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PCR assay of *Toxocara canis* from soil isolates in and around Hyderabad, Telangana state, India

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Abstract
The soil borne parasites extract a huge toll on the health and well-being of global population and are a major source of morbidity and mortality affecting millions of people as well as livestock. Among soil borne parasitic zoonoses, Toxocarosis is the major infection causing Visceral larva migrans. The presence of infective larva forms in the soil is an indicator of potential risk to both human and animals. About 230 soil samples were collected from four different locations in and around Hyderabad region of Telangana state, India. All the samples were processed for isolation and confirmed with Polymerase chain reaction (PCR). Of all the samples screened, *Toxocara canis* isolated from 39 soil samples of 380bp product size amplicons on agarose gel electrophoresis which is 16.95% prevalence and could detect as low as 0.781 ng of gDNA of *Toxocara canis* which was equivalent to even less than an egg DNA. By considering both the sensitivity and specificity it is concluded that the PCR assay standardized in the present study can be used as molecular diagnostic tool for specific detection of *Toxocara canis* egg DNA in soil samples, where visceral larva migrans is prevailing among human children.

Keywords: PCR, *Toxocara canis*, visceral larva migrans, specificity and sensitivity

Introduction
With burgeoning stray dog population, steep raise in companion animal population, poor socio economic conditions and unawareness of personal hygienic practices, the soil borne zoonotic parasites are disseminating to human dwellings at an alarming speed [1]. Children between 4-11 years old are the potential risk group for soil borne zoonotic parasites because of their playing habits and geophagia due to pica [2]. Among soil borne parasitic zoonoses, Toxocarosis is the major infection followed by Ancylostomosis [3] and cases of Visceral larva migrans [4] by *Toxocara* spp have been reported throughout India. PCR assay is a simple and reliable method for detection of *Toxocara* spp. and can be used for diagnostic and epidemiological purposes [5]. ITS-2 gene is the most useful and sensitive molecular marker for differentiation of *Toxocara* spp. so the present study is to determine species level identification from soil isolates using PCR assay [6].

Materials and Methods
Chemicals and kits
1. All the chemicals (for preparation of reagents and buffers) mentioned in this chapter were procured from Qualigens Pvt. Ltd., Mumbai and SRL Pvt. Ltd., Mumbai.
2. Kits for Soil DNA extraction were procured from DNeasy® power soil® kit Quaigen Pvt, Ltd, Delhi, India

Collection of worms: Adult *Toxocara canis* worms were collected from non descript pups maintained at Blue cross society of India, Hyderabad. The pups were dewormed with Piperazine hydrate (Virbac, India) at the dose rate of 100mg/kg. The adult worms expelled on the immediate next day were collected in Normal saline and transported on ice at 4 °C to the laboratory. Worms were cleaned for several times by gentle washing in PBS (pH 7.2) to remove host material. The gross morphology of *Toxocara canis* worms studied by visual examination and microscopic examination done by fixing worms in 70% alcohol for a day and the anterior and posterior ends of the worm were cut and cleared for species identification.

Isolation of genomic DNA from the eggs and female worm of *Toxocara canis*
DNA isolation is according to the procedure [7] with minor modifications.

The individual female worms in a petridish containing normal saline and dissected using a scalpel at the anterior one-third of the uterus and the eggs were released from the uterus by applying gentle pressure with forceps [8]. The number of eggs in 1ml of PBS (pH 7.2) thus collected was enumerated by ordinary microscopy. Take 400 ul of above solution and cell lysis buffer was added 600 u1 and incubated at 37 °C for 1hr followed by the addition of proteinase K (100 ug/ml) and incubation at 56 °C for 6hr with periodic swirling. The lysed cell suspension was then layered over with 1ml of Phenol: chloroform: Isoamyl alcohol (25:24:1) ratio to it and homogenized by gentle and incubated at room temperature for 5min and then centrifuged at 12000rpm for 15min, 4 °C. the separation of phases are seen in which the upper aqueous layer was transferred into was carefully into fresh 1.5ml ependroff tube. The aqueous phase was layered over an approximately equal volume of chloroform: isoamyl alcohol (24:1), incubated for 5min in room temperature and centrifuged 12000rpm for 15min, 4 °C. The final aqueous phase was carefully aspirated and the volume was measured. This was overlaid with equal volume (of original aqueous phase) of Isopropanol at room temperature. This was incubated overnight -20 °C or -80 °C. The precipitated DNA was then centrifuged at 12,000 rpm for 15min, 4 °C. The supernatant was carefully discarded and the pellet was washed twice again with 70% ethanol. After the final wash, the supernatant was removed. Before the pellet dried up completely. it was dissolved in l5-20 ul of TE buffer (pH 8). DNA samples were considered to be of sufficient purity if the ratio was 1.8 and above are used as reference positive template. Similarly genomic DNA was extracted from the female *Toxocara canis* worm and from *Ancylostoma caninum* eggs which were collected from the small intestine of a pup died on road side accident.

