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Case Report

Internal transcribed spacer sequence based molecular confirmation and drug efficacy assessment against *Toxascaris leonina* (Linstow, 1909) infection in Asiatic lions (*Panthera leo persica*)

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Summary

The eggs recovered during faecal screening of Asiatic lions (kept at MC Zoological Park, Punjab, India) were delineated as *Toxascaris leonina* eggs based on morphometric and molecular studies (polymerase chain reaction targeting internal transcribed spacer sequences). Therapeutic management with fenbendazole @10 mg/kg body weight, once daily orally for three consecutive days proved ineffective with maximum faecal egg count reduction (FECR) on day 3 post treatments (69.35 %). But, therapeutic intervention with extended period dose schedule (5 consecutive days) with fenbendazole (@10 mg/kg body weight) proved effective and showed a maximum FECR of 95.34 % at day 7 post treatments. But, when ivermectin (@100µg/kg body weight) was given orally on three alternate days, proved effective as FECR of 95.74 % was recorded at day 7 post treatments. Thus, present study highlights the molecular confirmation of *T. leonina* and its management using fenbendazole and ivermectin in Asiatic lions.

Keywords: Asiatic lion; fenbendazole; ITSs; ivermectin; *Toxascaris leonina*

Introduction

Toxascaris leonina, an ascarid nematode targets domestic and wild felines and canines as definitive hosts (Okulewicz *et al*., 2012). Various reports had also been documented for its concurrent infections with other ascarids including *Toxocara cati* (Labarthe *et al*., 2004; Dalimi *et al.,* 2006; Dubna *et al*., 2007; Reperant *et al*., 2007; Itoh *et al*., 2011) and *T. canis* (Reperant *et al*., 2007) in domestic and wild carnivores. The parasite possesses significant zoonotic potential and larvae of *T. leonina* can invade the tissues of laboratory animals (Despommier, 2003). There is only one report of Beaver & Bowman (1984) which described a larva of *T. leonina* from the eye of a child in East Africa.

The specific identification of the helminth parasites of wild felids and canids, belonging to family ascarididae is a prerequisite for studying their infection, population biology, systematics and epidemiology (Okulewicz *et al*., 2012). But, the morphological identification of the eggs from the faecal samples corresponding to a particular species is cumbersome job, which requires skill, experience and expertise (Moudgil *et al*., 2014a). The only characteristic which helps in differentiation of the eggs of *Toxocara* species from *T. leonina* is that egg shells of *Toxocara* species are pitted with different pit dimensions, but the eggs of *T. leonina* are smooth walled with translucent appearance (Okulewicz *et al*., 2012).

Recently, molecular techniques have provided alternative and apt approaches for the identification of eggs and adults of ascarid species. Various studies have demonstrated that the first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) act as reliable genetic markers for the identification of *T. canis*, *T. cati* and *T. leonina* in domestic and wild animals (Zhu *et al*., 2000; Li *et al*., 2007).

Various reports of *T. leonina* infection in captive wild felids includ-

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ing lions, Bengal tigers, jaguars, pumas and lynx from various zoological gardens had been documented from different parts of world from time to time (Okulewicz *et al.*, 2002; Okulewicz *et al.*, 2012). The therapeutic interventions for the control of *T. leonina* infection in wild felids had proved difficult and reoccurrence of infection was recorded after some time (Okulewicz *et al*., 2012). Okulewicz *et al*. (2012) also proposed that elimination of *T. leonina* and *Toxocara* species from the zoo environment is very difficult. Thus, the present study was envisaged with the objective of morphological and PCR-based molecular confirmation of *T. leonina* targeting internal transcribed spacer regions (ITSs) and therapeutic management with fenbendazole and ivermectin to control the ascarid infection.

Material and Methods

Collection of the samples

The faecal sample of 3 lions (2 males and 1 female), kept at MC Zoological Park, Chhatbir, Punjab, India were screened seasonally (from January 2013 to September 2014) at regular basis for any parasitic infestation. All the lions were kept individually in separate enclosures with wooden floors. The faecal samples of all the lions were collected carefully from the floor in separate polythene bags (with individual animal identity on it) and were transferred to the laboratory at the Department of Veterinary Parasitology, GADVASU, Ludhiana (India) at 4 °C for retrieval of parasitic eggs to carry out morphological and molecular analysis.

