducing allergic inflammation and visceral (VLM) and ocular larva migrans (OLM) in humans (Fillaux and Magnaval, 2013). In fact, *T. canis* was considered one of the most distributed worldwide especially in African which reflects its high public health significance by causing human toxocariasis and other zoonotic parasitic infections (El Menyawe and Abdel Rahman, 2007). Also, toxocariasis was endemic in the United states (Lee *et al,* 2014). The estimated prevalence rates in the WHO different regions ranged from 13% to 35%: Western Pacific (35%; 95% CI, 15-58%), Africa (27%; 95% CI, 11-47%), South America (25%; 95% CI, 13-33%), South-East Asia (21%; 95% CI, 3-49%), Middle East and North Africa (18%; 95% CI, 11-24%), Europe (18%; 95% CI, 14-22%), and North and Central Americas (13%; 95% CI, 8-23%).

A high prevalence was significantly associated with high geographical longitude (P = 0.04), low latitude (P = 0.02) and high relative environmental humidity (P = 0.04), indicating that public places were heavily contaminated with *Toxocara* eggs (Fakhri *et al,* 2018). Under favorable environmental conditions, eggs mature in the soil and can remain infectious for a long time (Overgaauw *et al,* 2009).

Toxocara canis infects all wild and domestic canids; more prevalent among puppies than older dogs. *T. cati* is found in wild and domestic felids of all ages, but slightly more common in kittens. Also, paratenic host ranges for both species encompass numerous birds and livestock; some human infections were linked to consumption of undercooked beef, lamb, chicken, and duck meat. Cockroaches and earthworms have been experimentally infected, and could serve as paratenic or transport hosts (CDC, 2019). But, humans are normally infected via accidental ingestion of embryonated eggs from contaminated soil (Okulewicz *et al,* 2012*)*. Dogs are the most successful pets, adapted to human habitation in most rural areas, they transmit also, *Echinococcus granulosis, Taenia* spp., *Dipylidium caninum*, *Ancylostoma* spp., *Giardia* spp., and *Cryptosporidium* spp., (Haridy *et al,* 2009). Also, *Toxocara* eggs persist in dog's fur (Hussein *et al,* 2021).

Species specificity determination of *Toxocara* larvae from animal and human tissues is very difficult due to their small size and few morphological features (Nichols, 1956). Ascaridoid nematodes are identified by morphological characteristics and their sites within hosts, but there are limitations in traditional methods (Gasser, 2013). To overcome these traditional limitations, many molecular techniques based on ribosomal and mitochondrial markers both for *Toxocara* spp., identification have been developed (Gasser *et al,* 2006). The PCR was developed for rapid and specific diagnosis, because of their ability to specifically amplify DNA from nematode eggs and thinly sections of larvae or adults (Smith *et al,* 2009).

The present study aimed to apply the mitochondrial cytochrome oxidase subunit I (COI) gene to molecularly identify *Toxocara* spp. among dogs in Tabuk Region, Saudi Arabia.

Material and Methods

Study and population: Tabuk Region is in the northwestern part of Saudi Arabia (28°23′ 50″N, 36°34′44″E). It covers an area of approximately 146,072km^2 and 760 meters above sea level with temperatures range from 10°C to 40°C, inhabited with 687,000 Saudis as well as immigrant employees (Abdulalim and El Damaty, 2022).

A total of 200 dogs fecal samples were collected from open public spaces in five areas in Tabuk district (north or industrial city, south, east, west or agricultural area, and central) to be examined for *Toxocara* eggs during the months of January to a Jun 2023. Fecal samples were collected from dogs either within the area accessed by free or tethered dogs.

Ethical approval: Sampling procedure didn't require any permissions Tabuk Health or Veterinary Authorizes, and the field studies did not involve endangered or protected species. Fecal samples about 50g were collected from each dog in labeled plastic boxes and stored at 4°C until examined.

Samples analysis: Fecal samples were examined as direct smears or sedimentation-formalin-ethyl acetate and centrifugal flotation with $ZnSO_4$-solution (Garcia, 2016). The eggs from all *Toxocara* positive samples detected by flotation/sedimentation were examined by one step PCR to determine *T. canis*. To concentrate eggs, the fecal samples were prepared with improved flotation method (Széll *et al,* 2014). When *Toxocara*-type eggs were identified microscopically, 1 ml of the flotation upper part of the centrifuge tube was transferred into a 15ml falcon tube. This step was repeated 4 times. The tube was filled with water and centrifuged at 2000x g 10min. Supernatant was discarded; sediment was re-suspended in 1ml water, transferred to a 2ml micro-tube, which was used for DNA purification.

Isolation of DNA & PCR: Genomic DNA from the *Toxocara* spp. eggs was extracted by using Pure Link Genomic DNA Kits (Invitrogen, Waltham, Massachusetts, USA), following the manufacturer's instructions. Samples were initially subjected to 3 freeze-thaw cycles of 20minutes each. Proteinase K digestion was performed overnight at 55°C under conti-

nuous shaking conditions (Borecka and Gawor, 2008) on the thermomixer compact at 450 rpm. Specific primers amplifying cytochrome oxidase CO subunit I(COI) of the mitochondrial DNA of the *T. canis* LCO1490: 5′GGTC AACA AATCATAAAGATATTGG-3′ as a forward primer & HCO-2198: TAAACTTC AGGGTGACCAAAAAATCA-3′ as a reverse primer were adopted (Folmer *et al,* 1994).