Polymerase Chain Reaction (Pcr) Assay

The region spanning ITS-2 of rDNA was amplified from gDNA of *Toxocara* spp. eggs by PCR using oligonucleotide primers *Tcan1* and *NC2* designed for the regions of the 5.8S and 28S genes [9]

Primers	Sequence
Forward (***Tcan1***)	5' AGTATGATGGGCGCGCCAAT 3'
Reverse (***NC2***)	5' TTAGTTTCTTTTCCTCCGCT 3'

PCR protocol: A 380bp fragment of region spanning ITS-2 in the rDNA gene was amplified by PCR using *Tcan1* and *NC2* primers. For conducting the PCR reaction with 2µl of template DNA, 2.5µl of 10x PCR buffer with magnesium chloride ($MgCl_2$) (Genei), 2.5µl of 25µM of dNTPs mix (Genei), 2µl (20 pmol) each of forward and reverse primers, 2 IU Taq DNA polymerase (Genei) and the reaction volume was made upto 25 µl with deionized autoclaved triple distilled water (ATDW). Thermocycling profile consisted of initial hold for 5 min at 94 °C followed by 35 cycles of 1 min denaturation at 94 °C, 30 seconds annealing at 53 °C and 1 min extension at 72 °C. The final extension was carried at 72 °C for 10 min and the amplified PCR products were stored at 4 °C until further use. Negative control in PCR was a mix inoculated with ultra pure water in substitution of DNA in equal volume. The gDNA extracted from *Toxocara canis* eggs DNA was used as positive control. Agarose gel electrophoresis run by 5ul of each PCR amplicon products of ITS-2 gene of *T. canis* eggs were run in ethidium bromide stained (0.5µg/ml) 2% (W/V) agarose gel in 1x TBE buffer at 100 volts for 35-35 min. Reconstituted DNA ladder (100bp ruler) was loaded in one of the wells. The sizes and quantities of PCR products were verified by comparison with DNA rulers and documented.

Analytical sensitivity of PCR assay: Appropriate descending double fold dilutions of template DNA of eggs of *Toxocara canis* made in ATDW, starting from a concentration of 25 ng up to 0.781 ng were run by PCR assay, as per the same conditions described above.

Specificitv of Primers in amplifying ITS-2 region of rDNA of *Toxocara canis* pure egg genomic DNA: Studies were conducted to test the specificity of primers targeted to ITS-2 region of rDNA of *Toxocara canis* earlier described for amplifying three separate PCR reactions consisting of template DNA of *T.canis* eggs, *A.caninum* eggs along with negative control (ATDW in place of template DNA) were set under the similar PCR conditions described earlier for amplifying *T.canis* egg DNA.

PCR Amplificatio Of *Toxocara Canis* Spp Eggs From Soil Samples

The genomic DNA was extracted from 230 soil samples by using DNeasy® power soil® kit. To enhance the extraction of gDNA from eggs, one ml of lysis buffer (0.5 M Tris Hcl, 1 mM EDTA, 2% SDS) was added to 2.5 grams of soil samples and kept for Proteinase K (80 µl) digestion overnight [10]. The remaining steps employed were according to the kit manufacturer's protocol except, obtained gDNA was eluted with 15 µl of nuclease free water instead of 100 µl buffer AE (supplied with kit).

Results

Gross morphological studies of *Toxocara canis* worms are large, creamy to pink colour roundworms. Male worm measures about 5.5±0.5 cm long and female is longer than male measures about 8.5±1.5 cm long (Figure 1).