Coprological examination and morphological analysis

Before starting the processing, the faecal sample was divided into two aliquots, one for morphological and other for molecular analysis. The morphological examination was performed with light microscopy after concentrating the eggs by Sheather's sugar floatation technique (Soulsby, 1982). The egg per gram (EPG) values to assess the intensity of infection was determined by McMaster technique (Soulsby, 1982) with faecal egg count sensitivity of 50 eggs. The morphometric analysis targeting the size of the eggs was performed as per Kazacos & Turek (1983). The length and breadth of the eggs (n = 10) was depicted in micrometers and was expressed considering mean ± standard deviation.

Genomic DNA extraction from eggs

The aliquots of the faecal material collected before and after deworming were subjected to Sheather's sugar floatation technique as described above. Approximately 50 µl of the supernatant containing concentrated material was aspirated using micropipettes and was transferred to 1.5 ml eppendorff vials (Traversa *et al*., 2004). Individual sample was then introduced to repetitive freeze -thaw process to induce complete disruption of the parasitic eggs. Genomic DNA from the eggs was extracted by using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) by following manufacturer's protocol. Finally, DNA was eluted by using 100 µl of elution (AE) buffer.

Primers used and PCR Procedure

For molecular confirmation, the PCR protocol of Li *et al*. (2007) was followed, targeting ITS-1 and ITS-2 ribosomal DNA (rDNA) genetic markers by using the forward (5'-ATA TCG GAA AAG GAC GCA CA-3') and reverse (5'-TTA GTT TCT TTT CCT CCG CT-3') primers. The PCR protocol for amplification of the templates was performed in a 25 µl total reaction volume (containing 12.5 µl of premix, 1.5 µl of each forward and reverse primers, 1 µl of MgCl2, 3.5 µl of nuclease free water and 5 µl of DNA) with following conditions in sequential order: (i) Initial denaturation (94 °C for 5 min.), (ii) Denaturation (35 cycles of 94 °C for 30 sec.), (iii) Annealing (35 cycles of 57 °C for 30 sec.), (iv) Extension (35 cycles of 72 °C for 1 min.), and (v) Final extension (72°C for 5 min.). For complete confirmation of the eggs belonging to the same parasite, the eggs retrieved from the faecal sample at day 1 post deworming were also used for DNA extraction and PCR amplification process. The DNA from the eggs of *T. canis* was also run simultaneously as control to rule out the presence of any other related species.

Therapeutic management

The animals targeted in the present study were not dewormed regularly. The anthelmintics used earlier for the treatment of ascarid infections in Asiatic lions of MC Zoological Park were ivermectin and pyrental pamoate (Singla *et al.*, 2003; Singh *et al*., 2006). But, there was reappearance of eggs in the treated hosts after a month of treatment. Thereafter the animals were treated for ascarid infections only with fenbendazole (due to its wide safety margin), but the efficacy of the drug was not assessed in the wild captive animals. In the present study, the therapeutic management was carried out thrice (orally in the meat) during the study period *i.e.* from January, 2013 to September, 2014. For the very first time (March, 2013) the treatment was given with fenbendazole @10 mg/kg body weight, once daily for three consecutive days. Therapeutic intervention with extended period dose schedule (5 consecutive days) with fenbendazole (@10 mg/kg body weight) was carried out during October, 2013. But, ivermectin @100 µg/kg body weight, once daily for three alternate days was used for therapeutic management during the third treatment schedule *i.e.* in March, 2014.

Assessment of drug efficacy

The faecal egg count (FEC) is the most widely performed test to assess drug efficacy (Cabaret & Berrag, 2004) and had been used in captive-wild animals (Goossens *et al.*, 2005; Young *et al.,* 2000). Thus, FEC was employed on the faecal samples collected from Asiatic lions at day 1, 3, 7 and 21 post-treatment by using the formula of Young *et al.* (2000).

$$\text{Percent efficacy} = \frac{(\text{Pre-treatment mean EPG - Post-treatment mean EPG}) \times 100}{\text{Pre-treatment mean EPG}}$$

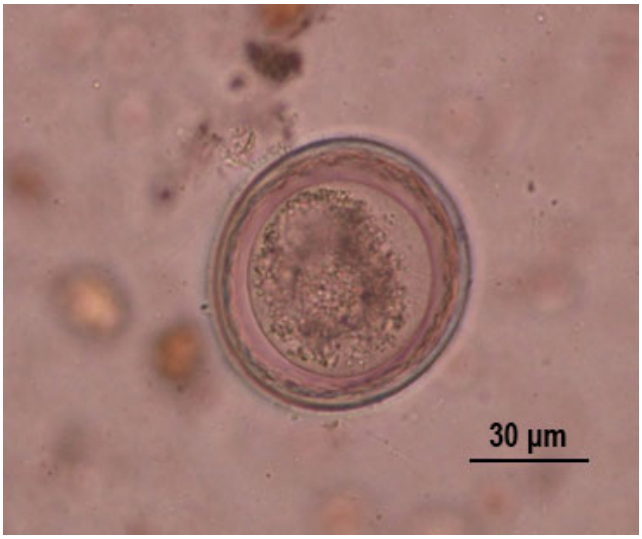

Fig. 1. Photomicrograph of unembryonated *Toxascaris leonina* egg.