Each PCR reaction was performed with 25 µl of Master Mix 2X (*i*-Taq, *iNt*RON, Seongnam, Korea), 1.5 of each primer (20 µM), 300 ng of genomic DNA, and DNase/RNase free water (*i*-Taq, *iNt*RON, Seongnam, Korea) to complete one volume of 50µl. Temperatures employed were as follows: initial denaturation at 95°C (5min.), followed by 35 cycles of denaturation at 95°C (30s), annealing at 58°C (30s), extension at 72°C (30s), and final extension at 72°C (10 min), and cooled to 4°C. Electrophoresis was performed with a volume of 5µl on 2% agarose gel containing ethidium bromide in 1X Tris-acetic acid-EDTA buffer and observed in an UV trans illuminator.

Sequence analysis: PCR products were purified by using a Macrogen reagent (Seoul, Korea). Single strand DNA sequencing was performed, after which nucleotide sequences of *T. canis* COI were aligned.

Phylogenetic analysis: Data sequences were assembled by using Chromas Pro 1.5 beta (Technelysium Pty., Tewantin, QLD, Australia). New COI sequences *T. canis* were compared to those available in Gen-Bank, using Basic Local Alignment Search Tool (BLAST) available at http://blast.ncbi.nlm.nih.gov/blast.cgi. Muscle alignment was used to align the sequences by MEGA 11.0 software. Kimura's two-parameter as used to calculate the sequence divergences (K2P) (Kimura, 1980).

To illustrate the *Toxocara* species divergence patterns, N.J. trees used the Tamura 3 parameter method (Tamura, 1992). The boot strapping was performed in the MEGA 11.0 with 1000 replications (Kumar *et al,* 2004).

Results

Dog feces showed that 25/200(12.5%) contained eggs of *Toxocara* spp. Determining the prevalence of *T. canis* in dogs from each of the five Tabuk regions showed highest prevalence in the west (agricultural area 15, 60%) and lowest in the north (industrial area 3, 12%). *Toxocara canis* was identified in 5 (20 %) of the 25 *Toxocara* egg-positive samples by PCR using COI primer, showed 505-bp fragments from *T. canis*, without mixed infections.

Details were given in table (1) and figures (1 & 2).

Table 1: Detection of *Toxocara* eggs in five areas of Tabuk region.

Area	No. of examined fecal samples	positive	%
North (industrial city)	45 (22.5%)	3	12
East	30 (15%)	-	-
West (agricultural area)	50 (25%)	15	60
Central	30 (15%)	-	-
South	45 (22.5%)	7	28
Total	200	25	100

The evolutionary distances were computed using the Tamura-Nei method and are in the units of the number of base substitutions per site. This analysis involved 13 nucleotide sequences (12 donor sequences were evaluated from NCBI, including one sequence that acted as an outgroup (MK684069.1). Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. There were total of 419 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

Also, *T. canis* (nucleotide sequences from the current study) showed a clear relationship (Figure 2) with species identified from Poland (MH937708.1), Sri Lanka (JN593098.1) and Russia (KX365898.1) with an evolutionary distance of approx. 0.00123.

The minimum haplotype spanning network

represented the haplotype and geographical location of the 13 nucleotide sequences evaluated in the present work, revealed the haplotype diversity and change throughout geographical differences. Nucleotide diversity showed P= 1.22605; number of segregating sites was 66, while 36 parsimony-informative sites were observed. The Tajima's D statistic was 12.5817 with P (D >= 12.5817) = 0.

Discussion

In the present study, *T. canis* eggs were found in 12.5% of dog feces. This agreed with Öge *et al.* (Öge *et al*., 2019) in Turkey, they found eggs in 9.5% of dogs, but less than *Chattha et al.* 2009) and Shah *et al*. (2018) in Pakistan, who reported 30% & 26% respectively. Abbas *et al*. (2023) in Egypt reported among 21 dogs (35.9%) had eggs/oocysts of eight different parasites including *T. canis* (19.2%). The huge toxocariasis and echinococcosis attracted the Public Health and Veterinary Authorities to this risky zoonosis (Kantarakia *et al,* 2020). In Saudi Arabia *Echinococcus granulosus* was reported in livestock in Al-Medina (Al-Mutairi *et al,* 2020), and man in Riyadh (El Marsfy and Morsy, 1975).

In the present study, *Toxocara canis* was identified in 5 (20%) of the 25 *Toxocara* egg-positive samples by PCR. But, the efficacy of PCR was low when compared to sedimentation/flotation. PCR results clearly indicate an increased number of false positive cases by microscopy due to closely related morphology of eggs in present and past studies. The important finding in this study was that only five of 25 microscopically positive samples were PCR-positive *T. canis*. PCR efficacy depends on the number of eggs in feces. PCR may not be able to detect DNA of ascarids because of low DNA concentration.

In the present study, COI sequences obtained from *T. canis* was found to be identical by 100.0% to sequences from *T. canis* recorded in Poland in the Gen-Bank. The identical sequences may be caused by the similarity in the environment and the climatic conditions reported abroad to those in Tabuk Region (Al-Mutairi, 2022). So, humans are at high risk for to zoonotic *T. canis* that spreads easily via stray dogs as in Taif City (Al-Tayib, 2013) and Riyadh District (Al Malki, 2021). Also, Omar *et al*. (2021) added that lack of knowledge among practicing medical community lead to misdiagnosis and misguided treatment.

Conclusion

Zoonotic dog diseases that can occur by contact pose risks to pets, their owners and to their bond. This may be first report in Tabuk District on the prevalence differentiation of *T. canis* using molecular method, PCR which was sufficiently sensitive to detect and identify in dog feces. There are many stray dogs in various parts of the study area. Awareness as to the contamination risk with zoonotic toxocarasis, echinococcosis and other diseases may be very limited even among pet owners.

Consequently, there must be an urgent need to plan adequate programs to control such infection, as well as stray dogs in the region.

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Explanation of figures

Fig. 1: Neighbor-Joining phylogenetic evolutionary tree.
Fig. 2: Minimum haplotype spanning network.