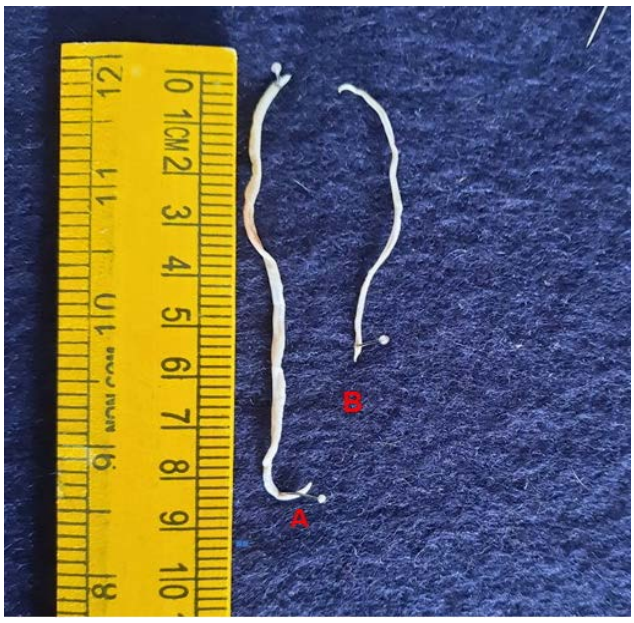

Fig 1: Adult male and female worms of *Toxocara canis* eggs

The eggs of *Toxocara canis* are subglobular with thick, finely pitted shells and measures about 90 to 75µm (Figure 2).

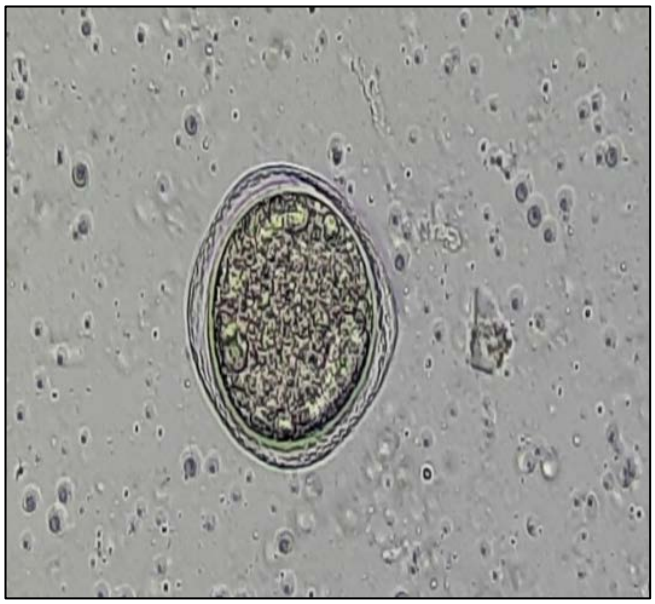

Fig 2: *Toxocara canis* egg (75µm)

Upon microscopic examination the anterior end of these worms were bent ventrad and has Cervical alae is large (Figure 3).

The mouth of the worm is surrounded by three fleshy lips one on dorsal and two sub ventral each one equipped with small papillae. The Tail end of the worm is curved ventrally and finger shaped. The posterior end of male worms has a terminal narrow appendage with caudal alae. Tail end is bluntly pointed end provided with two spicules (Figure 4).

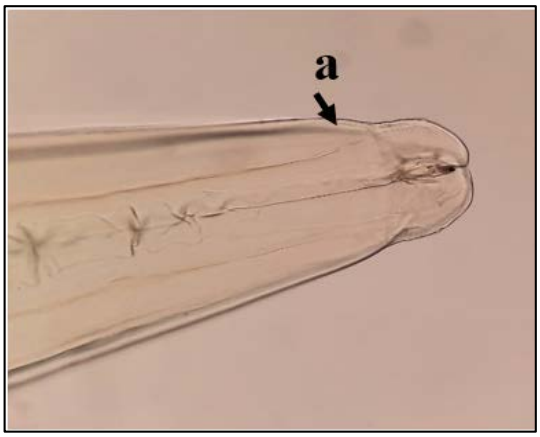

Fig 3: Anterior end of *T. canis* a) Cervical alae

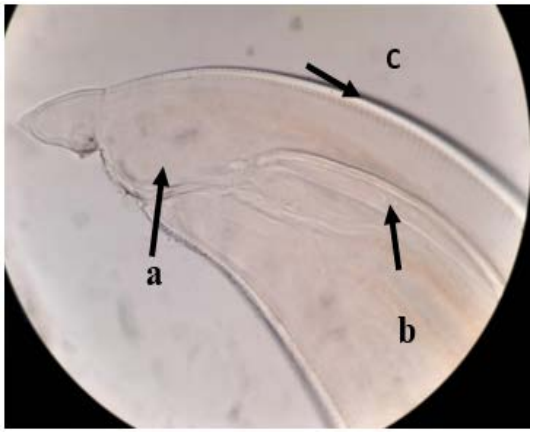

Fig 4: Posterior end of *Toxocara canis* a) Terminal appendage b) Spicules c) Caudal alae