Statistical analysis

One-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences (SPSS) 20.0 software was applied on eggs per gram values on different days (1, 3, 7 and 21) post treatment for the assessment of drug efficacies.

Results and Discussion

Morphological studies and parasitic intensity assessment

On detailed microscopic morphological examination, translucent ascarid eggs recovered were round to oval in shape with smooth walls (Fig. 1). Detailed morphometric studies (n=10) revealed the size of the eggs to be 81.78 ± 5.90 µm (74.8 – 86.2 µm) × 65.8 ± 4.90 µm (57.0 – 71.2 µm) (length × breadth). The results were in concordance with the findings of Gonzalez *et al*. (2007) and Okulewicz *et al.* (2012), who reported *T. leonina* eggs with smooth walls and without any albuminous coat.

The lions showed high egg per gram values (1033.33 ± 251.66, 1433.33 ± 251.66 and 783.33 ± 175.59) for *T. leonina* eggs before the therapeutic interventions during the three treatment schedules, which was further reduced with appropriate anthelmintics used (fenbendazole and ivermectin).

Molecular confirmation

With the optimized cycling conditions, the DNA extracted from the faecal samples of the Asiatic lions before and after deworming, amplified a product of approximately 380 base pairs (Fig. 2) confirming the infection of *T. leonina* only, which was almost in concordance with the findings of Li *et al.* (2007). No product was amplified from DNA extracted from *T. canis* eggs, determining the specificity of the primers.

Anthelmintic efficacy assessment

Anthelmintic efficacy against *T. leonina* in three lions was evaluated using a benzimidazole group drug, fenbendazole and a macrocyclic lactone, ivermectin. Fenbendazole used for three consecutive days schedule was proved ineffective to eliminate the infection in lions and showed a maximum egg reduction to 69.35 % at day 3 post treatments (Table 1). The reason could be inappropriate drug dosage given to the animals due to unclear picture of the actual body weights of the animals under study. Fenbendazole used for extended period of time during second treatment period and ivermectin used during third treatment period proved effective with 95.34 and 95.74 % of egg reduction in lions, respectively (Table 1). Ivermectin had earlier been also used by Singla *et al.* (2003) to check piperazine resistant toxocarosis in lions at MC Zoological Park, Chhatbir, Punjab. But, in the present study the egg per gram values again increased significantly to 516.67 ± 102.07 and 100 ± 32.28 ($P<0.05$), respectively after treatment II and III on day 21 post treatments (Table 1). This could be attributed to the housing conditions of the zoo animals, especially the wooden floors where the faecal material remained clogged in the wooden planks. The eggs survived for a longer time even under harsh conditions, contributing to generation of a perpetual source for the transmission of infection (Bowman, 1999; Singh *et al*., 2006). Due to the direct life cycle of *T. leonina* and development of infective stages inside the eggs within a shorter period (around 9 days) favour the earlier completion of the life cycle (Moudgil *et al*., 2014b). This could be considered as a reason for persistence of *T. leonina* infection in well sanitized cages (Bowman, 1999; Singh *et al*., 2006). The similar findings were observed by Singh *et al*. (2006) and Dehuri *et al*. (2013) while carrying out the assessment of efficacy of pyrental pamoate and ivermectin, respectively, against *T. leonina* infection in lions. On the other hand, the pour-on administration of ivermec-

Table 1. Drug efficacy assessment against *T. leonina* infection in Asiatic lions (n = 3).

EPG / Animals	Pre treatment	Post treatments (FECR %)			
		Day 1	Day 3	Day 7	Day 21
Treatment I	1033.33 ± 251.66^{a}	533.33 ± 152.75^{b} (48)	316.67 ± 76.37^{b} (69)	416.67 ± 28.86^{b} (59)	500 ± 100^{b} (51)
Treatment II	1433.33 ± 251.66^{a}	783.33 ± 86.60^{b} (46)	366.67 ± 76.37^{bc} (74)	66.67 ± 76.37^{c} (95)	516.67 ± 102.07^{b} (64)
Treatment III	783.33 ± 175.59^{a}	366.67 ± 104.08^{b} (53)	116.67 ± 28.86^{bc} (85)	33.33 ± 57.73^{c} (96)	100 ± 32.28^{bc} (87)

Values expressed as Mean ± Standard deviation

'n' indicates number of animals

Different 'superscripts' indicate values varying significantly at $P < 0.05$.