Standardisation of PCR assay of region spanning ITS-2 of ribosomal DNA of *Toxocara canis* spp. with respective *Tcan1* and *NC2* designed for the regions of the 5.8S and 28S genes primers in 25 µl reaction under cycle conditions described previously yielded a single band of 380 bp length with out any amplicon in the negative control when run in ethidium bromide stained (0.5 µg/ml) agarose (2%) gel electrophoresis (Figure 5).

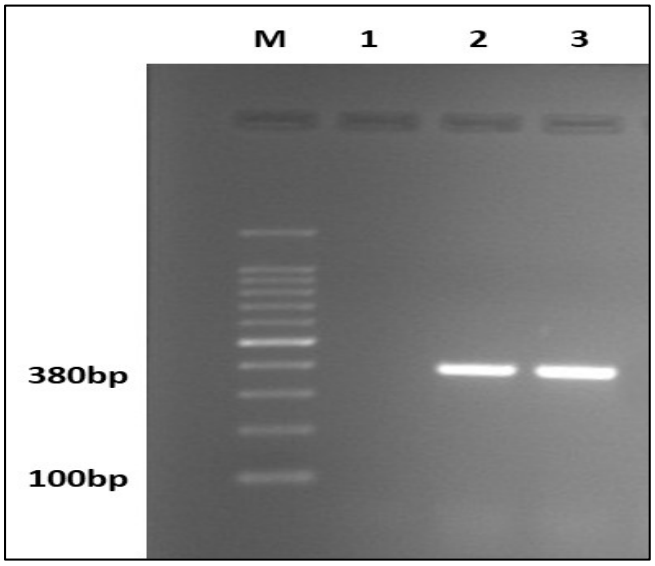

Fig 5: Standardisation of PCR assay for the diagnosis of *Toxocara canis* M : Gene Ruler of 100 bp DNA ladder 1: Negative Control 2: *Toxocara canis* egg DNA 3: *Toxocara canis* Adult worm

Trails were conducted to evaluate lowest quantum of egg DNA that could produce a visible signal by using the DNA isolated from the eggs of *Toxocara canis*. The PCR could detect the DNA as low as 0.781 ng from template DNA of eggs of *Toxocara canis* (Figure 6).

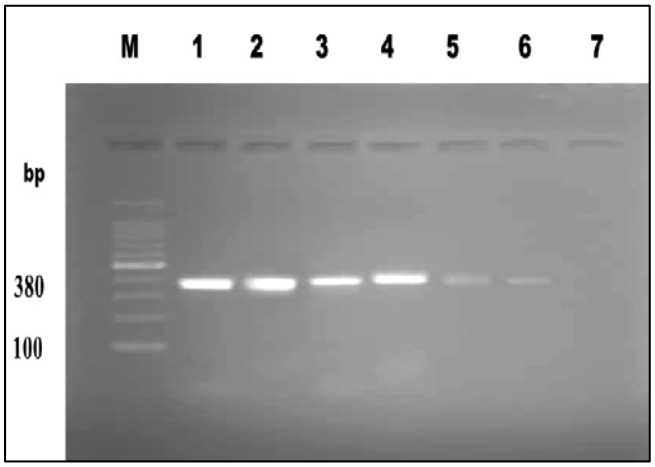

Fig 6: Analytical Sensitivity of PCR assay M : Gene ruler of 100 bp DNA ladder Lanes : 1to 7, double dilutions of *T.canis eggs* genomic DNA 1) 25 ng 2) 12.5 ng 3) 6.25ng 4) 3.125 ng 5) 1.562 ng 6) 0.781 ng

The PCR assay standardized to detect *T.canis* was tested for its specificity in detecting the DNA of *Ancylostoma caninum* egg DNA showed no band formed with out any amplicon when run in ethidium bromide stained (0.5 µg/ml) agarose (2%) gel electrophoresis (Figure 7).

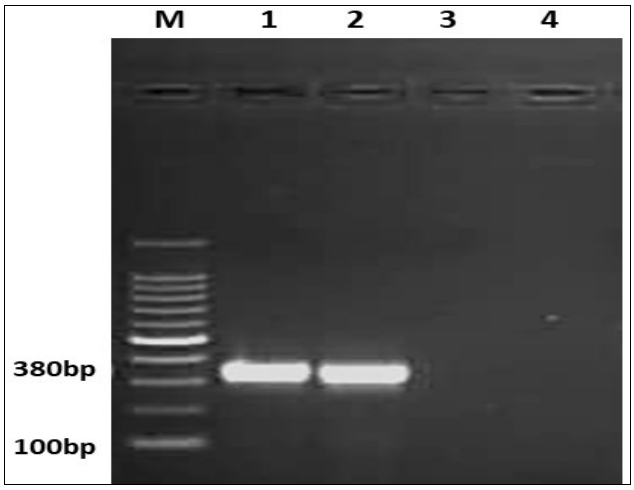

Fig 7: Analytical Sensitivity of PCR assay M : Gene ruler of 100 bp DNA ladder Lanes : 1to 7, double dilutions of *T.canis eggs* genomic DNA 1) 25 ng 2) 12.5 ng 3) 6.25ng 4) 3.125 ng 5) 1.562 ng 6) 0.781 ng

The gDNA was extracted from 230 soil samples by using DNeasy® power soil® kit. The gDNA of *Toxocara canis* eggs was served as positive control. Among them 39 soil samples were found to be positive of 380bp product size amplicons on agarose gel electrophoresis which is 16.95% percent prevalence (Figure 8).

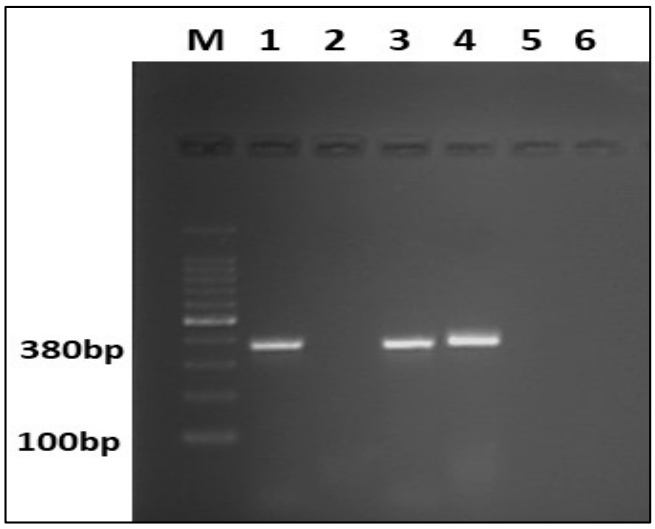

Fig 8: Diagnosis of *T.canis* infection in soil samples by PCR assay M : Gene Ruler of 100 bp DNA ladder 1: *Toxocara canis* pure egg DNA 2: Negative control 3, 4 : *Toxocara canis* positive soil samples 5,6 : *Toxocara canis* negative soil samples

Discussion

The differentiation of *Toxocara* spp. eggs from soil by ocular microscopy is extremely difficult due to the similarity in morphological characteristics of *T canis* and *T. cati* eggs. To overcome the problems caused by these traditional approaches, molecular techniques are gaining importance [11]. Study of the variation in spacer regions can be helpful to identify species or strains, Among these spacer regions transcribed spacers are more conserved and useful in the study of closely related species [12].

PCR assay is a simple and reliable method for detection of *Toxocara* spp and can be used for diagnostic and epidemiological purposes [5]. ITS-2 gene is the most useful and sensitive molecular marker for differentiation of *Toxocara* spp. for species level identification of *Toxocara canis* [6].

The present study is in agreement with the studies designed specific Primers to unique regions of the ITS-2 Sequences of the three species of *Toxocara* spp (*Toxocara canis*, *Toxocara cati*, *Toxocara leonine*) for use in PCR amplification assay [9]. By using those species specific primers *Tcan1,NC2* of *Toxocara canis*, a 380 bp product of *T. canis* was isolated from stray dogs of Iran [13]. Trails were conducted to detect Analytical sensitivity of PCR assay in detecting lowest quantum of *T. canis* egg DNA. PCR assay performed with 25 µl reaction mixture consisting of double dilutions of template DNA with the other conditions remained unchanged yielded a detectable band of 380 bp with 0.781 ng of DNA which is slightly less compared [14] who detected 0.13-0.54 ng is the minimum amount of DNA that could give a detectable band of 380bp during PCR assay. These variation might be due to the lab to lab in variations.