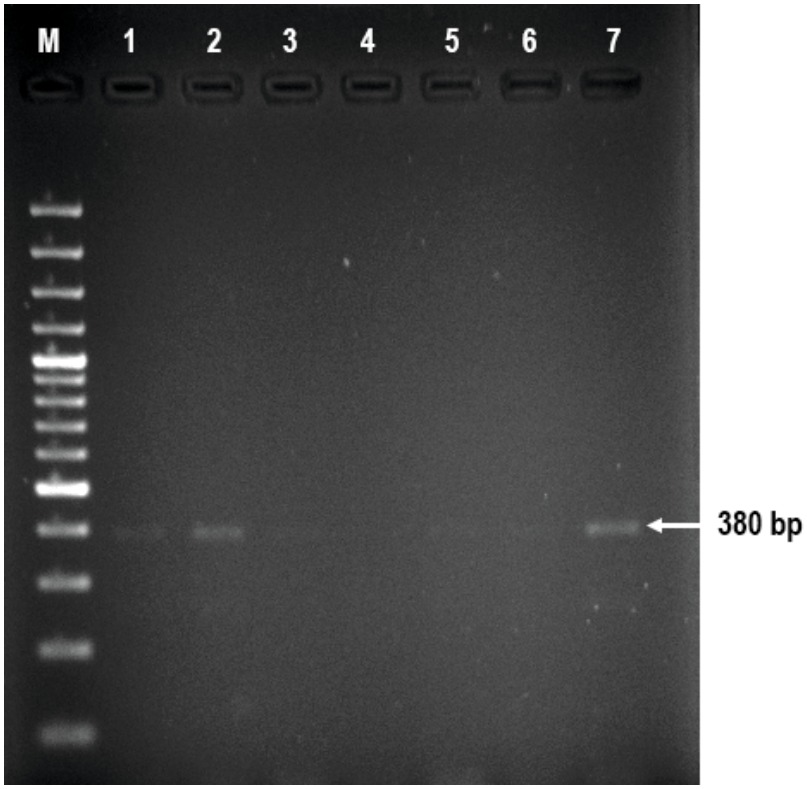

Fig. 2. PCR amplification targeting ITSs: M – 100bp Gene Marker, 1 & 2 – PCR product of eggs collected before deworming, 3 & 4 – Negative control, 5 – Negative control containing PCR products of *Toxocara canis* eggs, 6 – Non template control (without amplicon), 7 – PCR product of eggs collected after deworming.

tin in dogs against *T. leonina* infection proved 98.54 % effective even after 28 days of treatment (Khayatnouri & Garedaghi, 2012). The reason could be attributed to the fact that actual body weight of dogs could easily be assessed for preparing the total dosage of anthelmintic, whereas it could not be assessed accurately in case of wild animals. The vehicle of drug to captive wild animals (especially wild felines) plays an important role in administering exact dosage, as in order to cover the wastage of drug a relatively higher dosage is given in certain cases and overconsumption of such drugs over and again may lead to toxicity or development of anthelmintic resistance. In order to prevent toxicity of drugs in case of precious wild animals kept at zoological gardens, relatively lower dosage regimen is generally followed, which is a limiting factor in exact assessment of drug efficacy and may further lead to development of anthelmintic resistance due to under and repetitive dosing of drug.

The management conditions should be given utmost importance in Zoological Parks as they play an important role in controlling the ascarid infections, because parasitic eggs possess the potential to withstand the harsh environmental conditions and also it is difficult to remove *T. leonina* and *Toxocara* species from the zoo environment due to passage of large number of eggs everyday by the infected individuals. The present findings associated with the effective ascarid treatment with fenbendazole over an extended dosage schedule, involving repeated dosing highlighted the first case of fenbendazole resistance in Asiatic lions. Coles *et al*. (2001) mentioned that resistance against benzimidazoles develop in stages and in the initial phase the individual parasites respond to repeated doses of the drug, as was observed in present study. Although, ivermectin exhibited more than 95 % faecal egg count reduction on day 7 post treatments, but also at day 21 post treatments there was recorded slight resistance against the drug. The observation could be attributed to the management conditions such as wooden floors of the enclosure. Further, a combination of drugs (avermectins and benzimidazoles) or alternative anthelmintics such as moxidectin could be adopted for successful treatment of the ascarid infections in precious wild animals. Further studies on this aspect are warranted.

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