The specificity of PCR assay targeted to *T. canis* egg DNA was cross checked with the *A. caninum* egg DNA. *A. caninum* egg DNA was extracted from the eggs collected directly from female worms recovered from a pup died on road side accident. The pure DNA was collected and DNA has yielded good amount of DNA with a 260/280 OD ratio of 1.72 and single band seen without any smearing when run in 0.7% (W/V) agarose gel electrophoresis.

The PCR assay with *A.caninum* egg DNA as template DNA with all other conditions remained unchanged did not yield any detectable band when run in 2% (W/V) agarose gel electrophoresis indicating the specificity of primers to *T. canis* egg DNA.

Conclusions

It is concluded that by considering both the sensitivity and specificity of ITS-2 region of rDNA of *Toxocara canis*, PCR assay is the most sensitive molecular diagnostic method for detecting species specific soil borne parasitic zoonotic infections. Hence the PCR assay standardized in the present study can be applied as molecular diagnostic tool for specific detection of *Toxocara canis* egg DNA in soil samples where visceral larva migrans is prevalent in human children.

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References

1. Traub RJ, Robertson ID, Irwin PJ, Mencke N, Thompson RA. Canine gastrointestinal parasitic zoonoses in India. Trends in parasitology 2005;21(1):42-48.
2. Worley G, Green JA, Frothingham TE, Sturner RA, Walls KW, Pakalnis VA *et al*. *Toxocara canis* infection: clinical and epidemiological associations with seropositivity in kindergarten children. Journal of Infectious Diseases 1984;149(4):591-597.
3. Jutang BAT, Sumeeta K, Abhishek M, Rakesh S, Ankita S. Clinical and laboratory characteristics of patients with toxocariasis encountered at a tertiary care centre in North India. Indian Journal of Medical Microbiology 2018;36(3):432-434.
4. Hegde SH, Maiya PP, Dandekar CH, Vishwanath D, Rao SG, Benakappa NA. Visceral larva migrans. Indian Pediatrics 1995;32(11):1245-1246.

5. Chen J, Zhou DH, Nisbet AJ, Xu MJ, Huang SY, Li MW *et al*. Advances in molecular identification, taxonomy, genetic variation and diagnosis of *Toxocara* spp. Infection, Genetics and Evolution 2012;12(7):1344-1348.
6. Mahdy OA, Mousa WM, Abdel Maogood SZ, Nader SM, Abdel Radi S. Molecular characterization and phylogenetic analysis of *Toxocara* species in dogs, cattle and buffalo in Egypt. Helminthologia 2020;57(2):83.
7. Sambrook J, Fritsch EF, Maniatis T. Molecular cloning*: a* laboratory manual (No. Ed. 2). Cold spring harbor laboratory press 1989.
8. Thomas D, Jeyathilakan N, Basith SA, Senthilkumar TMA. *In vitro* production of *Toxocara canis* excretory-secretory (TES) antigen. Journal of Parasitic Diseases 2016;40(3):1038-1043.
9. Jacobs DE, Xingquan Zhu, Gasser RB, Chilton NB. PCR-based methods identification of potentially zoonotic ascaridoid parasites of the dog, fox and cat. Acta Tropica 1997;68:191-200.
10. Borecka A, Gawor J. Modification of gDNA extraction from soil for PCR designed for the routine examination of soil samples contaminated with *Toxocara* spp. eggs. Journal of Helminthology 2008;82(2):119-122.
11. Fogt-Wyrwas R, Jarosz W, Mizgajska-Wiktor. Utilizing a polymerase chain reaction method for the detection of *Toxocara canis* and *T. cati* eggs in soil. Journal of helminthology 2007;81(1):75-78.
12. Hillis DM, Dixon MT. Ribosomal DNA: molecular evolution and phylogenetic inference. The Quarterly review of biology 1991;66(4):411-453.
13. Khademvatan S, Rahim F, Tavalla M, Abdizadeh R, Hashemitabar M. PCR-based molecular characterization of *Toxocara* spp. using faeces of stray cats: A study from southwest Iran. PLOS One 2013;8(2):1-6.
14. Li M W, Lin RQ, Chen HH, Sani RA, Song HQ, Zhu XQ. PCR tools for the verification of the specific identity of ascaridoid nematodes from dogs and cats. Molecular and cellular probes 2007;21(5, 6):349-354.